

The University of Nottingham

UNITED KINGDOM · CHINA · MALAYSIA

DISSECTING THE MECHANISM OF GROWTH INHIBITION AND TUMOUR REVERSION MEDIATED BY OOCYTE EXTRACT IN HUMAN BREAST CANCER CELLS

BY

NORAZALINA SAAD, BSc, MSc

School of Veterinary Medicine and Science
University of Nottingham, Sutton Bonington Campus

Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy

September 2016

Acknowledgments

The work presented herein was funded by Vice-Chancellor's Scholarship for Research Excellence (International), University of Nottingham. I also gratefully acknowledge the funding received from University of Putra Malaysia, Malaysia that facilitated my PhD journey to seek a wider knowledge.

Firstly, I would like to express my sincere gratitude to my wonderful supervisor Dr. Cinzia Allegrucci who has patiently and continuously guided and encouraged me throughout this PhD study. I would not have done it without you and thank you for putting up with me as your student. I would also like to thank my other great supervisor, Dr. Anna Grabowska. Thank you for your advice, support, insightful comments and critical discussion. My sincere thanks also go to Dr. Ramiro Alberio and Professor Andrew Johnson who were always there whenever I needed help. You have taught me the passion and responsibility that one should have in research.

I would especially like to thank research technicians in Vet school for their kind help and advice with my research. I am indebted my colleagues Mansi, Leong and Ryan for giving me all the help and support. To all members of A43 lab and all my friends in Vet School, I am so grateful for all the support and cheerful comments I had during the past 3 years of my study.

Special thanks go to my family. Words cannot express how grateful I am to my beloved mother, father-in-law, sister and all family members for all the sacrifices, continues prayers and motivation in these past few years. Last but not least a truly thank for my loving, supportive, encouraging and patient husband Hisyam for his faithful support throughout this study.

Declaration

I hereby declare that the present thesis has been written by myself and has not been previously submitted for any other degree. All procedures and work described in this thesis were performed by me.

Norazalina Saad

Date:

Abstract

Axolotl oocyte extract (AOE) has the ability to reprogram the breast cancer cell epigenome by modifying DNA methylation and histone modifications at the promoter region of silenced tumour suppressor genes, resulting in loss of cellular tumourigenicity (Allegrucci et al., 2011). The present study aimed to determine the signalling pathways and associated chromatin modifications involved in tumour reversion mediated by AOE in an attempt to identify specific oocyte molecules responsible for the reprogramming of breast cancer cells. Microarray analysis of reprogrammed tumours revealed a cell cycle block that was confirmed by BrdU labelling of xenograft tumours. The phenotype was recapitulated *in vitro* cultures up to 4 days after reprogramming, with AOE-treated cells showing a significant decreased entry in S phase of the cell cycle. Reduced expression of JUN and up-regulation of CDKN1B (p27) were measured, together with a reduction of phosphorylation of p44/42 MAPK signalling pathway. CDKN1B protein also showed a nuclear localisation in reprogrammed tumour xenografts compared to untreated controls. The reprogramming of breast cancer cells at genome-wide level was associated with a decrease in H3K9me2, H3K9me3 and H4K20me1 and in increase in H3K4me3, H3K27me3, H3K9Ac, H4K20me2 and H4K20me3 immediately after reprogramming (6 hours) and the pattern of changes was generally maintained at the time point of injection into xenografts (12 hours after reprogramming). Specific histone modifiers inhibitors were used during AOE treatment to dissect histone modification activities during cells reprogramming. Altogether, the results obtained suggest that axolotl oocytes contain complex activities that may cooperate to remodel the epigenetic and signalling landscape of breast cancer cells leading to reversion of malignancy by inducing cell cycle arrest and a state of quiescence. Therefore, this work has the potential to develop new therapeutic approaches based on oocyte molecules that could be tested in different types of breast cancer, especially those with poor prognosis and patient outcome.

TABLE OF CONTENT

		PAGE
ACKNOWLEDGEMENTS		i
DECLARATION		ii
ABSTRACT		iii
TABLE OF CONTENTS		iv
LIST OF FIGURES		vii
LIST OF TABLES		x
LIST OF ABBREVIATIONS		xi
CHAPTER 1.0	INTRODUCTION	1
1.1	Thesis rational, hypothesis, aims and objectives	1
1.2	Breast cancer	2
1.2.1	Breast cancer epidemiology and etiology	2
1.2.2	Breast cancer classification	3
1.2.3	Intrinsic subtypes and gene expression profiling of breast cancer	6
1.3	Hallmarks of cancer	9
1.4	Breast cancer signalling pathways and the hallmarks of cancer	12
1.5	Cancer and cell proliferation	14
1.6	Cell cycle and breast cancer	16
1.7	Cancer genetics	25
1.7.1	Breast cancer genetics	
1.8	Cancer epigenetics	29
1.8.1	Breast cancer epigenetics	34
1.9	Interplay between genetics and epigenetics in cancer	38
1.10	Erasing the epigenetic landscape: cellular reprogramming	42
1.10.1	Somatic Cell Nuclear Transfer	43
1.10.2	Cell Fusion	44
1.10.3	Induced Pluripotent Stem Cells (iPSC)	45
1.10.4	Cell and oocyte extracts	47
1.11	Cancer reprogramming and tumour reversion	50
1.12	Aims and Objectives	57
CHAPTER 2.0	MATERIALS AND METHODS	58
2.1	Microarray gene-expression profiling	58
2.2	Cell line and culture	60
2.2.1	Maintenance of cell line	60
2.2.2	Freezing of cell line	60
2.2.3	Thawing of cell line	61
2.3	Cell permeabilisation	61
2.4	Preparation of Oocyte extracts	61
2.5	Treatment of cells in oocyte	62
2.6	Matrigel culture (3D on-top assay)	62
2.7	3D Imaging Cell Proliferation	63
2.8	Cell Recovery from Matrigel	64

2.9 EdU Staining and cell cycle analysis 64
2.10 Immunohistochemistry 65
2.11 Preparation of protein sample and quantification for cell signalling western blotting 65
2.12 Acid Histone Extraction for histone western blotting 66
2.13 SDS-PAGE and Western blot analysis 67
2.14 Membrane Stripping and Re-probing 71
2.15 RNA extraction 71
2.16 cDNA preparation 72
2.17 Real Time Polymerase Chain Reaction 72
2.17.1 Optimisation of Internal Control and Calibrator Genes 73
2.17.2 Quantitative PCR Data Analysis 73
2.18 Statistical Analysis 74
CHAPTER 3.0 GENE EXPRESSION PROFILING OF BREAST CANCER CELLS AFTER TREATMENT WITH AXOLOTL OOCYTE EXTRACT: MICROARRAY STUDY OF TUMOUR XENOGRAFTS 75
3.1 Introduction 75
3.2 Results 80
3.2.1 Validation of total RNA quality 80
3.2.2 Gene expression profiling 82
3.2.3 Gene Ontology analysis 83
3.2.4 Canonical pathway analysis 88
3.2.5 Top diseases and functions analysis 97
3.2.6 Gene networks analysis 99
3.2.7 IPA upstream analysis 103
3.2.8 Validation of differentially expressed genes related to cell cycle arrest 103
3.2.9 Validation of cell cycle block *in vivo* 105
3.3 Discussion 106
3.4 Conclusions 115
CHAPTER 4 MODULATION OF CELL SIGNALLING BY AOE EXTRACTS AND EFFECT GROWTH INHIBITION 117
4.1 Introduction 117
4.2 Results 126
4.2.1 Development of the reprogramming process in vitro 126
4.2.1.1 Optimisation of permeabilisation and viability of MCF-7 cells 126
4.2.1.2 Cell cycle analysis after reprogramming
4.2.2 Expression of genes involved in cell cycle regulation 128
4.2.2.1 Optimisation of reference genes 133
4.2.2.2 Expression of cell cycle genes in MCF-7 cells after AOE treatment 132
120
121
4.2.3 Cell signalling pathways involved in the inhibition of G1 to S transition 138

4.2.3.1 Effect on phosphorylation levels of the MAPK family (p44/42 MAPK, p38 MAPK and JNK) 138
4.2.3.2 Effect on phosphorylation levels of Akt/mTOR signalling pathways 141
4.2.3.3 Effect on phosphorylation levels of TGFβ pathway 143
4.3 Discussion 144
4.4 Conclusions 151
CHAPTER 5 EPIGENETIC REMODELLING OF HISTONE MARKS BY AOE 153
5.1 Introduction 153
5.2 Results 160
5.2.1 Optimisation of western blotting for the study of histone marks 160
5.2.2 Effect of AOE on repressive histone marks 160
5.2.3 Effect of AOE incubation on active histone marks 163
5.2.4 Effect of AOE on cell proliferation- and quiescence-associated active and repressive histone marks 165
5.2.5 Effect of epigenetic inhibitors on associated histone marks 167
5.2.5.1 H4K20me1 after treatment with by Daminozide (PHF8 histone demethylase inhibitor) 167
5.2.5.2 H4K20me2 and H4K20me3 after treatment with A196 (SUV420H1/H2 histone methyltransferases inhibitor) 168
5.3 Discussion 171
6.4 Conclusion 177
CHAPTER 6 GENERAL DISCUSSION 179
6.1 Summary of work 179
6.2 Cell cycle arrest mediated by oocyte extracts 180
6.3 Inter-relationship of cell signalling and epigenetic regulation of cell cycle 183
6.4 Cell cycle arrest and quiescence: therapeutic opportunities 188
6.6 Conclusions 190
6.5 Future work 190
REFERENCES 197
APPENDIX

LIST OF FIGURES

		Page
Figure 1.1	The breast tissue is constituted of glandular ducts and lobules, connective tissue, and fat	4
Figure 1.2	Classification of breast cancer subtype based on to histological characteristics	5
Figure 1.3	The Hallmarks of cancer	9
Figure 1.4	Phases of the cell cycle; G1, S (DNA replication), G2 and M (mitosis)	15
Figure 1.5	Mechanism of cell-cycle transition from G_1 to S phase	17
Figure 1.6	Mitotic cell cycle progression is accomplished through a reproducible sequence of events, DNA replication (S phase) and mitosis (M phase) separated temporally by gaps known as G1 and G2 phases	21
Figure 1.7	Genetic changes in cancer	23
Figure 1.8	Role of epigenetic modifications for chromatin	31
Figure 1.9	Combination of genetic and epigenetic alterations leading to malignant changes	40
Figure 1.10	Different approaches used in reprogramming (A) Somatic cell nuclear transfer (SCNT) (B) Cell fusion (C) Cell extract (D) Induced Pluripotent Stem Cells (iPSC)	43
Figure 1.11	Timeline of tumour reversion	51
Figure 2.1	Steps involve in the process of analysing gene expression using microarray	59
Figure 3.1	Steps involved in microarray experiments	78
Figure 3.2	Electropherograms of RNA integrity for four samples with two untreated and treated xenografts	81
Figure 3.3	Volcano plot illustrating differential gene expression between control and AOE treated groups	82
Figure 3.4	GO enrichment analysis of all differentially expressed genes visualised as a scatter plot using REVIGO	84
Figure 3.5	Treemap distribution of all differentially expressed genes among biological processes generated by REVIGO	85
Figure 3.6	GO enrichment analysis of upregulated genes visualised as a scatter plot using REVIGO	86
Figure 3.7	Treemap distribution of all upregulated genes among biological processes generated by REVIGO	86
Figure 3.8	GO enrichment analysis of downregulated genes visualised as a scatter plot using REVIGO	87
Figure 3.9	Treemap distribution of all downregulated genes among biological processes generated by REVIGO	88
Figure 3.10	Canonical pathway analysis of genes regulated by AOE treatment	90
Figure 3.11	Best match canonical pathways	93

Figure 3.12	Graphical representations of network of key genes associated in Cell Cycle: G1/S Checkpoint Regulation pathway	96
Figure 3.13	Illustration of diseases and functions affected by AOE treatment	98
Figure 3.14	Interaction between networks for identified molecules with specific functions	102
Figure 3.15	Expression of cell cycle related genes in AOE-treated tumours compared untreated controls	104
Figure 3.16	BrdU staining of nuclei in AOE-treated and untreated tumour xenografts	105
Figure 3.17	Immunoflorescence of p27 expression in AOE-treated and untreated tumour xenografts	106
Figure 4.1	Illustration of interacting signalling pathways comprising the MAPK family, PI3K/Akt and mTOR and downstream signalling pathways in response to phosphorylation of the receptor tyrosine kinase (RTK)	120
Figure 4.2	TGF-β signaling cascade	123
Figure 4.3	Unpermeabilised cells and digitonin permeabilised cells	127
Figure 4.4	Number of cells recovered and flowcytometric analysis of EdU staining and cell cycle phases in untreated and AOE treated cells for 6 hours and extract-free culture overnight (12 hour after reprogramming)	129
Figure 4.5	Morphology of permeabilised MCF-7 cells cultured on plastic (2D) after 4 days	130
Figure 4.6	Number of cells recovered and flowcytometry analysis of EdU staining and cell cycle phases in untreated and AOE treated cells after four days in 2D culture	131
Figure 4.7	Crossing point (CP) value for five selected reference genes in control and AOE groups	132
Figure 4.8	Expression of key regulatory genes of cell cycle after reprogramming of MCF-7 cells with AOE at 6 and 12 hours	135
Figure 4.9	Expression of cell cycle modulating genes after reprogramming of MCF-7 cell with AOE at 6 and 12 hours	137
Figure 4.10	Phosphorylation of p44/42 MAPK, p38 MAPK and JNK pathways after AOE treatment at four different time points (30 minutes, 1 hour, 3 hour and 6 hour)	139
Figure 4.11	Level of phosphorylation of p44/42 MAPK, p38 MAPK and JNK pathways after AOE treatment at 6 hours.	140
Figure 4.12	Phosphorylation of Akt and mTOR pathways after AOE treatment at four different time points (30 minutes, 1 hour, 3 hour and 6 hour)	141
Figure 4.13	Level of phosphorylation of Akt and mTOR pathways after AOE treatment at 6 hours	142
Figure 4.14	Analysis of phospho-Smad2 and total Smad 2/3 in permeabilised MCF-7 cell incubated with AOE	143

Figure 5.1	Epigenetic regulators involved in post-translational modification of histones	155
Figure 5.2	Levels of two different histone marks in MCF-7 cells with different amount of histone proteins loaded	160
Figure 5.3	Relative repressive histone modification changes in MCF-7 cells after 6 and 12 hours of reprogramming with AOE	162
Figure 5.4	Relative active histone modification changes in MCF-7 cells after 6 and 12 hours of reprogramming with AOE	164
Figure 5.5	Relative histone modification changes associated to cell proliferation and quiescence in MCF-7 cells after 6 and 12 hours of reprogramming with AOE	166
Figure 5.6	Morphology of MCF-7 cells in absence and presence of 10 µM Daminozide.	167
Figure 5.7	Relative histone modification changes of H4K20me1 in MCF-7 cells after 6 hours incubation in AOE in the presence of 10 µM Daminozide (PHF8 inhibitor)	168
Figure 5.8	Morphology of MCF-7 cells in absence and presence of 10 µM A-196	169
Figure 5.9	Relative histone modification changes of H4K20me3 in MCF-7 cells after 6 hours incubation in AOE in the presence of 10 µM A-196 (SUV420H1/H2 inhibitor)	170
Figure 5.10	Relative histone modification changes of H4K20me3in MCF-7 cells after 6 hours incubation in AOE in the presence of 10 µM A196 ((SUV420H1/H2 inhibitor)	171
Figure 6.1	Key molecules participating during G1/S phase transition in the cell cycle	181
Figure 6.2	Working hypothesis on relationship of signalling pathways and histone modifications involvement during G1/S transition	184
Figure 6.3	Growth morphology of permeabilised MCF-7 cells after four days culture in 3D on-top assay	193
Figure 6.4	EdU incorporation was visualized by staining with Alexafluor 488 (green)	193
Figure 6.5	Number of cell recovered before flow cytometric analysis of EdU staining and cell cycle phases in untreated and AOE treated cells after four days cultured in 3D matrigel.	195

LIST OF TABLES

		Page
Table 1.1	Summary of the breast tumour molecular subtypes	7
Table 1.2	Descriptions of several histone methyltransferase and demethylase inhibitors	33
Table 1.3	Summary of different approaches to cancer cell reprogramming	54
Table 2.1	Primary and secondary antibodies used in western blotting for epigenetic modifications	69
Table 2.2	Primary and secondary antibodies used in western blotting for signalling pathways	70
Table 2.3	List of TaqMan® gene expression assays with a FAM™ dye label used	73
Table 3.1	Analysis of RNA integrity using Agilent RNA *pico* labchip	80
Table 3.2	List of biological processes enriched in all differentially expressed genes obtained by GO analysis using REVIGO	83
Table 3.3	Summary of top 5 canonical pathways	91
Table 3.4	List of associated genes in Cell Cycle: G1/S Checkpoint Regulation pathway	95
Table 3.5	The top 10 diseases and functions affected by AOE treatment	97
Table 3.6	Networks of genes identified differentially expressed in AOE-treated tumours by microarray analysis	100
Table 3.7	Most significant upstream regulators of differentially expressed genes	103
Table 4.1	Percentage of permeabilisation of MCF-7 cells treated with digitonin (20µg/ml) for 1.45 minutes	127
Table 4.2	Percentage of MCF-7 cells recovered after treatment with digitonin as measured by counting number the cells that survived the permeabilisation	127
Table 4.3	Descriptive analysis of five selected housekeeping using BestKeeper Software	133

LIST OF ABBREVIATIONS

Abbreviations	Description
%	Percent
°C	Degree Celsius
µg	Microgram
µl	Microlitre
ml	Millilitre
ng	Nanogram
mM	Milimolar
ATCC	American Type Culture Collection
BrdU	Bromodeoxyuridine
CO_2	Carbon dioxide
CDK	Cyclin-dependent kinase
CDKI	Cyclin-dependent kinase inhibitor
cDNA	Complementary DNA
cRNA	Complementary RNA
DMSO	Dimethyl sulfoxide
DNMT	DNA methyltransferases
EdU	5'ethylnyl-2'-deoxyuridine
ER	Estrogen receptor
EGFR	Epidermal Growth Factor Receptor
FACS	Fluorescence activated cell sorter
FCS	Fetal Calf Serum
GSK-3	Glycogen synthase kinase 3
HDAC	Histone deacetylase
HDM	histone demethylase
HER2/neu	Receptor tyrosine-protein kinase erbB-2
HMT	Histone methyltransferases
IHC	Immunohistochemistry
iPSC	Induced Pluripotent Stem Cells
kDa	Kilodalton
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
miRNA	Micro RNA
M	Molarity
MAPK	Mitogen-activated protein kinases
N	Normality
PBS	Phosphate-buffered saline
PR	Progesterone receptors
PI	Propidium iodide
qPCR	Real-Time Polymerase Chain Reaction
RNA	Ribonucleic acid
Rb	Retinoblastoma

RPMI	Roswell Park Memorial Institute
RT	Room temperature
SCNT	Somatic cell nuclear transfer
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate

1.0 Introduction

1.1 Thesis rational, hypothesis, aims and objectives

Cancer is a disease characterised by unrestrained cell proliferation and conventional therapies are directed to halt cell division by interfering with DNA replication and mitosis. Novel therapies instead aim to target molecular mechanisms at the basis of tumorigenesis either by affecting oncogenes or restoring the function of tumour suppressor genes (Thompson, 2009). Another approach for the development of novel therapies is to study the mechanisms of tumour reversion, a biological process by which tumour cells lose or escape malignancy (Telerman and Amson, 2009). The idea of tumour reversion or cancer reprogramming was put forward more than 20 years ago with the queries of how normal cells transform to cancer cells and how tumour cells can be reverted. The field of tumour reversion started with initial findings revealing that ovarian teratoma cells are capable to develop to non-tumorigenic embryonic germinal layers and tissues after introduction into a mouse blastocyst as the normal embryonic differentiation could take place and tumorigenicity was lost (Telerman and Amson, 2009). This highlighted that epigenetic reprogramming in embryonic environments can revert a malignant phenotype to normalcy, despite cancer genetic abnormalities. The recent finding that epigenetic defects are reversible and they influence activation or silencing at critical cancer gene promoters is directing research in epigenetics to be at forefront of cancer research (Hardwick and Philpott, 2015).

Previous findings from our laboratory revealed the ability of amphibian oocyte extracts to epigenetically reprogram breast cancer cells by modifying DNA methylation and histone marks, further participating to the remodelling of somatic cell chromatin and loss of cell tumorigenicity both *in vitro* and *in vivo* (Allegrucci et al., 2011). The aim of this project was to dissect the mechanisms of tumour reversion as well as the molecular basis of the tumour reversed phenotype observed in the described mouse xenografts. To this end, the objectives of the project were to analyse the expression profile of the reprogrammed tumour xenografts by

microarray and to perform functional studies for selected signalling pathways and epigenetic modifications which could be involved in the initiation of the reprogramming after treatment of cancer cells with the axolotl oocyte extracts. Based on current knowledge of cellular reprogramming and the epigenetic remodelling activity of oocytes, the present project was based on the hypothesis that the epigenetic remodelling of cancer cell chromatin by axolotl oocyte extracts would make breast cancer cells susceptible to the effects of signalling pathways that arrest cell proliferation to inhibit malignant growth.

1.2 Breast cancer

1.2.1 Breast cancer epidemiology and etiology

Breast cancer was ranked as the cancer with the highest incidence in women in 2008 with an estimation of nearly 1.4 million women diagnosed and almost 459,000 women dead from the disease worldwide (Youlden et al., 2012). GLOBOCAN statistics reported an increased prevalence of breast cancer in 2012, with 18% increase in breast cancer occurrence as compared to 2008. It is predicted that breast cancer will reach nearly 3.2 million new cases per year by 2050 (Ferlay et al., 2015). In the UK, the most common cancer registered in 2013 was malignant breast cancer, with 44,540 cases recorded. Breast cancer accounted for 31.2% of the total female cancer registrations (www.ons.gov.uk). The reported statistics highlight the critical importance of breast cancer incidence, which affects society globally and therefore calls for urgent preventive and treatment actions (Tao et al., 2014).

Several important epidemiological factors that increase the risk of breast cancer are well-established (Kluttig and Schmidt-Pokrzywniak, 2009). Risk factors related to elevated estrogen levels include age at menarche, age at menopause and obesity as well as hormonal factors. Increased breast density is considered a potent risk factor for breast cancer, even though it is still doubtful whether a decrease in breast density could instead influence the reduction of breast cancer (Barlow et al., 2006). Several adaptable risk factors, such as alcohol consumption, sedentary lifestyle, obesity, and

hormone replacement therapy use have been identified to in enhance the risk of breast cancer. Thus, as the incidence of identified risk factors is considerably high with an estimation of the population-attributable risk to almost 40% of breast cancer cases, strategies applied or targeted on these particular risk factors could be implemented to prevent breast cancer (Kluttig and Schmidt-Pokrzywniak, 2009).

1.2.2 Breast cancer classification

Traditional classification of breast cancer refers to the clinical-pathologic analysis of tumours, with classes of breast cancer defined by histopathologic features, including pattern of architectural growth and nuclear grade (low, intermediate, or high) (Yersal and Barutca, 2014). Treatment choices are selected according to tumour size, local invasion, lymph node contribution and distant metastases, as described by the American Joint Committee on Cancer's TNM staging classification (Singletary and Connolly, 2006). Survival rates correlate best with tumour size and the existence of axillary metastasis, but breast cancer patients at the same stage of disease can have markedly different clinical courses and clinical outcomes (Weigelt et al., 2005).

Histologically, the breast tissue consists of a branching ductal system that connects lobuli and terminates in terminal ducts known as ductal-lobular units (TDLUs). This system is embedded within an interlobular matrix consisting of fat and fibrous tissues and composed of adipocytes and stromal fibroblasts, respectively (Figure 1.1) (Cardiff and Wellings, 1999).

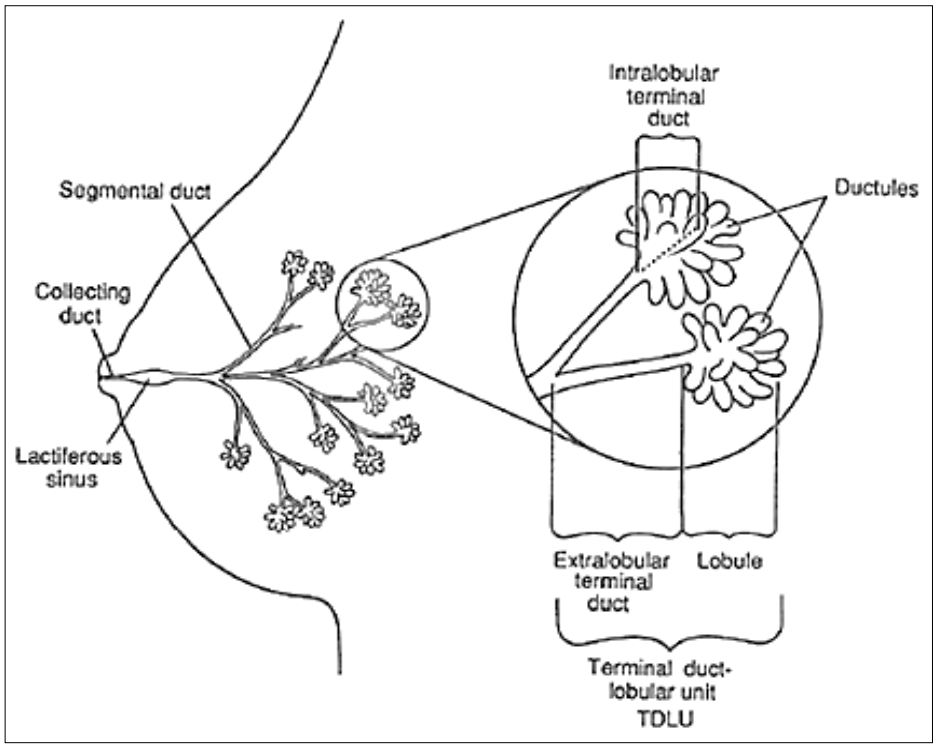


Figure 1.1. The breast tissue is constituted of glandular ducts and lobules, connective tissue, and fat. Figure from (Hindle, 1999).

The majority of breast cancers begin in the ductal-lobular units which are composed by luminal epithelial cells, myoepithelial cells, and progenitor/stem cells lining a basement membrane (Smalley and Ashworth, 2003, Dimri et al., 2005). Uncommonly, breast cancer is initiated in the stromal tissues, which incorporates fatty and fibrous connective tissues. Progression of the ductal hyperproliferation involves the *in situ* and invasive carcinoma and eventually metastasis (Monslow et al., 2015).

Therefore, breast cancer is classified into *in situ* carcinoma and invasive (infiltrating) carcinoma. Breast carcinoma *in situ* is further sub-categorised to either ductal or lobular; growth patterns and cytological features differentiate these two groups (Malhotra et al., 2010). Ductal carcinoma *in situ* (DCIS) has higher occurrence compared to the lobular carcinoma *in situ* (LCIS) equivalent and represents a heterogeneous group of tumours. DCIS is conventionally sub-classified to reflect the architectural characteristics of the tumour into five well-established categories: Comedo, Cribiform, Micropapillary, Papillary and Solid (Malhotra et al., 2010) (Fig. 1.2).

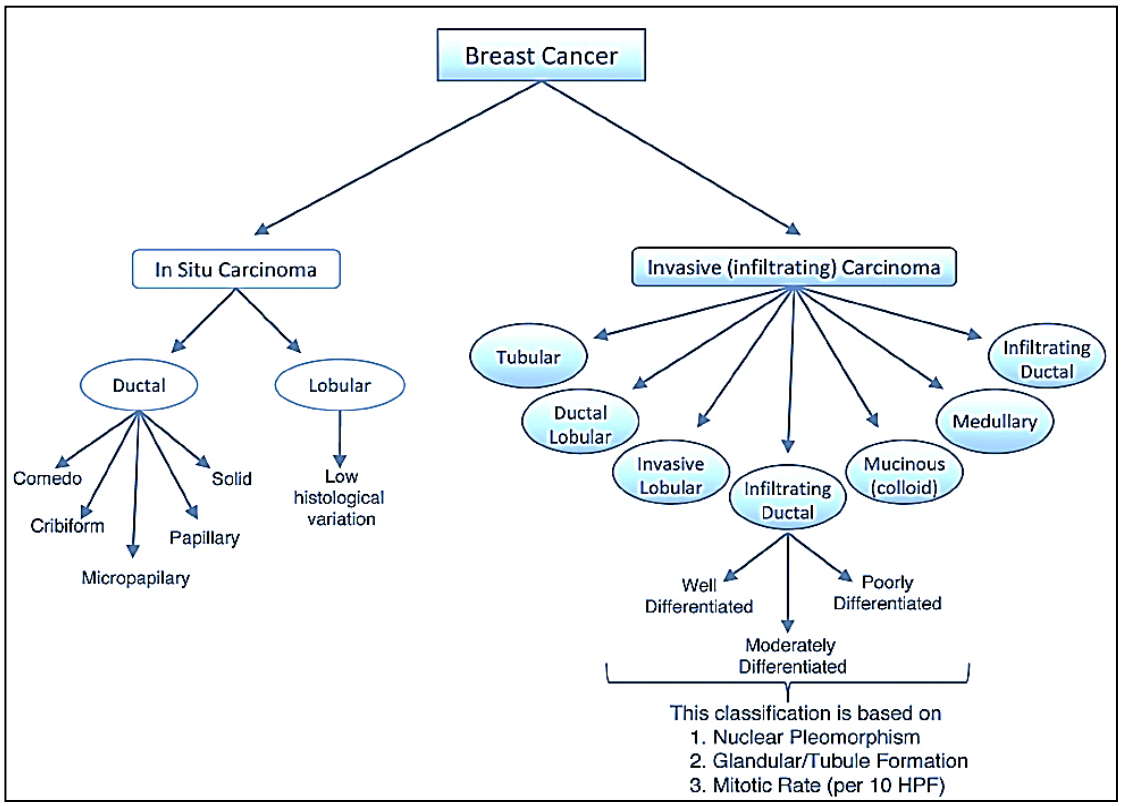


Figure 1.2. Classification of breast cancer subtype based on to histological characteristics. Figure from (Malhotra et al., 2010).

Invasive tumour types are divided into infiltrating ductal, invasive lobular, ductal/lobular, mucinous (colloid), tubular, medullary and papillary carcinomas (Fig. 1.2) (Malhotra et al., 2010, Cava et al., 2015). Infiltrating ductal carcinoma (IDC) is the most common subtype with almost 70–80% categorised as invasive lesions. IDC is further classified to well-differentiated (grade 1), moderately differentiated (grade 2) or poorly differentiated (grade 3) according to the degree of nuclear pleomorphism, glandular/tubule formation and mitotic index.

The latest classification systems integrate molecular markers, for example estrogen receptor (ER), progesterone receptors (PR), ErbB2 (Her2/neu) and p53 to establish an accurate definition of patients with good prognosis or relative risk of relapse. The utilisation of ER, PR and HER2/neu is acceptable for IDC and applicable to all invasive carcinomas. At present, the prominent use of these markers helps the stratification of patients who can respond to targeted treatments (Malhotra et al., 2010).

1.2.3 Intrinsic subtypes and gene expression profiling of breast cancer

Although the traditional classification of breast cancer remains relevant in clinical practice, it has been improved in the last two decades by advancement in the field of molecular biology as tumours are now being analysed based on expression of particular biomarkers (Trop et al., 2014). With the development of microarrays, gene expression profiling has been utilised for breast cancer prognosis, purposely identifying patients with satisfactorily good quality diagnosis to justify the use of adjuvant chemotherapy (Dai et al., 2015). The rational underlying such classification is that the differences underlying the gene expression patterns among cancer subtypes is reflected in the fundamental differences of the tumours at the molecular level (Reis-Filho et al., 2006).

Besides, the application of the latest high-throughput sequencing technologies has driven to gain much attention in utilising mutational profiling. Table 1.1 summarises five intrinsic molecular subtypes according to an IHC-defined subtype (Holliday and Speirs, 2011, Dai et al., 2015), except for the normal-like tumours which shares a similar IHC status with the luminal A subtype and are characterized by a normal breast tissue profiling. This particular category is highly associated with intrinsic gene expression microarray classification (Onitilo et al., 2009).

Table 1.1. Summary of the breast tumour molecular subtypes.

Intrinsic subtype	IHC status	Grade	Outcome	Prevalence

Luminal A	[ER+|PR+] HER2- KI67-	1|2	Good	23.7% (Cheang et al., 2009)
Luminal B	[ER+|PR+] HER2- KI67+	2|3	Intermediate	38.8% (Cheang et al., 2009)
HER2 over-expression	[ER-PR-] HER2+	2|3	Poor	11.2% (Cheang et al., 2009)
Basal	[ER-PR-]HER2-, basal marker+	3	Poor	12.3% (Cheang et al., 2009)
Normal-like	[ER+|PR+] HER2- KI67-	1|2|3	Intermediate	7.8% (Sorlie et al., 2003)

Luminal tumours are the most common subtype among breast cancer, with luminal being the majority. The luminal-like tumours express hormone receptors together with expression profiles indicative of breast luminal epithelial cells (Yersal and Barutca, 2014). Luminal A tumours present elevated expression of ER-associated genes and lower expression of proliferative genes compared to luminal B cancers, with luminal B tumours presenting higher grade compared to luminal A tumours. Luminal tumours are of better prognosis, responding well to hormone therapy but poorly to conventional chemotherapy (Dai et al., 2015). However, treatment response differs between luminal subtypes as only luminal A type can be effectively treated with endocrine therapy. The luminal B tumours which are more proliferative can instead benefit from combination therapy with chemotherapy and hormonal treatment. Other targeted approaches such as anti-angiogenic strategies are suggested to be effective for luminal tumours as well (Dai et al., 2015, Yersal and Barutca, 2014).

The HER2 over-expressing tumours are characterised by over-expression of genes in the HER2 amplicon; GRB7 and PGAP3 (Dai et al., 2014). Almost 40% to 80% of these tumours harbour TP53 mutation. Although HER2 overexpressing breast tumours hold a poor prognosis, they are highly proliferative but sensitive to anthracycline and taxane-based neoadjuvant chemotherapy, with significantly elevated response compared to luminal breast tumours (Dai et al., 2015). Molecular targeted agents such as anti-HER2 monoclonal antibody, trastuzumab, are available for the treatment of HER2 over-expressing cancers (Dai et al., 2015).

The basal subtype consists of ER-PR-HER2- (triple negative) tumours with the expression profiles mimicking basal myoepithelial cells (Dai et al., 2015). Basal tumours account almost 60% to 90% of triple negative cases (Swenson et al., 2009). They are deficient in expression of hormone receptors and HER2, and present high expression of basal markers and genes associated with proliferation (Yersal and Barutca, 2014). The tumours are categorised by basal cytokeratin expression and are most likely to have low BRCA1 (Abd El-Rehim et al., 2005) expression and harbour TP53 mutations (O'Brien et al., 2010). Their aggressiveness and deficiency of any form of targeted therapy has drawn particular interest on this tumour subtype. These tumours are related with younger patient age, more common to develop among African-American women as well as between pre-menopausal women (Carey et al., 2006). Additionally, these tumours are associated with a lower survival and a higher possibility of relapse (Dai et al., 2015).

The normal-like category are poorly characterised and have been grouped into intrinsic subtypes with fibroadenomas and normal breast samples. Due to the negative expression of ER, PR and HER2, these tumours are grouped as triple-negative however they are not considered as basal-like cancers since they are negative for CK5 and EGFR (Yersal and Barutca, 2014). The expression of genes characteristic of adipose tissue also describes an intermediate prognosis between luminal and basal-like cancers which normally do not respond to neoadjuvant chemotherapy. Since the study on this subtype is very limited, their clinical significance continues to be debated. Besides, their presence as a breast cancer subtype is doubtful as some scientists consider them as a technical artefact occurred because of contamination with normal tissue whilst analysing by microarrays (Weigelt et al., 2010). Moreover, when a large series of samples were selected to isolate neoplastic cells by microdissection, no cases of normal breast-like subtype were observed, further supporting this theory (Yersal and Barutca, 2014).

1.3 Hallmarks of cancer

During cancer progression, tumours can acquire biological capabilities that give them a selective advantage in the host and allow tumour growth and metastatic dissemination. These comprise supporting proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis and activating invasion and metastasis, reprogramming of energy metabolism and evading immune destruction. These indicators can be distinctive and/or complementary to each other and provide the mechanisms that underpin our understanding of cancer behaviour (Hanahan and Weinberg, 2011) (Figure 1.3).

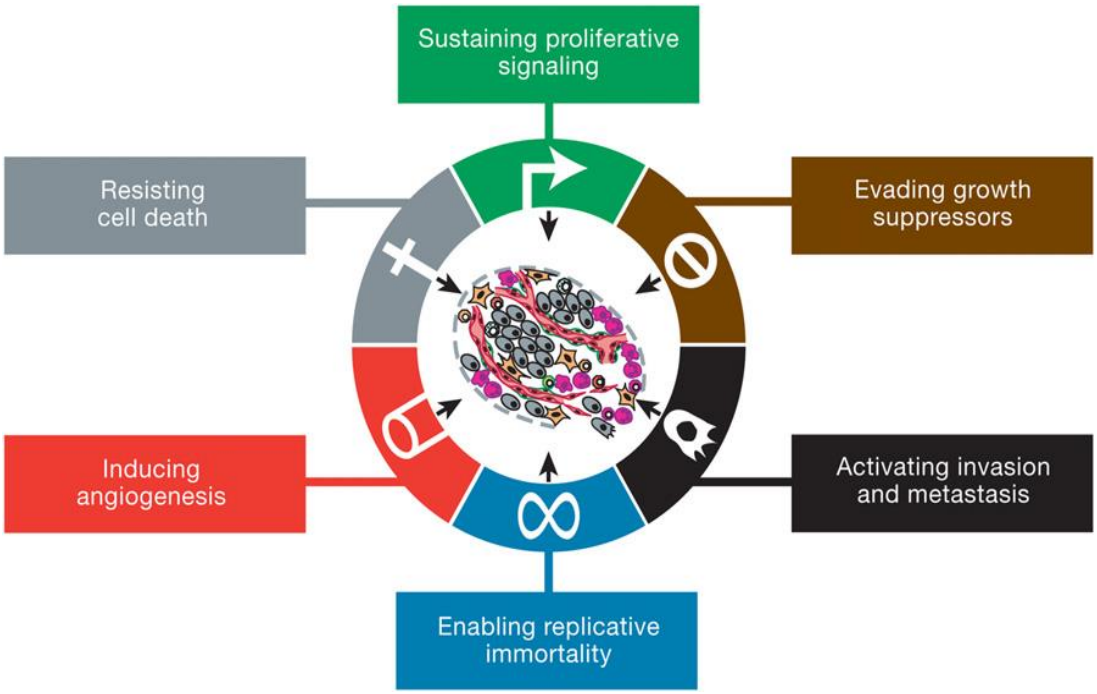


Figure 1.3. The Hallmarks of cancer. All six identified hallmarks of proficiency originally proposed in 2000 perspective. Figure from (Hanahan and Weinberg, 2011).

In normal tissues, the production and distribution of growth-inducing signals important for cell growth and development are carefully controlled. The supporting signals are primarily carried by growth factors that bind to cell-surface receptors, generally of the tyrosine kinase family. Notably, little is known how the mitogenic signals are regulated within normal tissues. The bioavailability of growth factors is controlled by requisitioning in the pericellular space and outer matrix, and by the

activities of proteases, sulfatases and other enzymes that release them in a precise manner (Hanahan and Weinberg, 2011).

In contrast, more is known about mitogenic signalling in cancer cells. Cancer cells have the capability to respond and enhance proliferative signalling in several ways. They might release growth factor ligands themselves, to which they are able to react through the expression of related receptors. These signals then activate cells of the tumour-related stroma that respond by providing the cancer cells with numerous growth factors that potentiate cancer growth (Trichopoulos et al., 2008, Bhowmick et al., 2004). Receptor signalling is also potentially activated by increasing amounts of receptors expressed on the cell surface or by intrinsic activation resulting from structural alterations causing signalling in the absence of growth factors (Hanahan and Weinberg, 2011).

Evasion of growth suppression is another important feature of cancer. Two main tumour suppressor genes are the RB (retinoblastoma-associated) and TP53 proteins (Hanahan and Weinberg, 2011). They function as 'gatekeepers' of the cell cycle and defects in their pathways are common in cancer cells, leading to a loss of critical cycle regulation and persistent cell proliferation. Stress and inhibitory signals are received differently by the tumour suppressors: RB transduces growth-inhibitory signals from the outer cell, whereas TP53 mediates responses from intracellular stress and DNA damage. If there are signals demonstrating disturbing or irreversible destruction to such cellular compartments, TP53 can initiate apoptosis (Hanahan and Weinberg, 2011).

Apoptotic-inducing stresses result from high levels of oncogene signalling and DNA damage related to excessive proliferation (Hanahan and Weinberg, 2011). The apoptotic mechanism is poised by upstream regulators and downstream effectors (Adams and Cory, 2007). The regulators can be activated by extracellular death-stimulating signals (the extrinsic apoptotic program, including the Fas ligand/Fas receptor), or other sensing signals of intracellular death (the intrinsic program). The effectors are molecules that inevitably cause destruction of the nucleus, cellular

matrix and membrane. The "apoptotic trigger" that delivers signals among the regulators and effectors is structured by balancing pro- and anti-apoptotic associates genes of the Bcl-2 regulatory proteins (Hanahan and Weinberg, 2011).

In general, cancer cells need unrestricted replicative ability to produce macroscopic tumours. This is different to the characteristics of normal cells that are only capable to go through certain restricted amount of continuous cell division cycles. This restriction is operated by senescence, an irreversible non-proliferative but viable state, and crisis that includes apoptosis. Nevertheless, infrequently cells can bypass these restrictions and show unrestricted replicative potential possibility that is referred as immortalization (Hanahan and Weinberg, 2011).

Telomerase, the DNA polymerase that maintains telomere length at the end point of chromosomes, is nearly lacking in normal ageing cells. However, the level of expression is very much increased (~90%) in immortalized and cancer cells. Existence of telomerase activity is associated with a resistance to senescence and crisis or apoptosis (Hanahan and Weinberg, 2011). Together with proliferation, other hallmarks of cancer are sustained angiogenesis and invasion. During normal development, the process of angiogenesis addresses the requirement of food and oxygen in addition to the capacity to eliminate metabolic wastes and carbon dioxide. However, the blood vessels created surrounding tumours by chronically stimulated angiogenesis are abnormal: tumour neovasculature is characterised by developed capillary sprouting, complicated and unnecessary vessel branching, distorted and increase size of vessels, irregular blood flow, microhemorrhaging, leakiness, and unusual levels of endothelial cell proliferation and apoptosis (Nagy et al., 2010, Baluk et al., 2005).

As cancer cells evolved from normal epithelial tissues to pre-neoplastic tissue and malignant tumours, they develop modifications in their morphology and interaction to other cells, also to the extracellular matrix (ECM). The most described adjustment involves the destruction of E-cadherin expression, a critical cell-to cell adhesion molecule (Hanahan and Weinberg, 2011). E-cadherin is normally down-regulated by

mutation or epigenetic silencing in human carcinomas, thus functioning as an important tumour suppressor (Berx and van Roy, 2009). Furthermore, expression of genes encoding for other cell-to-cell and cell-to-ECM adhesion molecules is changed in aggressive carcinomas, with those mediating cytostasis characteristically down-regulated. On the other hand, adhesion molecules that are generally connected with the cell migration arising throughout embryogenesis and inflammation are up-regulated in those cancers (Hanahan and Weinberg, 2011).

As consequence of the activation of invasion pathways and deregulation of cell-cell/matrix proteins, cancer cells can acquire the capability of invade and metastasize to neighbouring and distant tissues (Cavallaro and Christofori, 2004). Invasion and metastasis are products of a transient step-by-step progression (Talmadge and Fidler, 2010, Fidler, 2003). Even though important progresses have been achieved in understanding the essential mechanisms of these complex cancer hallmarks, there are still much more needs to be discovered (Hanahan and Weinberg, 2011).

1.4 Breast cancer signalling pathways and the hallmarks of cancer

Different signalling pathways are involved in breast cancer, influencing cell survival, proliferation, migration, differentiation and apoptosis (Aziz and Aziz, 2013). During breast cancer development, the abnormal activation of Epidermal Growth Factor Receptor (EGFR) that is a transmembrane receptor tyrosine kinase of the ErbB family is frequently observed. The ErbB family comprises four associated receptors: EGFR (HER-1/ErbB-1), HER-2 (ErbB2/c-neu), HER-3 (ErbB3), and HER-4 (ErbB-4) (Masuda et al., 2012). EGFR and HER2 are significantly involvement during breast development as they are expressed preferentially in the mammary glands of younger women (Lo et al., 2006). Moreover, these receptors are activated in the stroma and epithelium. In the absence of EGFR , pubertal ductal development is impaired, further demonstrating its critical contribution to mammary growth (Aziz and Aziz, 2013).

Two main signalling pathways coordinated by EGFR pay a crucial function during breast carcinogenesis: the Ras/Raf/MEK/ERK and the PI3K/AKT pathways (Guille et

al., 2013). The Ras/Raf/MEK/ERK signalling pathway consists of a kinase cascade that is coordinated through phosphorylation and de-phosphorylation by particular kinases, phosphatases as well as GTP/GDP exchange proteins, adaptor proteins and scaffolding proteins. As a wide range of substrates are affected in the activation of ERK1/2 kinases, suppression of MEK and ERK activities impacts strongly on breast cell development (Aziz and Aziz, 2013). Hence, abnormally regulation of this pathway leads to abnormal cellular proliferation and differentiation leading to cancer. In addition, several major apoptotic effector molecules such as Bcl-2, Bad, Bim, CREB, Foxo, and Caspase-9 are regulated by ERK phosphorylation. Deregulation of apoptosis is crucial in breast cancer and the involvement of important molecules the in apoptotic cascades is responsible for the loss of growth inhibition mediated by this pathway (Lo et al., 2006).

The other pathway involved in breast cancer is the PI3K/AKT pathway. This can be categorised into three groups, according to their structural and substrate characteristics (Aziz and Aziz, 2013). Upregulation of Receptor Tyrosine Kinases (RTK) which is mainly activating the JUN/MAPK pathway further activates the PI3K/AKT/mTOR pathway (Guille et al., 2013). Once stimulated by RTKs and G protein coupled receptors (GPCRs), the signals from several growth factors and cytokines further transducing the intracellular signal by producing phospholipids (Aziz and Aziz, 2013). This leads to the activation of AKT. PI3Ks are involved in several cellular processes which are crucial in cancer development such as metabolism, cell survival, proliferation, differentiation and motility (Liu et al., 2009).

Other signalling pathways are very important during metastatic progression. For instance the activation of Myc is at the crossroad of multiple signalling pathways that can be activated by different oncogenes that can drive the expression of advanced metastasis-specific genes (Blanco and Kang, 2011). In the metastatic process the activity of Src, H-Ras, E2F3, Myc, β-catenin, TCF/Wnt, and transforming growth factor (TGF)-β, have been implicated. For instance, Src signalling is implicated in reducing patient long term survival and subsequent outgrowth of clinical and experimental bone metastasis (Blanco and Kang, 2011). TGF-β activity enhances meta-static

spreading to the lung, instead of bone tissue (Drabsch and ten Dijke, 2011). Thus far, *ANGPTL4 has* been successfully identified as a novel TGF-β-responsive lung metastasis gene (Katz et al., 2013). WNT signalling has been also found promoting metastasis and used to make prognosis for breast cancer patients (Blanco and Kang, 2011). Finally, the VEGF pathways is crucially important to promote angiogenesis during breast cancer metastasis (Eroles et al., 2012).

1.5 Cancer and cell proliferation

The replication of DNA and cell division is driven by a tightly controlled cell cycle progression. The cell cycle comprises four consecutive stages beginning with quiescence (G0 phase), followed by proliferation (G1, S and M phases) and return again to quiescence (Diaz-Moralli et al., 2013). The progression from one cell cycle stage to the next follows an organised sequence and it is controlled by important cellular proteins. The cyclin-dependent kinases (CDKs), members of the serine/threonine protein kinases, act as the key regulatory proteins and they are triggered at particular stages of the cycle (Figure 1.4). To date, nine CDKs have been recognised, with five of them active in the cell cycle (Vermeulen et al., 2003). On the other hand, cyclin-dependent kinase inhibitors (CKIs) function to halt the cell cycle and therefore are essential as negative regulators (Park and Lee, 2003).

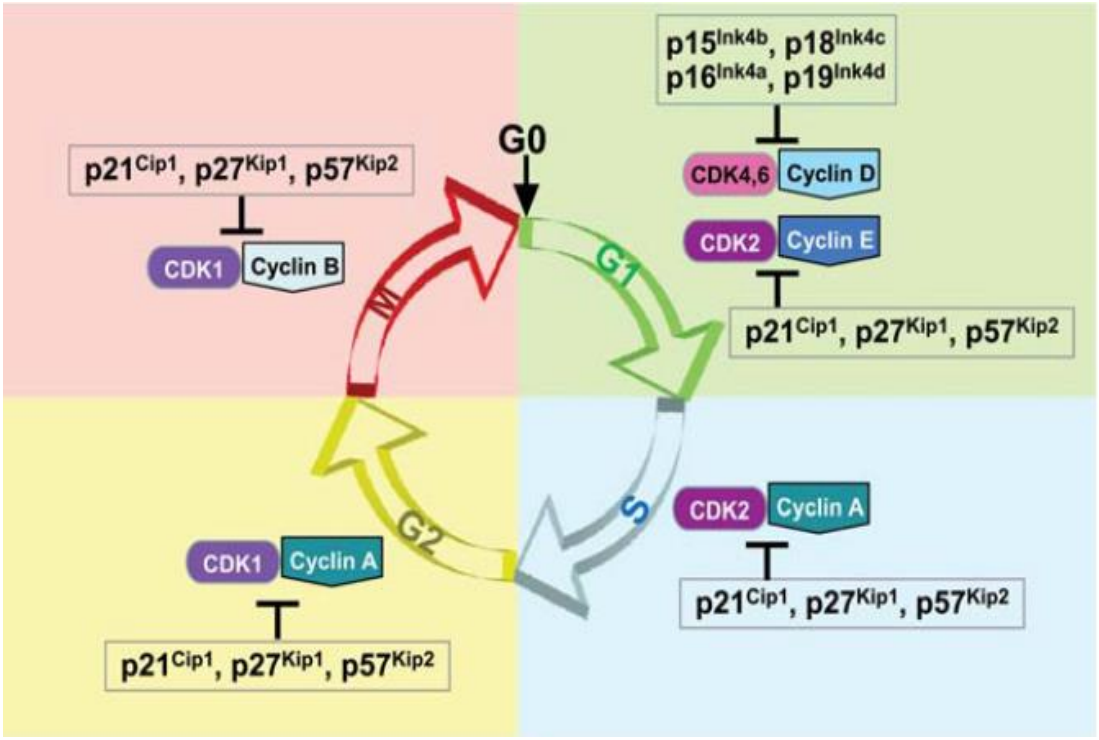


Figure 1.4. Phases of the cell cycle; G1, S (DNA replication), G2 and M (mitosis). The sites of activity of CDK/cyclin complexes and CKIs are also specified. Figure from (Fuster et al., 2010).

Two groups of CKIs have been characterised according to their specific activity: the INK family, which includes p15 (INK4B), p16 (INK4A), p18 (INK4C), and p19 (INK4D), and the CIP/KIP family, which shows homology at the N-terminal CDK inhibitory domain. This family includes p21 (CDKN1A or CIP1/WAF1), p27 (CDKN1B or KIP1), and p57 (CDKN1C or KIP2). Throughout the cell cycle, CDK proteins are normally stable. In contrast, binding partners (such as cyclins) and post-translational regulators (containing kinases and phosphatases) run through cyclic degradation to control DNA synthesis and cell division (Caldon et al., 2006).

The transition of one cell cycle phase to the next is controlled at particular positions known as checkpoints. They function as sensor mechanisms to respond to the cellular environment and also check that suitable circumstances have been achieved before decided to progress further in a new cell division cycle (Garrett, 2001). In case of DNA damage, checkpoints arrest the cell cycle in relation to give time for DNA repair. There are two main DNA damage checkpoints with the first one positioned before the cell proceeds into S phase (G1-S checkpoint). The next checkpoint is placed after

DNA replication (G2-M checkpoint) to perform a DNA damage checkpoint in S and M phases (Vermeulen et al., 2003). G2/M checkpoint also functions to monitor the fidelity of DNA replication (Garrett, 2001).

Deregulation of cell cycle is clearly observed during tumour progression (Maya-Mendoza et al., 2009). In human tumours, molecular analysis demonstrated that cell cycle regulators are mutated. The abnormal function of cyclins, CDKs and CKIs is commonly connected with mutations or deviations in expression. The most recurrent defect in human cancer is the increased expression of cyclins D1, E and A1 that occurs in breast cancer, colorectal cancer, squamous carcinoma of the head and neck, and prostate cancers. Amplification and overexpression of CDKs and their regulators are infrequently identified in human tumours (Park and Lee, 2003).

As for CKIs, p21 expression is stimulating by p53. The p53 gene is frequently mutated in cancer and the loss of function of this important gate keeper of the cell cycle is critical for uncontrolled cell proliferation in cancer (Pucci et al., 2000). The expression of p27 is instead often triggered by TGF-β. The decreased expression of p27 is typically identified in most human cancers (Elliott and Blobe, 2005). Modifications and loss of INK4A genes are commonly seen in human malignancies and transformed cells. These consist of melanoma, acute lymphocytic leukemia, osteosarcoma, lung, brain, breast, head and neck, bladder and ovarian cancers. The common loss of INK4A promotes uncontrolled cell proliferation due to bypass of senescence (Park and Lee, 2003).

1.6 Cell cycle and breast cancer

Cyclins and CDK inhibitors are comprehensively considered regulators of the cell cycle in breast cancer. They function as possible oncogenes or tumour suppressor genes. In breast cancer cells they are controlled by mitogenic stimuli that activate receptor tyrosine kinases and steroid hormone receptors. Particularly critical for breast cancer development is the transition from G1 to S phase. Cyclin D1 is a crucial regulator for G_1/S progression that occurs via Rb phosphorylation and p21/p27 titration (Figure 1.5). It is activated by several mitogenic factors during G_1 and during S phase is

omitted from the nucleus and promptly degraded via phosphorylation by GSK-3β (Caldon et al., 2006).

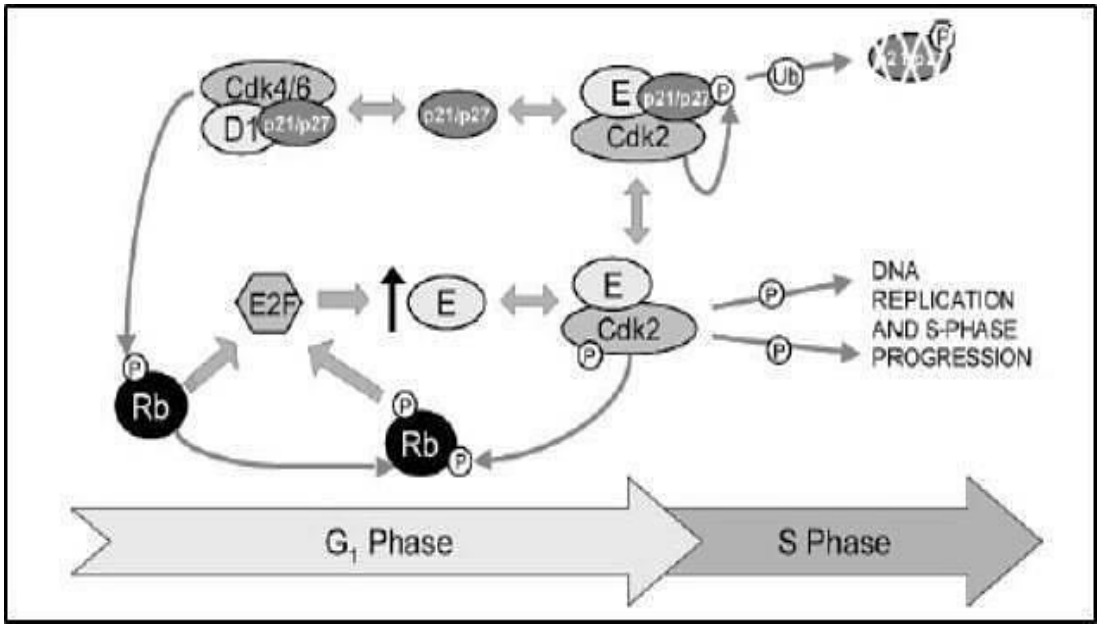


Figure 1.5. Mechanism of cell-cycle transition from G_1 to S phase. The levels of these CDK inhibitors are in part regulated by their ubiquitin-mediated degradation, D1: cyclin D1; E cyclin E; Ub: ubiquitin. Figure from (Caldon et al., 2006).

CDK4 and CDK6 show regulated substrate specificity compared to other CDKs. The most important identified substrates are Rb and other groups of the pocket protein family that in their phosphorylated form block the E2F-mediated transcription of genes important for DNA synthesis. Both of p21 and p27 are capable to bind cyclin D1-CDK4/6 complexes allowing their kinase activity in functional conditions. The cyclin E1 and E2 are closely connected and co-expressed. CDK2 is triggered via cycling E, and through phosphorylation of target proteins. CDK inhibitors p21, p27, and p57 negatively modulate CDK1 complexes in G1 phase and CDK2 (CDC2) complexes. Regulation of p27 expression occurs at the transcriptional and translational level, by modulating protein expression and subcellular localisation (Caldon et al., 2006). Even though transcription of the p27 gene is controlled by AFX-like Forkhead transcription factors, its accumulation is mainly regulated post-transcriptionally (Ahmad et al., 2012, Caldon et al., 2006). In contrast, the CDK inhibitor p21 is transcriptionally controlled following DNA damage and senescence. The four members of INK4 family

of CDK inhibitors also function as tumour suppressors. Indeed, the cyclin-dependent kinase inhibitor p16 (INK4A) has a crucial function in transformation of mammary epithelial cells (Caldon et al., 2006). A detailed schematic of cell cycle, regulatory proteins and signalling pathways is shown in Figure 1.6. Several signalling pathways are identified to regulate the conversion from G1 to S phase. These include P42/44 MAPK (ERK), P38 MAPK, Jun N-terminal kinase (JNK), TGF-β and Wnt.

The mitogen-activated protein kinases (MAPK), which are classified under serine/threonine kinases, are mediators of intracellular signals in response to several mitogenic stimuli. Activation of this signalling pathway drives progression of the cell cycle and entry of cells in G1 phase. Many genes stimulated by the Ras–ERK pathway participate in cell proliferation and differentiation (e.g., AP1, c-Myc). Thus, aberrant activation of this pathway results in sustained proliferative signals and continuous cell proliferation. This pathway also cooperates with PI3K–AKT–mTOR to encourage cell cycle progression by elevating the expression of cyclin D1 phosphorylation of Rb and downregulation of p27 (Neuzillet et al., 2014).

A continuous proliferative signalling in breast cancer can be mediated by HER-2 activation which is part of the human epidermal receptors (HER) family. This pathway is able to stimulate three major pathways including Ras/Raf/MAPK, JAK/Stat and PI3K/AKT/mTOR (Eroles et al., 2012). Triple negative breast cancer also shows upregulation of phospho-MEK and phospho-ERK, despite the low incidence of Ras and Raf mutations (Neuzillet et al., 2014) . Another pathway that has been ascertained as a target for breast cancer is IGF1R/PI3K/AKT/mTOR pathway. This pathway is important for cell growth and metabolism regulation. It is stimulated in normal conditions while nutrients are available for lipid, proteins and glycogen synthesis (Eroles et al., 2012). After stimulation by IGF1, the activated pathway mediates phosphorylation of tyrosine and serine residues of the insulin receptor substrate (IRS) and Src. It then activates the MAPK and PI3K/AKT/mTOR pathways (Baserga et al., 2003, Gonzalez-Angulo and Meric-Bernstam, 2010).

In breast cancer this pathway is self-sustained by initiating mutations of the membrane receptor and the catalytic subunits of PI3K and AKT. Besides, loss of control to negative regulators, such as PTEN and TSC 1/2, has been characterised (Tabernero et al., 2008). Up-regulation of these pathways in ER positive tumours has been hypothesised conferring resistance to conventional anti-estrogen therapy (Baselga, 2011). Furthermore, mutations occurring at different levels in these pathways have been connected with resistance to anti-HER2 drugs (Berns et al., 2007).

Wnt signalling is also an important pathway regulating cell proliferation and metastasis in breast cancer (Jang et al., 2015) . As Wnt is bound, Frizzled stops the protein kinase (GSK-3) in its role to stimulate the degradation of β-catenin and to activate the cyclin D1 promoter. The degradation of β-catenin is induced by phosphorylation mediated by the protein kinase GSK-3. GSK-3 is also able to phosphorylate β-catenin once it bound to APC protein, which is required to hold the protein in check. The loss or inactivation of APC is also linked to cancer progression (Howe and Brown, 2004).

Other signalling pathways are associated with cell cycle inhibition and their alteration prevents their action at checkpoints resulting in an impairment of cell cycle arrest or apoptosis. p38 MAPK activation is caused by stress signals, inflammation, DNA damage and leads to G_1/S checkpoint which results in increased p53 and p21 expression (Zhou et al., 2011). Similarly, JNK can be also activated by environmental stresses to halt cell cycle transition and induce apoptosis (Whyte et al., 2009). The cytokine TGF-β exerts a cytostatic effect on breast cells by inhibiting G_1/S transition via induction of CDK inhibitors and modulation of CDK action. TGF-β signals through a network of transmembrane serine/threonine kinase receptor and activated SMAD complexes to upregulate p21 and p15 (INK4B), whilst supressing the expression of c-Myc, cyclin D1 and Cdc25A. Subsequently, upregulation of p15 induces degradation of cyclin D1-CDK4 and p27 re-distribution. In addition, cyclin E-CDK2 activity is blocked via amplified binding of p21 and loss of Cdc25A stimulation (Caldon et al., 2006).

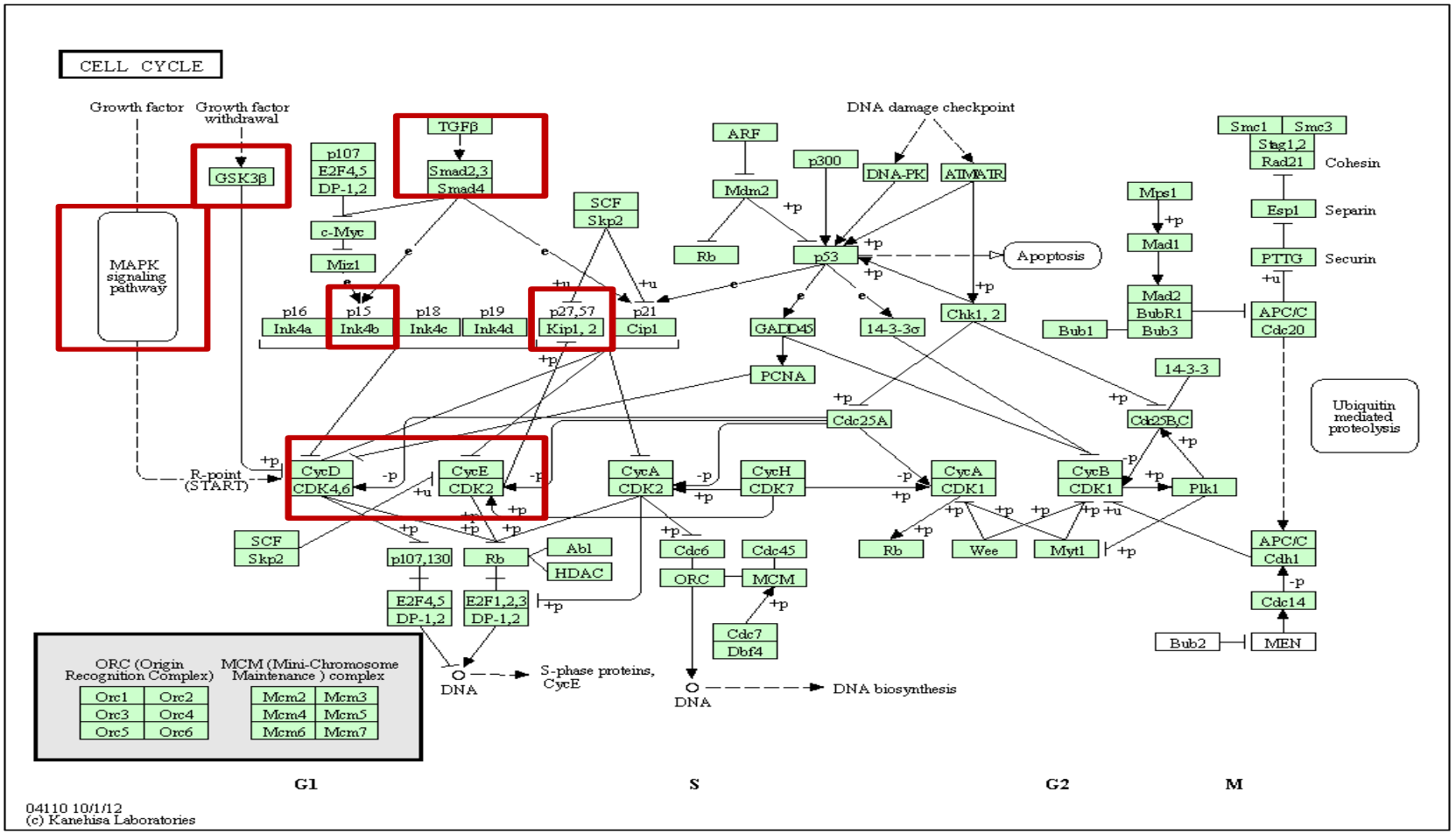


Figure 1.6. **Mitotic cell cycle progression is accomplished through a reproducible sequence of events, DNA replication (S phase) and mitosis (M phase) separated temporally by gaps known as G1 and G2 phases**. The highlighted red boxes indicate pathways involved in G1 to S transition which are part of this study. Figure from KEGG: Kyoto Encyclopedia of Genes and Genomes web.

1.7 Cancer genetics

During cancer development and progression, genetic mutations occurring at oncogenes, tumour suppressor genes and DNA repair genes are associated with genome instability (Semi et al., 2013). Mutation in cancer cells comprises small alterations in nucleotide sequences that occur via point mutation and large modifications which incorporate up to millions of base pairs including translocations, deletions and amplifications (Choong and Tsafnat, 2012). Several genes that are heritably changed in human cancer cells have been recognised in the last 20 years. To date, almost 100 oncogenes have been identified. Subsequently, knowledge towards understanding the mechanism behind genetic changes in human cancer cells has advanced upon the isolation of tumour suppressor genes (Yokota, 2000).

Deactivation of TP53 and Rb, activation of Ras (or any members of that pathway), and constitutive expression of *hTERT* have been proposed as the minimum collection of mutations necessary for oncogenic transformation in humans. These genes are involved in controlling cellular functions that avoid unrestrained proliferation (Figure 1.7) (Dixon and Kopras, 2004). In human cancers, the most prevalent mutations occur in the tumour suppressor genes TP53 and Rb, with both base substitutions and gene deletions (Hanahan and Weinberg, 2011). As Rb protein functions as a fundamental regulator of the cell cycle, loss of function will initiate sustained cell proliferation and defects in terminal differentiation. Moreover, the p53 protein is essential for cellular reactions to stress, regulating DNA repair, cell cycle checkpoints and cell death. Consequently, defects in these two suppressor genes direct to a higher cell proliferation and impaired cell death (Dixon and Kopras, 2004).

Promotion of cancer progression can also occur via mutations that stimulate the expression of proto-oncogenes that control cell proliferation (Hanahan and Weinberg, 2011). Ras mutations are seen in almost 20% of tumours, such as colon, lung, breast, and bladder cancer. The consequence of stimulation of these proto-oncogenes is to promote cell proliferation, inducing the growth of altered cell

populations and enhancing the effects of losing the control of tumour suppressor genes (Dixon and Kopras, 2004).

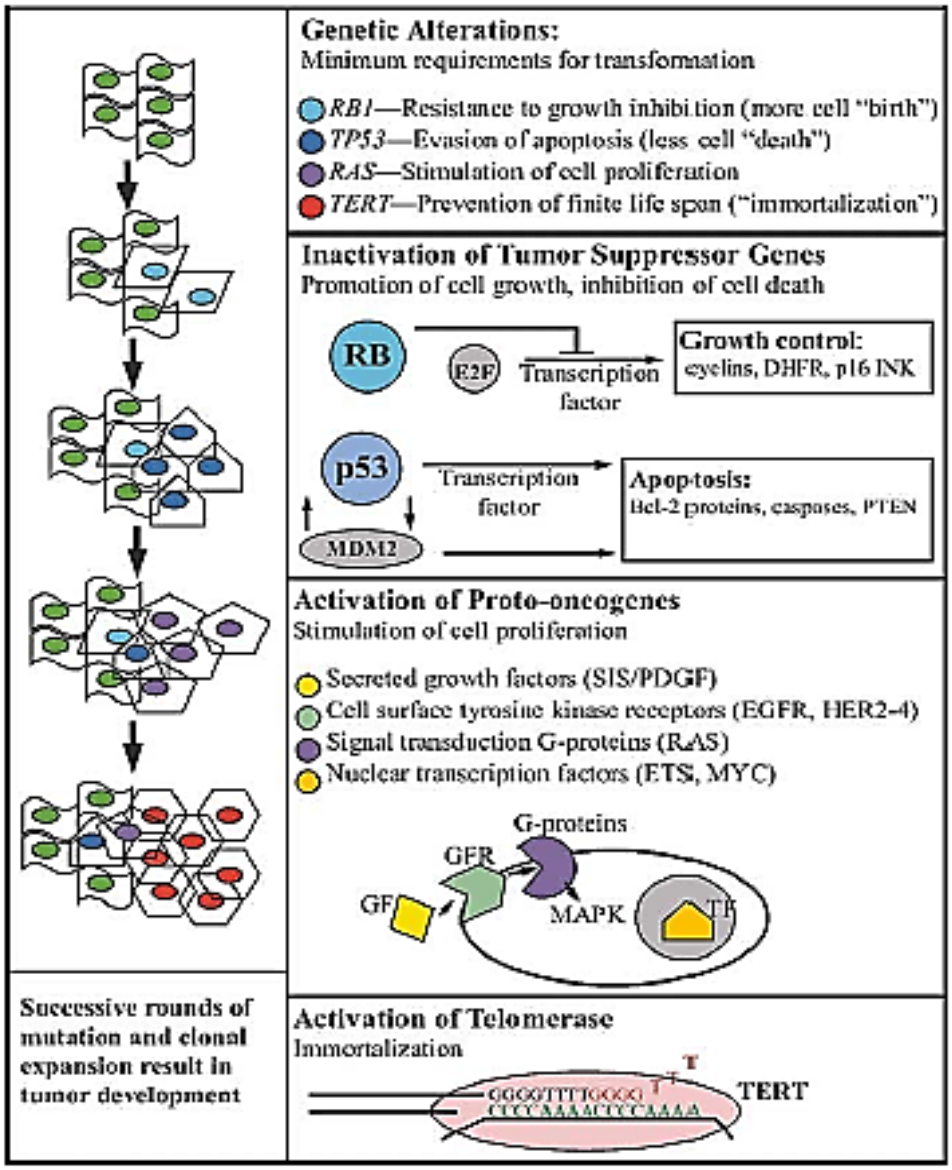


Figure 1.7. Genetic changes in cancer. Progression of cancer over time results from sequential mutation and growth of mutant clones. Figure from (Dixon and Kopras, 2004).

In addition, cancer development also requires cell "immortalization". Normal cells are capable to have a limited number of cell divisions towards extent "crisis" and death. This is due to shortening of telomeres length up to the point that further cell replication would lead to chromosome degradation and end-to-end fusions. In tumour cells, the gene (hTERT) telomerase (enzyme that sustains tolemerase length)

will switch on to overcome this restriction and allow cancer cells to sustain indefinite cell division (Cech, 2004).

With the advent of genome-wide analysis via sequencing, the genomic landscape becomes more comprehensive. It was identified that almost ~140 genes, changed by intragenic mutations, can drive cancer development (Vogelstein et al., 2013). The driver versus-passenger mutations theory was initially applied to discriminate mutations that affected a selective growth advantage from the total of mutations contained in a cell (Thiagalingam et al., 1996). The "drivers" comprise cancer-related genes (oncogenes and tumour suppressors) which are responsible for unrestrained proliferation and loss of organisation (McFarland et al., 2014). It is believed that in a typical solid tumour, about two to eight of these “driver gene” exhibit mutations while the remaining mutations are passengers (Vogelstein et al., 2013). “Passengers” that are randomly dispersed throughout the genome confer no immediate beneficial effect (Lawrence et al., 2014). Initially, passengers were expected to be neutral and did not contribute to cancer research. However, the latest evidence propose that they could be potentially destructive to cells and involved in both cancer development and clinical consequences (McFarland et al., 2014).

For low-frequency mutations, it is quite challenging to discriminate between “driver mutations” and “passenger mutations”. Even though the difference between drivers or passengers is still controversial, it is crucial to determine the most important driver genes and driver gene mutations. A driver gene is affected by river gene mutations but also might have passenger mutations. In the larger driver gene APC, only mutations that encode the protein in its N-terminal 1600 amino acids are categorised as driver gene mutations. Whereas, the other missense mutations contained in the gene (mutation occur in C-terminal 1200 amino acids) are described as passenger gene mutations (Vogelstein et al., 2013).

As described above, a gene that does not hold driver gene mutations are not included as driver gene. However, there are a huge number of genes that have very little or

no driver gene mutations that have been considered as driver genes in the literature. These contain genes that are overexpressed, underexpressed, or epigenetically modified in tumours, or those that increase or inhibit some characteristics of tumorigenicity when their expression were tested. Therefore to resolve this dilemma the genes assumed to increase the selective growth advantage of tumour cells can be classified as either "Mut-driver genes" or "Epi-driver genes." Mut-driver genes have an adequate number or type of driver gene mutations to clearly differentiate them from other genes. Furthermore, epi-driver genes are expressed abnormally in tumours, but not commonly mutated. They are modified via several mechanisms such as changes in DNA methylation or chromatin modification that persevere as the tumours cell proliferate (Vogelstein et al., 2013).

1.7.1 Breast cancer genetics

Breast cancer develops as a sequence of mutations including both driver and passenger genes. BRCA1 and BRCA2 are the most important driver genes which are also involved in familiar-liked breast cancer, but a number of less penetrating mutations are also found correlated with increased risk of developing breast cancer or cancer progression (Naga Anusha, 2011). Important diver genes are those that control DNA repair.

BRCA1 is classified as tumour suppressor gene and it is involved in repairing damaged DNA. The mutation of one of these genes has been found in approximately 3%-8% of all women with breast cancer (Brody and Biesecker, 1998). The mutations hold an 11-base pair deletion, a 1-base pair insertion, a stop codon, a missense substitution, and an inferred controlling mutation. The BRCA1 gene is expressed in the breast and ovary, and encodes a predicted protein of 1863 amino acids (Miki et al., 1994). In early-onset hereditary breast cancer, mutations in the BRCA2 is implicated (Martin and Weber, 2000). Protein-truncating mutations of BRCA2 are frequently destructive and enhance the incidence of breast cancer for almost 80% over a lifespan (Petrucelli et al., 2010). Some missense mutations in BRCA2 are predicted to have an equally

high penetrance, apart from more well-known neutral polymorphisms (Gorski et al., 2005).

BARD1 that also known as "BRCA1-associated RING Domain 1" acts to support the growth and function of cells and has been correlated to breast cancer occurrence (Naga Anusha, 2011). BARD1 is involved in stabilising BRCA1 and subsequently promoting the ubiquitin E3 ligase complex which is important in DNA repair by regulating apoptosis (Christou and Kyriacou, 2013).

Also, mutation of BRIP1 or "BRCA1 interacting protein C-terminal helicase 1" can develop breast cancer. The mutations occur in one copy of the gene and results in the generation of an aberrantly short, non-functional form of the BRIP1 protein (Frank et al., 2007). This leads to improper and incomplete interaction with BRCA1 protein to repair damaged DNA. Thus, the accumulation of DNA defects could initiate the cells to divide uncontrollably and form a tumour (Naga Anusha, 2011).
Other genes involved in DNA repair are implicated in breast cancer.

"Ataxia Telangiectasia Mutated" or ATM acts to instruct protein production inside the nucleus which is important for the regulation of cell division. This protein identifies the damaged DNA and fixes it by recruiting enzymes on the DNA strands (Ahmed and Rahman, 2006). The presence of only one copy of the ATM gene can increase breast cancer development by leading to ineffective repair of DNA damage, and therefore inducing further mutations in other genes and allow carcinogenesis (Naga Anusha, 2011, Anwar and Khan, 2008).

NBN or "Nibrin" is crucial to regulate important cellular functions and DNA repair by interacting with two proteins (MRE11A and RAD50) forming a large protein complex (Naga Anusha, 2011). The complex works together with the protein made from ATM gene which acts to recognise broken strands of DNA. The deleterious mutation of this gene is considered to increase breast cancer incidence by 2-3 folds (Seemanova et al., 2007).

"Check point Kinase 2" or CHEK2 instead functions to translate a tumour suppressor protein by phosphorylating p53 and BRCA1 in response to DNA damage (Naga Anusha, 2011). These proteins are important to regulate cell division. A single nucleotide mutation in theCHEK2 gene directed leads to the formation of an abnormal and non-functional CHEK2 protein (Ramanujan et al., 2010). This results in the loss of the ability to regulate cell division and also accumulation of DNA damage (Naga Anusha, 2011).

PALB2 or "partner and localizer of BRCA2" has been identified with 10 mutations in people with familial forms of breast cancer (Naga Anusha, 2011). It functions to translate a protein called partner and localizer of BRCA2 which interacts with a protein generated from the BRCA2 gene (Naga Anusha, 2011). Monoallelic PALB2 truncating mutations that occur in one copy of the gene results an aberrant short version of the PALB2 protein (Guenard et al., 2010). This leads to a failure of DNA repair .Therefore, cells continue to proliferate uncontrollably with 2-fold increase in breast cancer risk (Naga Anusha, 2011).

Finally, RAD50 known as "RAD50 homolog (S. cerevisiae)" binding to MRE11A and NBN, functions in repairing damaged DNA (Naga Anusha, 2011). RAD50 mutations able to direct the generation of an aberrantly small, non-functional form of the RAD50 protein (Naga Anusha, 2011). RAD50 protein works together with several proteins such as BRCA1 and BRCA2 and the BRCA2 protein regulates the activity of the RAD50 protein by translocating it to sites of DNA damage in the nucleus (Mi et al., 2011). A number of alterations in the RAD50 gene have been correlated to an increased risk of developing breast cancer (Thacker, 2005).

Other genes mutated in breast cancer instead regulate cell adhesion and cell proliferation.

Another gene associated with breast cancer is CDH1 that functions to direct synthesis of cadherin or E-cadherin in epithelial cells (Pinheiro et al., 2012). Cadherins are a group of proteins involved in cell adhesion. Thus, inherited mutations in the CDH1

gene highly correlates with enhanced risk of developing breast cancer that initiates in the milk-producing glands (lobular breast cancer) (Naga Anusha, 2011). The incidence of lobular carcinoma of the breast accounts for 10-15% of breast cancer with distinguished histology characterised a diffuse growth pattern as a consequences of damaging cell cohesiveness (Sawyer et al., 2014). E-cadherin-mediated adhesion is important for sustaining normal epithelium, as its dysfunction is closely associated to malignancy (Sarrio et al., 2004). CDH1 is classified as tumour suppressor and its loss revealed invasion of tumours. The existence of a single nucleotide polymorphism (SNP) positioned at the -160 site of CDH1 promoter has been defined as influencing E-CD transcription *in vitro* (Hozyasz et al., 2014). Mutations of PTEN/MMAC1/TEP that functions as tumour suppressor gene in several types of sporadic tumours as well as breast cancers have also been reported. The PTEN gene encodes a protein phosphatase able to dephosphorylate t membrane phosphatidylinositals phosphorylated by PI3K (Naga Anusha, 2011). Loss of PTEN function results in enhanced phosphorylation by PI3K signalling cascade wich leads to increased survival and breast carcinogenesis (Lu et al., 1999).

TP53- "Tumour Protein 53" has been identified to contribute to breast cancer with 50% of all tumours holding p53 mutations (Teresi and Waite, 2008). It functions to regulate the cell cycle and acts as a tumour suppressor. The most familiar deregulation in primary human cancer is a greater expression of the nuclear phosphoprotein p53 (Rivlin et al., 2011). The mechanism involved is a result of point mutation occurring in a conserved area of the p53 gene which then encodes for a mutant, more stable protein (Davidoff et al., 1991). Several studies revealed that p53 is responsible for inhibition of apoptosis protein survivin. Hence, the mutation in p53 drives the overexpression of survivin that prevents apoptosis and contributes to tumour progression (Vegran et al., 2007).

"Androgen Receptor" encoded by the AR gene also has been identified to contribute to the development of breast cancer. This receptor is responsible for the regulation of testosterone (Naga Anusha, 2011). The length of a segment in the AR gene (CAG) in DNA which is repeated several times is believed to be correlated with breast cancer

development. Moreover, mutations occurred in the androgen receptor (AR) gene have been associated to male breast cancer (Jasmine and Yogeshwer, 2008).

"DIRAS family, GTP-binding RAS-like 3" or DIRAS3 is classified as a RAS gene family which regulates cell growth and maturation (Ramanujan et al., 2005). However, the DIRAS3 protein in RAS family represses the growth of cells (Naga Anusha, 2011). It is severely downregulated in breast cancer, therefore contributing to cancer growth (Naga Anusha, 2011).

Faults or mistakes during the replication process results in several copies of a gene on a chromosome (gene amplification) , which might underlie the development and growth of tumour cells depending on which gene is amplified (Kingsley et al., 2009). In breast cancer, amplification of the ERBB2 (HER2) or "v-erb-b2 erythroblastic leukaemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog" has been identified in 25% of cases, with the extra copies of this gene causing too much of the ErbB2 receptor protein to be made in the cell (over expression)(Naga Anusha, 2011). The elevated ErbB2 protein signals drive the cells to proliferate and divide uncontrollably, thus driving the progression of cancerous tumours. Overexpression of ErbB2 is correlated with aggressive breast tumours that are prone to metastasis (Naga Anusha, 2011).

1.8 Cancer epigenetics

The word "epigenetics" defines the study of mitotically or meiotically hereditary modifications in gene expression devoid of alteration of the DNA sequence (Rodriguez-Paredes and Esteller, 2011). Epigenetic modifications include DNA methylation, histone modifications and RNA-mediated silencing. Alteration of any these epigenetic mechanisms can lead to incorrect gene expression, affecting cancer progression and other 'epigenetic diseases' (Egger et al., 2004, Feinberg et al., 2006). Epigenetic events can inactivate the expression of tumour suppressor genes, activate that of oncogenes, and/or alter imprinting patterns without genomic instability (Choong and Tsafnat, 2012). Epigenetic regulation of gene expression consist of DNA

methylation at CpG islands of gene promoters and chromatin remodelling, histone modification, and silencing by non-coding RNA (e.g miRNA and long non coding RNA) (Handy et al., 2011).

Methylation of DNA is a type of covalent modification in which a methyl group is inserted into a cytosine in the genome (Figure 1.8). It occurs at the 5' position of the cytosine ring within CpG dinucleotides and it is maintained by DNA methyltranferase (DNMT) enzymes (Meng et al., 2015). Modified expressions of DNMTs partially explains the irregular patterns of genomic methylation seen in cancer cells (Herceg and Hainaut, 2007). Methylation is crucial for silencing of genes on the inactive X-chromosome, on imprinted genes, and repetitive DNA. In a normal genome approximately 70-80% of all CpGs are methylated. Nevertheless, CpGs islands (CpG-rich regions usually obtained in the promoters of gene) are normally unmethylated. Lack of methylation permits genes to be active and expressed to regulate physiological processes (Choong and Tsafnat, 2012).

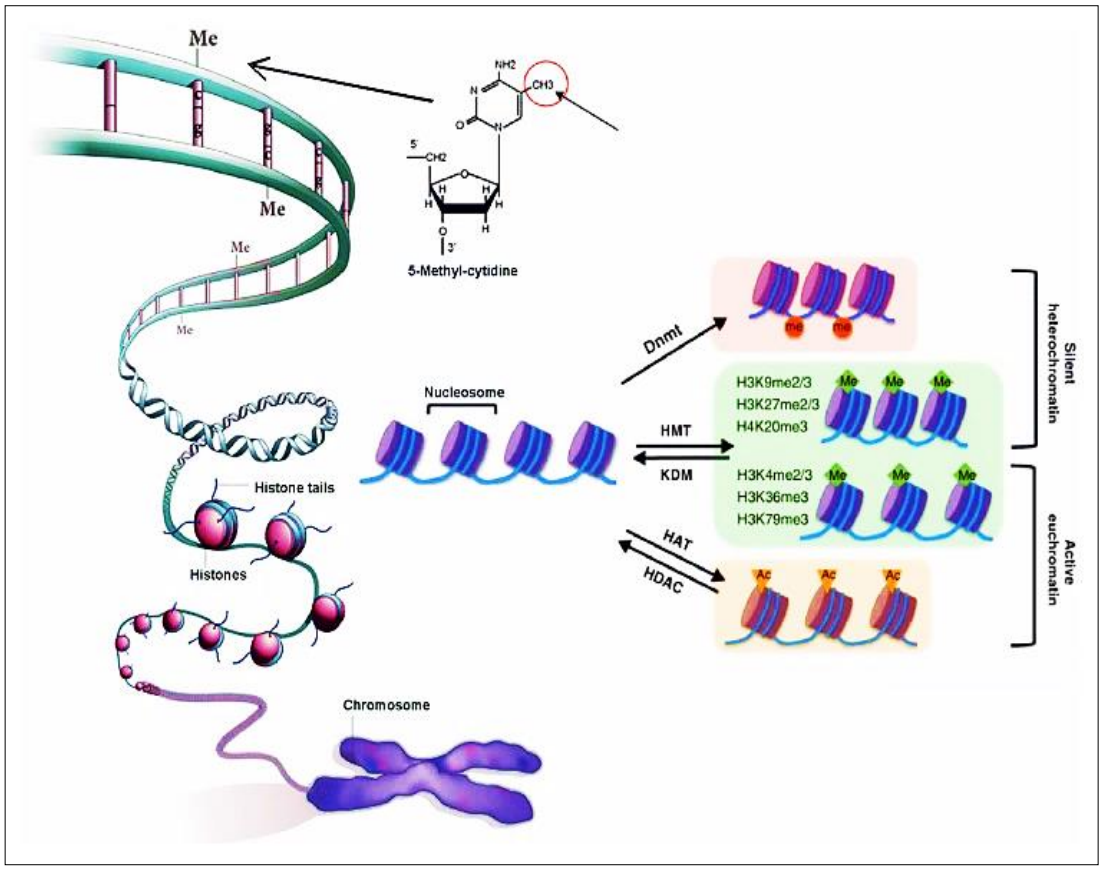


Figure 1.8. Role of epigenetic modifications for chromatin. Figure from (Maleszewska and Kaminska, 2013).

In cancer cells, two forms of DNA methylation alterations take place: loss in methylation at repetitive sequences (global hypomethylation) and gene promoter related hypermethylation (CpG island-specific) (Herceg and Hainaut, 2007). Several types of cancer such as stomach, kidney, colon, pancreas, liver, uterus, lung and cervix have been found associated to the extensive range of hypomethylation. Three possible mechanisms have been suggested to link hypomethylation to progression of cancer, including enhanced genomic instability, reactivation of transposable elements and loss of imprinting which possibly account for the activation of several proto-oncogenes (Hirst and Marra, 2009, Rodriguez-Paredes and Esteller, 2011).

In contrast, de novo hypermethylation in CpG islands of tumour suppressor genes and microRNA (miRNA) genes leads to deactivation of tumour suppressor genes that occurs via silencing of promoter regions (Sharma et al., 2010). During cancer progression, formation of de novo aberrant methylation of CpG islands has been

defined as one of the precipitating events, as it is connected with silencing of tumour suppressor genes and molecules related to differentiation (Kalari and Pfeifer, 2010). The occurrence of breast cancer is associated to DNA hypermethylation directing epigenetic silencing of tumour suppressor genes and further disrupting genomic stability (Jones and Baylin, 2007, Stearns et al., 2007).

Beside DNA methylation, alterations in histone modifications also play an important function in cancer. Histones are proteins that are found in cell nuclei and function to pack and order the genomic DNA into the structural units of nucleosomes (Figure 1.4). Post-translational modifications of histones that occur via acetylation, methylation, phosphorylation, ubiquitylation, sumoylation and rybosilation affect the activity of transcription of surrounding DNA (Semi et al., 2013). Hence, numerous important roles in cellular processes such as gene transcription, DNA repair, recombination and DNA replication are related to histone modification (Herceg and Hainaut, 2007). Most of the research done on histone modifications focuses on histone acetylation or deacetylation, with more studies currently published on methylation and demethylation (Huang et al., 2011). Several histone tails are identified to be acetylated including H3K9, H3K14, H3K18, H4K5, H4K8 and H4K12 Dokmanovic and Marks, 2005) as well as single-site modifications, H4K16ac (Marks et al., 2004). Histone methylation occurs instead on lysine residues, characterised as mono, di or tri-methylated. It involves H3K4, H3K9, H3K27, H3K36, H3K79, and H4K20, with methylation of histone H3 lysine 4 (H3K4me), H3K36, or H3K79 related to active transcription, while methylation of H3K9, H3K27, or H4K20 connected to gene silencing (Dokmanovic and Marks, 2005, Jenuwein and Allis, 2001).

The relevant enzymes involved in histone modification are histone acetyltransferases (HATs), histone methyltransferases (HMTs), histone deacetylase (HDACs) and histone demetylases (HDMs) (Sharma et al., 2010). In human malignancies, there is evidence of the involvement of histone acetylation and protein complexes (HATs) (Herceg and Hainaut, 2007). HDACs, responsible for loss of acetylation, are normally seen overexpressed in different types of cancer. Cancer cells also present extensive modifications in the patterns of histone methylation. Studies conducted by (Nguyen

et al., 2002) and (Valk-Lingbeek et al., 2004) revealed the modifications of H3K9 and H3K27 methylation that are associated with abnormal gene silencing in numerous types of cancers. Lysine specific-demethylases (LSD1) that work in concert with HMTs to maintain total histone methylation patterns is also involved in cancer development (Sharma et al., 2010).

A number of HDAC inhibitors have been developed to modify chromatin structure, and thus induce re-expression of aberrant silenced genes to stop cancer growth and induced apoptosis (Khan and La Thangue, 2008, Keen et al., 2003). For cancer treatment, several HDAC inhibitors are used in the clinic and are still under phase I/II trials to be applied for the treatment of breast cancer patient. They include Vorinostat (SAHA), romidepsin (FK228), MS-275 (entinostat) and LBH-589 (panobinostat) (Pathiraja et al., 2010, Huang et al., 2011). A number of inhibitors that have been established for targeting histone methyltransferases and demethylases are presented in Table 1.2.

Table 1.2. Descriptions of several histone methyltransferase and demethylase inhibitors.

Inhibitor	Major targeted enzymes	Changed histone marks	References
Chaetocin1	Suv39h1, G9a	H3K9	(Greiner et al., 2005)
DZNep	EZh2	H3K27	(Tan et al., 2007)
Novobiocin	SMYD3	H3K4	(Luo et al., 2010)
Trancylpromine, clorgyline, pargyline	LSD1, LSD2	H3K4, H3K9	(Lim et al., 2010, Lee et al., 2006, Metzger et al., 2005)
Polyamine analogs	LSD1	H3K4	(Huang et al., 2007, Huang et al., 2009)
N-oxalylglycine	JMJD2A, JMJD2C	H3K9, H3K36	(Chen et al., 2007)

1.8.1 Breast cancer epigenetics

In breast cancer, aberrant histone modifications together with DNA hypermethylation are related to epigenetic silencing of tumour suppressor genes and genomic instability (Sadikovic et al., 2008). During development of breast cancer, estrogens have been identified as the main hormones involved. Two key isoforms of intracellular estrogen receptors (i.e ERα and ERβ) are involved in regulating activities of estrogens which is controlled by transcription of ER (Abba et al., 2005). Moreover, response to hormonal therapy with ER antagonists is determined by ER expression. Many ER-positive tumours lose ER expression as breast cancer progresses due to epigenetic silencing (Vo and Millis, 2012).

In ductal carcinoma in situ (DCIS) and stage I breast tumours, more than 100 CpG islands have been identified with aberrant methylation. It has been observed an increased methylation of the CpG islands of the tumour suppressor p16INK4a (Lustberg and Ramaswamy, 2009). In addition, a corresponding increase in cyclin-D1 was seen in intraductal proliferative lesions of the breast, with highest levels in DCIS (Liu et al., 2008). Increased promoter hypermethylation have been described including increased hypermethylation of the promoter of the tumour suppressor RUNX3 in DCIS, as well as invasive ductal carcinomas (Subramaniam et al., 2009). A higher frequency of RUNX3 hypermethylation is seen with estrogen and progesterone expression (Suzuki et al., 2005). NES1 is another tumour suppressor that is hypermethylated in DCIS and may serve as a predictive marker for invasive carcinoma at the time of surgery (Yunes et al., 2003).

Promoter hypermethylation of BRCA1 and resulting gene inactivation occurs in a minority of sporadic cancers but is more common with loss of heterozygosity in certain tumor subtypes such as medullary and mucinous carcinomas (Esteller et al., 2000). BRCA2 promoter methylation has also been shown in 4% of sporadic ovarian cancers (Dhillon et al., 2004). Conflicting data are reported instead regarding promoter methylation of BRCA2 in breast cancer (Collins et al., 1997). The more predominant way of epigenetic regulation of BRCA2 is via formation of repressor complexes and chromatin remodelling (Leu et al., 2004). The Tudor domain 'Royal Family', epigenetic repressor family of chromatin remodelling factors has benn

demonstrated to interact with BRCA2 in sporadic cancers (Lapidus et al., 1996). Hereditary breast and ovarian cancers have variable penetrance even in family members with identical mutations (Ford et al., 1998). Modifications of risk by single nucleotide polymorphisms, as well as by epigenetic alterations, including hypermethylation of the promoter CpG island have been described (Kontorovich et al., 2009).

A study by (Kontorovich et al., 2009) demonstrated BRCA1 promoter hyper methylation present at a low frequency (5%) in BRCA1 and BRCA2 carriers with and without breast cancer as well in healthy controls in an Israeli Jewish study population. The findings obtained in this study further confirmed and suggest that BRCA promoter methylation is not a definitive risk modifier in hereditary breast cancers and a more complex epigenetic cascade may yet need to be elucidated (Lustberg and Ramaswamy, 2009).

For BRCA1, methylation is detected in sporadic breast cancers. It is possible that BRCA1 methylation is found in infrequent subtypes of basal-like origin (Turner et al., 2007). Even though breast cancer could be classified into distinctive histological sub classifications, no studies reported differences in DNA methylation patterns among ductal and lobular breast cancers (Fackler et al., 2003; Bae et al., 2004). Distinctive epigenetic profiles can be recognised when dividing breast tumours into categories reflected by hormone receptor status (Feng et al., 2007; Widschwendter et al., 2004) and candidate genes scrutinised are usually less methylated in ER-negative tumours.

Epigenetic and genomic changes that impact the X chromosome stability in BRCA1 and basal-like tumours has been investigated (Vincent-Salomon et al., 2007). The role of X inactive-specific transcript RNA coating, or XIST RNA, in BRCA1 tumours and its contribution to epigenetic instability have been debated (Lustberg and Ramaswamy, 2009). Earlier work on small numbers of BRCA1 tumours showed decreased XIST in these tumours, although a more recent study showed that XIST RNA coating of the unaffected X chromosome is not affected by the BRCA1 mutation (Vincent-Salomon et al., 2007).

Methylation of tumour-suppressor gene RARb is also found in breast cancer and in situ lesions from lobular and ductal cancers. Methylation of the other tumour suppressor genes such as RASSF1A is also found in breast cancer (Dammann et al., 2000, 2001). This is considered an initial epigenetic event in DCIS and LCIS (Lehmann et al., 2002; Fackler et al., 2003). Additionally, inhibitors of cell growth, migration and invasion such as HIN1 are normally silenced by DNA methylation in breast cancer (Krop et al., 2005, 2001).

However, several genes are hypo methylated in primary breast tumours including endonucleases FEN1, the N-acetyltransferase NAT1 (Kim et al., 2008), cadherin CDH3 (Paredes et al., 2005). Genes that are hypomethylated in breast cancer cell lines are not always found hypomethylated in primary tumours, such as the metastasis gene PLAU (Pakneshan et al., 2004) breast cancer-specific gene 1 (BCSG1)(Gupta et al., 2003) and ARH1 (Yuan et al., 2003). With the latest high resolution assessment of DNA hypomethylation in breast cancer, many hypomethylated regions with approximately 1500 areas hypomethylated in a cancer-specific type have been recognised (Shann et al., 2008).

Mutations in the TP53 gene that have been associated to variable methylation patterns in breast cancer where tumours with TP53 mutations are hypomethylated (Feng et al., 2007; Toyota et al., 2000). As mutations in TP53 and loss of ER expression are strongly connected to the basal-like expression subtype it is anticipated that tumours with a basal-like phenotype are hypomethylated in a cancer specific type. The relationship between these epigenetic modifications on well-established prognostic factors including tumour size, lymph node status, distant metastasis, grade, hormone receptor status, and TP53 status and breast tumour expression subgroups is starting to be established (Jovanovic et al., 2010).

The tumour microenvironment might also endure epigenetic modifications that regulate the conversion of in situ to aggressive breast cancer. Myoepithelial cells and stromal fibroblasts undergo epigenetic alterations in early breast cancer

carcinogenesis (Lustberg and Ramaswamy, 2009). Increasing evidence suggests that signals from the stromal microenvironment, including the breast cancer-associated fibroblasts, may serve as triggers for the initiation of epigenetic modifications which promote early disease progression (Lin et al., 2008). Distinctive epigenetic variations are present in the stromal cells of breast cancers. Promoter hypermethylation of the cystatin-M (CST6) tumour suppressor due to aberrant AKT activation is one of the epigenetic changes that occur when breast epithelial cells are co-cultured with cancer fibroblasts (Lin et al., 2008, Lustberg and Ramaswamy, 2009). Additional studies are ongoing to investigate other triggers of epigenetic silencing in the tumour microenvironment.

As for histone modification changes, elevated relative levels of global histone acetylation and methylation were connected to good prognosis and identified in nearly all luminal-like breast tumours (93%) (Elsheikh et al., 2009). On the other end, basal carcinomas and HER-2-positive tumours show moderate to low levels of lysine acetylation (H3K9ac, H3K18ac, and H4K12ac), lysine (H3K4me2 and H4K20me3), and arginine methylation (H4K3me2) (Chervona and Costa, 2012). By using clustering analysis, three groups of histone modifications presenting distinct patterns in breast cancer, have distinctive associations to known prognostic factors and clinical outcome (Elsheikh et al., 2009).

A study by (Chervona and Costa, 2012) also reported a strongly significant association between total histone modifications status, tumour biomarker phenotype, and clinical results after assessment of a well-defined series of human breast carcinomas. This analysis discovered a loss of H4K16ac in most breast cancer cases, indicating that this alteration could signify a primary indication of breast cancer. Furthermore, the global level of histone modifications in breast cancer seems to demonstrate gene specific histone modifications. Chromatin-immunoprecipitation (ChIP) analysis of breast cancer tissues has shown that centromeric satellites (SAT2) level were enriched with H3K9me3, a mark of transcriptional repression, and with H4K20me3, a mark that involves in gene silencing and indexing of pericentric heterochromatin (Leszinski et al., 2012).

1.9 Interplay between genetics and epigenetics in cancer

The development of most cancer types is generally initiated by deregulation of gene expression due to genetic mutations and epigenetic alterations (Choi and Lee, 2013). The interaction between these two mechanism is becoming more evident as it has been realised that mutations of a number of epigenetic modifier genes are highly connected to human cancers (Rodriguez-Paredes and Esteller, 2011). Currently, it is largely acknowledged that human cancer cells encompass global epigenetic aberrations and that epigenetic modifications play a fundamental role in tumour initiation and progression (Baylin and Jones, 2011, Sandoval and Esteller, 2012, Sharma et al., 2010). Moreover, modifications occurring in epigenetic mechanisms are able to affect genetic mutations and on the other hand genetic mutations in epigenetic regulators direct change of the epigenome (You and Jones, 2012).

The mechanisms involved in genetic changes comprise mutation of tumour suppressors and/or oncogenes affecting either a loss or gain of function and abnormal expression (Choi and Lee, 2013). However, epigenetic alterations can involve different epigenetic variations such as DNA methylation, histone variants and modifications, and nucleosome remodelling, also small non-coding regulatory RNAs (Sharma et al., 2010). Each stage in cancer development involves irreversible genetic modifications and reversible epigenetic alterations. Both mechanisms are intertwined and take advantage of each other during cancer development. They are also complementary mechanisms that are susceptible to external factors, including carcinogens exposure, environmental conditions, diet and lifestyle (Figure 1.9) (Herceg and Hainaut, 2007). Any change at the level of transcription as a consequence of genetic and/or epigenetic mutation will be further preserved via mitotic division. The genetic mutations will change the DNA nucleotide sequence whereas epigenetic mutations will disturb its 3-D organization and its chemical susceptibility to protein interactions (Choong and Tsafnat, 2012).

The abnormal gene expression and genomic instability in cancer can occur via mutation of epigenetic modifiers (e.g DNMT1, DNMT3A, DNMT3B, MBD1/2, TET1, MLL1/2/3, EZH2) responsible for aberrant DNA methylation, histone modifications and nucleosome positioning (Jin et al., 2011). Mutations of DNA methyltransferases (DNMTs) generally characterise a loss of function and have an important role in the progression of the disease (Jin and Robertson, 2013). Besides hypermethylation of tumour suppressor genes and hypomethylation of oncogenes, the spontaneous hydrolytic deaminations of 5-methylcytosine (5mC) to thymine instead of uracil can cause T:G mismatches difficult to repair, thus leading to mutations and single nucleotide polymorphisms or variant (SNP or SNVs) which found at methylated CpG sites. Global alteration in histone modification that are regulated by histone acetyltransferases (HATs), histone methyltransferases (HMTs), histone deacetylases (HDACs) and histone demethylases (HDMs) also influence aberrant genes expression and subsequently contribute to tumour development (You and Jones, 2012). Additionally, mutations in miRNAs are able to disrupt the recognition of binding targets and subsequently affect oncogene activation and/or tumour suppressor repression (You and Jones, 2012).

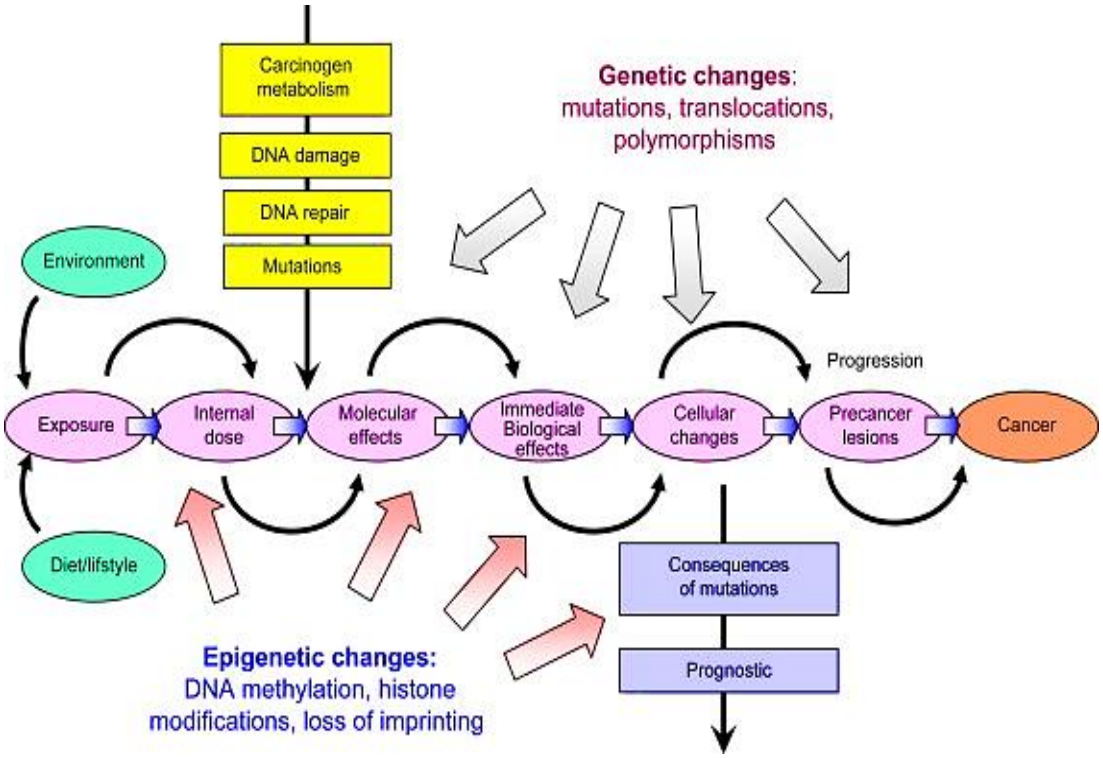


Figure 1.9. Combination of genetic and epigenetic alterations leading to malignant changes. Figure from (Herceg and Hainaut, 2007).

With the progresses of high-throughput whole genome and exome sequencing, the complex view of cancer genomes has been revealed, thus allowing a comprehensive overview of the molecular basis of breast cancer. The latest studies have incorporated extensive genomic data that redefine our thinking about the various types of breast cancers, by considering classification and treatment (Eroles et al., 2012). Tumours can be scrutinised simultaneously for copy number alterations (CNA), epigenetic modifications, miRNA and mRNA expression (Cava et al., 2015).

Several established cancer genes implicated in breast cancer progression have been identified. They include BRCA1, RB1, TP53, PTEN, AKT1, CDH1, GATA3, and PIK3CA, which are recognised to have somatic mutations (Byler et al., 2014). These genes have a function in apoptosis, cell-cycle control, and transcription regulation. Also, several genes involved in signal transduction hold somatic mutations such as s APC, ARID1A, ARID2, ASXL1, BAP1, KRAS, MAP2K4, MLL2, MLL3, NF1, SETD2, SF3B1, SMAD4, and STK11 (Zamanian-Azodi et al., 2015). Additional group of genes that were found mutated containing ARID1B, CASP8, MAP3K13, MAP2K13, NCOR1, SMARCD1, and CDKN1B (Byler et al., 2014). The extent of genetic mutations proved the intricacy and multiplicity of tumour classification based on genetic subtypes. CNAs will result in affected cells having an abnormal number of copies of one or additional parts of the DNA.

A study done by Curtis et al. (2012) reported the deletions in the PPP2R2A, MTAP, and MAP2K4 genes and CNAs in almost 40% of genes, which could result of either cis- or trans-acting CNAs. Cancer subtypes without CNAs occurrence had a better prognosis compared to those holding several CNAs. In addition, mutation of TP53, PIK3CA, and GATA3 have more than 10% occurrence of all breast cancer cases when analysing primary breast cancers by genomic DNA copy number, DNA methylation, exon sequencing, mRNA arrays, microRNA sequencing, and reverse-phase protein arrays (The Cancer Genome Atlas, 2012). Besides, the difference among the

molecular subtypes luminal A, luminal B, basal-like, and HER2- enriched can be observed by not only by mutation occurrence but also by the type of mutation (Byler et al., 2014). These include levels of copy number changes, including focal amplification of regions containing PIK3CA, EGFR, FOXA1 and HER2 genes, as well as focal deletions of regions comprising MLL2, PTEN, RB1, and MAP2K4 genes, associated with various mRNA subtypes (Byler et al., 2014).

At the epigenetic level, studies on miRNA expression have revealed miRNA signatures specific for the specific molecular subtypes of breast cancer (Byler et al., 2014). Other wide-ranging studies has recognised particular miRNAs with aberrant expression in breast cancer, further validating a variation in miRNA expression patterns comparing healthy breast tissue and breast cancer cells. miR-12b, miR-145, miR-21, and miR-155, were found to be down-regulated in several breast cancer types (Iorio et al., 2005). During cancer progression, (Volinia et al., 2006) identified miRNAs with significantly different expression in ductal carcinoma in situ (DCIS) as compared to invasive breast carcinoma. During the development from normal tissue to DCIS, three types of miRNAs such as let-7d, miR-210, miR-221 were found to be down-regulated, whereas afterwards turn to up-regulated during from DCIS to invasive carcinoma (Volinia et al., 2012). Interpreting the targets of these miRNAs allowed the relative function in carcinogenesis with the case of miR-210 which acts to target BRCA1 and E-cadherin, among other proteins (Byler et al., 2014).

In addition to miRNA, other epigenetic alterations have also been recognised being related with breast cancer. A study conducted by Elsheikh et al. (2009) proposed the modifications in histone acetylation and methylation patterns could reflect the onset of breast cancer. Changes from moderate-to-low levels of lysine acetylation and lysine and arginine methylation were associated to carcinoma subtypes with low prognosis such as basal carcinomas and HER2-positive subtypes (Elsheikh et al., 2009). Also, alterations in DNA methylation might be used as an indication of more aggressive metastatic breast cancer cells, compared to less metastatic cells as proved in a work done by (Rodenhiser et al., 2008). The results obtained revealed hyper-

methylation and hypo-methylation of genes that possibly implicated in carcinogenesis and also in metastasis (Byler et al., 2014).

The reversible nature of in epigenetic abnormalities, as compared to genetic alterations which are irreversible, has raised an emergent interest in cancer epigenetics (Meeran et al., 2010). The reversible nature of epigenetic alterations also has directed to the development of the strategy of epigenetic reprogramming that involves processes leading to the erasure of epigenetic modifications found in cancer cells (Sharma et al., 2010). Epigenetic drugs such as DNA methylation inhibitors and HDAC inhibitors are currently under clinical evaluation for treating breast cancer, either alone or as adjuvant therapies (Thurn et al., 2011, Connolly and Stearns, 2012).

1.10 Erasing the epigenetic landscape: cellular reprogramming

The principle of nuclear reprogramming implies the generation of one cell type from another distinct cell type. This process involves the re-instruction of the mechanisms that regulate the gene expression profile of a cell (Agarwal, 2006). The first experimental evidence of reprogramming was observed in frogs by transplantation of viable cell nuclei into an enucleated egg (Briggs and King, 1952). Afterwards, various techniques of reprogramming been developed to reprogram cells to a pluripotent state. They include somatic cell nuclear transfer (SCNT), cell fusion, cell extracts as well as induction of pluripotency mediated by defined factors (iPS cell) (Figure 1.10).

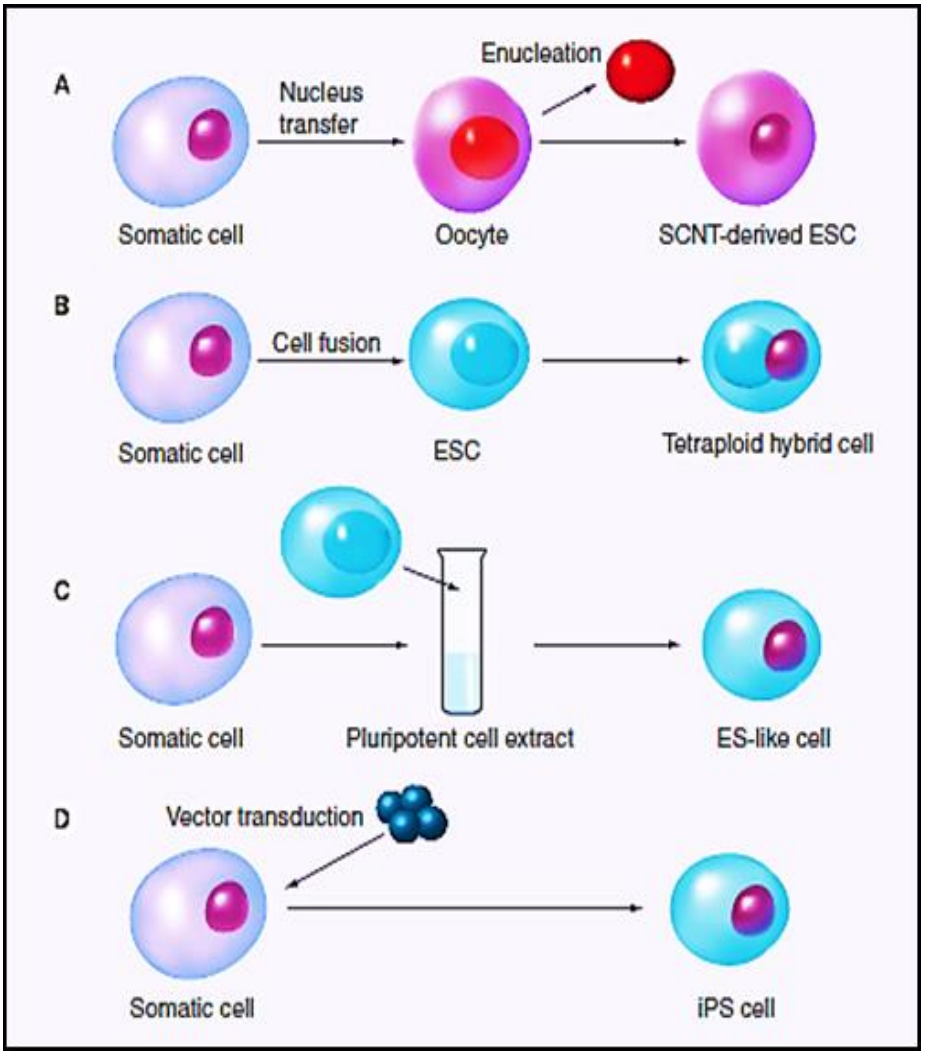


Figure 1.10. Different approaches used in reprogramming (A) Somatic cell nuclear transfer (SCNT) (B) Cell fusion (C) Cell extract (D) Induced Pluripotent Stem Cells (iPSC). Figure from (Han and Sidhu, 2011). ESC= embryonic stem cell.

1.10.1 Somatic Cell Nuclear Transfer

This approach involves the transplantation of a nucleus into an enucleated oocyte resulting in the generation of an entire individual. The nuclear transfer process is also known as reproductive cloning since it can produce a clone of the original somatic cell (Yamanaka and Blau, 2010). As a substitute, therapeutic cloning can be pursued when embryonic stem (ES) cells are isolated from the cloned blastocysts. Two major advances in this field have been achieved with the earliest evidence by Briggs and King (1952) revealing that SCNT could be used to clone frogs. The next main finding was seen in the production of a cloned normal adult sheep (Dolly). In this case, the

nuclei of cultured mammary glands form adult sheep were transplanted into enucleated sheep eggs (Gurdon and Melton, 2008).

Many studies revealed the use of SCNT-based reproductive cloning of bovine, mouse and pig species and the cytoplasm of oocytes from sheep, cow and rabbit is proficient of reprogramming somatic cells from other species in the production of interspecies-cloned embryos (Armstrong et al., 2006). Therapeutic cloning is believed to be the most relevant and ethically acceptable form of SCNT for humans with the capability of generating stem cells from a patient with a disease. Thus, synergistic application with gene therapy could be used for treatment that act via autologous cellular transplantation (Armstrong et al., 2006).

Several technical hurdles and restrictions limit this approach for human cell therapy. Besides the constraint on resources as the method require large amount of oocytes, human SCNT embryos at the blastocyst stage are also very difficult to attain (Sumer et al., 2011). Moreover, faulty reprogramming causes fatality of most clones promptly after implantation or birth of clones with severe defects (Jaenisch and Young, 2008). However, studies conducted by Brambrink et al. (2006) and Wakayama et al. (2006) demonstrated that there is no molecular or biological difference between ES cells derived from inseminated embryos or else through nuclear transfer (Jaenisch and Young, 2008).

1.10.2 Cell Fusion

This process requires hybridisation of a somatic cell with a pluripotent cell to generate a tetraploid cell hybrid. In hybrids, the features of the less-differentiated cells overrides the phenotype of the more-differentiated cell (Sumer et al., 2011). The earliest study revealed that the fusion of pluripotent teratocarcinoma cells with primary thymocytes produced pluripotent hybrids which shared same characteristics with the parental embryonal carcinoma (EC) cells, together with the possibility to develop tumours (Miller and Ruddle, 1976). The domination of pluripotent cells upon differentiated cells was then observed in cell hybrids generated among somatic cells

and murine embryonic germ (EG) and ES cells. This reprogramming ability is also preserved in human ES cells (Hochedlinger and Jaenisch, 2006).

Nevertheless, the elimination of ES cell derived DNA is needed in order to able to use the reprogrammed cells as autologous pluripotent cells. This is due to the ES-somatic cells tetraploids hybrids generated showing incompetency to contribute to chimeras (Matsumura et al., 2007). The effective reprogramming of a somatic cell in a heterokaryon before nuclear fusion followed by consequent enucleation of the ES nucleus has been proposed (Rodolfa, 2008). The other method comprises the deletion of the particular ES cell derived chromosomes that important for self-recognition in the ES-somatic cell hybrid produced a hybrid aneuploidy cell holding an immune resembled to the somatic cell (Sumer et al., 2011).

1.10.3 Induced Pluripotent Stem Cells (iPSC)

A significant discovery has been described by Takahashi and Yamanaka who have identified factors that are fundamental to the conservation of pluripotency of ES cells and in the reprogramming process (Jaenisch and Young, 2008). The identified transcription factors are Oct3/4, Sox2, Klf4 and c-Myc which potentially to reprogram mouse somatic fibroblast cells to a pluripotent state (Sumer et al., 2011). The combination of the same four factors or the mix of Oct4, Sox2, Nanog and Lin28 have been used for reprogramming human somatic cells as well as monkey, rat and pig cells (Semi et al., 2013). However, limited use of this technology in a clinical setting may occur due to the variable reprogramming generated by using viruses and random integration of selected transgenes into the host genome (Sumer et al., 2011).

As a consequence, numerous approaches towards avoiding this issue have been undertaken such as generation of iPS cells by using several different viral constructs, and/or minimizing viral incorporation via replacing combinations of essential reprogramming factors with chemical compounds or by using identified populations of somatic cells endogenously expressing one of more of the crucial pluripotency factors (Han and Yoon, 2011). Expression of the pluripotency factors from non-

integrating viruses, transposons that can be excised producing effective reprogramming, transfection of episomal plasmids, distribution of reprogramming proteins, delivery of microRNA or mRNA can be used (Sumer et al., 2011). It has been reported that The iPSC possess an 'epigenetic memory' from the somatic cell from which they are derived (Vaskova et al., 2013). As a consequence some cell types are more efficient in reprogramming than others, depending on their epigenetic makeup.

Indeed, blood-derived iPSC are more proficient than fibroblast-derived iPSC at differentiation to hematopoietic lineages (Huang and Wu, 2012). The epigenetic variations of iPSC initiated from distinct lineages might be eradicated when the iPSC are derived in the presence of chromatin modifying compounds or after several continuous passaging. The identification of possible factors participating during the reprogramming process is continuously researched with the purpose of directly generate lineage-specific reprogramming from somatic cells, without comprehensive reprogramming to a pluripotent state (Sumer et al., 2011).

Together with improvement of iPSC production techniques, some approaches using iPSC for disease treatment were also established. The most significant approach for clinical applications of iPSC relates to the combination of iPSC technology and targeting editing of the iPSC genome. This combination allows the clinical application of iPSC, particularly for patients with genetic disorders (Van Pham, 2015).

1.10.4 Cell and oocyte extracts

Another approach in reprogramming requires the treatment of differentiated cells or nuclei with cell extracts prepared from oocytes, or extracted cell from mammalian embryonal carcinoma and ES cells. Importantly, the extracts are available for purification of protein complexes contributed in reprogramming (Hochedlinger and Jaenisch, 2006). The process is started with the permeabilisation of differentiated cells which can be achieved by using chemicals such as streptolysin-O (SLO) or digitonin. Then, the permeabilised cells are exposed to the cell extracts and this allows the cells to be reprogrammed to an embryonic state (Sumer et al., 2011).

A study conducted by Hakelien et al. (2002) proved the earliest reprogramming effect of mammalian cell extracts in human embryonic kidney cells. Subsequently, research showed that reprogramming with cell extracts comprises ATP-dependent chromatin remodelling (Collas and Hakelien, 2003). Studies on HEK293T cells and immortalized NIH/3T3T cells mouse fibroblasts showed that cells attained the properties of pluripotent stem cells while introduced into extracts from a pluripotent human carcinoma cell line (NCCIT). Increased expression of several pluripotency genes and decreased expression of somatic genes, such as lamin A, were identified up to 4 weeks subsequent of exposure (Taranger et al., 2005). Notably, phenotypic alterations in the reprogrammed cells were the result of epigenetic changes mediated by chromatin remodelling factors, histone acetylation, expression of particular genes as well as protein synthesis (Alberio et al., 2006).

Oocytes are highly differentiated and unique cells that are capable to reprogram nuclei for the development of a totipotent embryo (Miyamoto et al., 2009, Fengxia et al., 2014). In normal development, the reprogramming of a differentiated cell state to a primary undifferentiated condition is stringently restrained once it has been initiated. However, a study conducted by Miyamoto et al. (2009); (2011), revealed that differentiated nuclei possibly reverted to an undifferentiated embryonic phase after nuclear transfer into enucleated eggs or oocytes. The availability in large quantities of extract, extremely large of size and easy provision of amphibian oocytes together with reprogramming capacities parallel to those of mammalian oocytes make them of particular interest (Miyamoto et al., 2009, Alberio et al., 2005, Bui et al., 2008). The versatility of amphibian oocytes has been demonstrated in several fundamental experimental systems such as determination of cell cycle mechanisms, cell metabolism, cell signalling and cell characteristics (Hardwick and Philpott, 2015). In addition, oocytes extracts are particularly interesting as they are able to provide homogenous signalling, and they allow manipulation of their composition by immunodepletion of particular maternal proteins, or addition of proteins or inhibitors (Crane and Ruderman, 2006).

In an experiment that utilised *Xenopus* oocyte extracts, sperm nuclei demonstrated the alteration in morphology and biochemistry together with progression of cell cycle in early embryonic development. This system served as a system for determination of MAP kinase's (Peng, 1991) function and linker histones (Ohsumi et al., 1993) during development. Similar approaches also allowed the assessment of endogenous targets of drug compounds potentially affecting cell cycle kinetics (Rosania et al., 1999). The extracts have been also important to characterise various stages of cell cycle and further investigate the alterations of protein stability in cell cycle transitions (Vosper et al., 2009, McDowell et al., 2014).

Previous studies also demonstrated that amphibian oocyte extracts retain the capability to alter DNA methylation and histone marks, and to remodel somatic cell chromatin (Allegrucci et al., 201). In addition, a study reported by Alberio et al. (2005) revealed the ability of oocyte and egg components to remodel nuclear lamina of somatic cells. Molecules contained in oocyte extracts decreased the total levels of repressive histone mark (H3K9me3), HP1alpha and DNA methylation of somatic cells, and also increased levels of active histone mark (H3K9ac). The analysis of particular genome sequences revealed that somatic cells treated with oocyte extracts had demethylation of LINE-1 repeats but Major Satellite repeats and the imprinted gene H19 was not unaffected. The increased demethylation of the Oct-4 promoter was also observed together with kinetics of induction of transcription factors Oct-4 and Nanog expression from *mouse embryonic fibroblasts* (*MEFs*) nuclei (Bian et al., 2009).

Several molecules were identified responsible for driving nuclear remodelling or dedifferentiation in oocyte reprogramming. They include ISWI (Kikyo et al., 2000), FRGY2a/b (Gonda et al., 2003), BRG1 (Hansis et al., 2004), Histone B4 (Jullien et al., 2010) and NPM (Tamada et al., 2006). BRG1 and nucleoplasmin, which are present in *Xenopus* extracts, have been proved to induce reprogramming of mammalian cells, further demonstrating that several reprogramming activities are very well-conserved among species (Tamada et al., 2006, Hansis et al., 2004).

Salamander oocytes extracts are also used for reprogramming. Urodele amphibians (salamanders) deviated from a urodele-like ancestor more than last 250 million years (Roy et al., 2000, Voss and Shaffer, 2000, Anderson et al., 2008). Embryological evidence indicates that the amphibian ancestor of amniotes was urodele-like and published work reveals that important molecular mechanisms governing development are conserved from urodeles to mammals (Dixon et al., 2010). The unique characteristic of urodele amphibians (salamanders) is the only vertebrates which able to regenerate complete organ systems makes them an essential model for embryological, physiological, biomedical and natural history research (Voss *et al*., 2001). Among urodeles, the Mexican axolotl (*Ambystoma mexicanum*) is of particular interest as this species has extensive history as research model (Voss et al., 2001).

A study conducted by Bian et al. (2009) demonstrated that axolotl oocyte extracts are valuable system for investigating epigenetic remodelling of somatic cells to a stem cell configuration, and to identify exact mechanism of oocyte by nuclear reprogramming. Another study done by Allegrucci et al. (2011) revealed the capability of axolotl oocytes in tumour reversion by epigenetic reprogramming. The incubation of permeabilised breast cancer cells with the oocyte extract for 6 hours resulted in reprogramming cancer cell chromatin, reversing epigenetic silencing and triggering expression of silenced tumour suppressor genes. Furthermore, long term suppression of tumour growth was also showed in a mouse xenograft model, suggesting a stable reversal of tumourigenicity. A similar outcome was obtained utilising bovine oocyte extracts to reprogram H460 human lung cancer cells. The silenced tumour suppressor genes were reactivated without increase level of pluripotency-related genes (Wang et al., 2013b, Wang et al., 2013a, Wang et al., 2014).

1.11 Cancer reprogramming and tumour reversion

Cancer reprogramming is the process by which a tumour cell reverses its malignant phenotype (Ge et al., 2011). It can also be defined at the molecular level by the generation of substitute pathways that enable prevailing genetic modifications in

cancer resulting in suppression of tumorigenicity (Tuynder et al., 2002). A timeline of tumour reversion research is summarized in (Figure 1.11). The earliest cancer reprogramming work was started by Askanazy at the beginning of the 20th century. Tumour cells have then been proved to differentiate into benign tissues by Pierce and colleagues (Pierce and Dixon, 1959, Kleinsmith and Pierce, 1964).

After that, the initiation of the differentiation of an epithelial tumour in newts and the regression of aggressive tumours and metastasis has been reported by Seilern-Aspang and Kratochwil (1962). The reversal of Ras-transformed cells has been successfully induced by Noda and colleagues in 1989 (Telerman and Amson, 2009). Subsequently, tumour reversion by down-regulation of the translationally controlled tumour protein (TCTP/*tpt1*) has been described by Telerman and Amson in 2009 (Bommer et al., 2009). Altogether, these seminal studies of tumour reversion and the involved pathways highlight a potential for novel discoveries in cancer treatment (Ge et al., 2011).

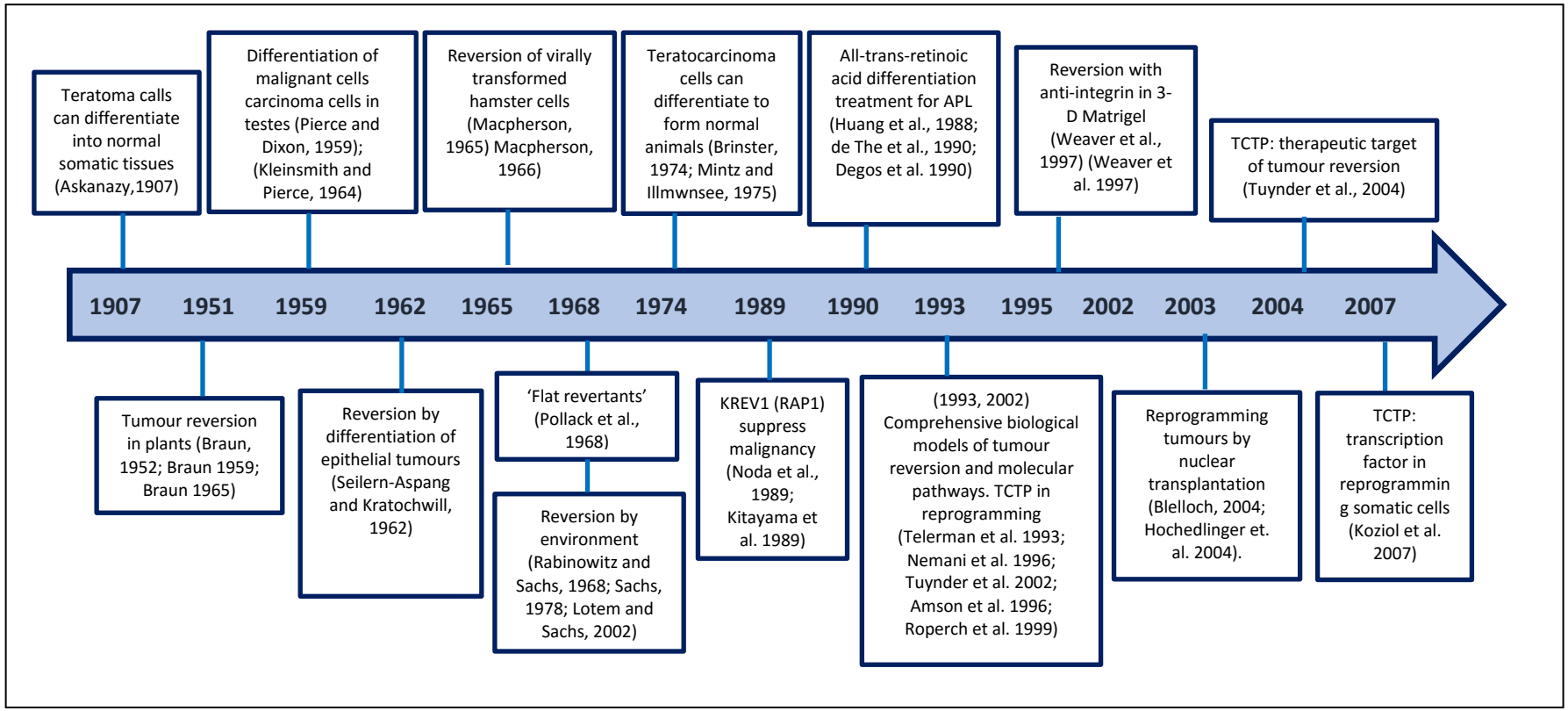


Figure 1.11. Timeline of tumour reversion. Figure from (Telerman and Amson, 2009)

Cell reprogramming that occurs in normal cells differs from reprogramming-like events identified in cancer cells. Firstly, it has been proposed that the genetic and epigenetic pathways involved in cancer cells have a different characteristic from those in normal cells. Second, normal cells generally undergo their differentiation program through alterations of reversible epigenetic modification, whereas in cancer cells it also involves irreversible genetic alterations.

Third, the establishment of four well-established transcription factors such as Oct4, Sox2, Klf4, and cMyc into cancer cells induces the expression of genes associated to an undifferentiated state and shows multi-potentiality by differentiation into three germ layers, even though they do not develop a teratoma, a mark of pluripotency. This suggests that the induced pluripotency might be partly limited by genetic mutations in cancer cells or that the cancer program originated in normal multi-potent cells may be different from the pluripotency program in normal cells (Nishikawa et al., 2012). In addition to identified genetic mutations that have been identified in most of human cancers, deregulated epigenetic modifications have been recognised to contribute significantly to the onset and progression of this diseases (Sadikovic et al., 2008).

All reprogramming approaches explored include modifications of the epigenome (Freberg et al., 2007). Several important events that occur during cell reprogramming include reversion to an immature, non-programmed (undifferentiated) state, and cells normally attain multi differentiation characteristics (pluripotency) resulting the loss and restoration of significant epigenetic marks, especially DNA methylation and histone acetylation. Different approaches that are used in cancer cell reprogramming, including nuclear transfer, embryonic stem cell (ESC) extract, microRNA, iPSC, embryonic microenvironment as well as oocyte extracts, are summarized in Table 1.3 (Nishikawa et al., 2012, Dewi et al., 2012, Yilmazer et al., 2015).

The summarised findings suggest that variations in epigenetic modifications contribute to a crucial role in the initial stages of cellular reprogramming and

incomplete epigenetic repression of key genes may be an obstacle in the transition to the acquisition of pluripotency. Nevertheless, because epigenetic alterations participate in the aggressive behaviour of cancer cells, reprogramming of these modifications for therapeutically intentions might be important (Nishikawa et al., 2012).

Table 1.3. Summary of different approaches to cancer cell reprogramming. Adapted from (Dewi et al., 2012) and (Nishikawa et al., 2012).

Methods	Type of Cancer	Characterisation	Reference
1. i. Nuclear Transfer	Medulloblastoma (primary culture, mouse, Ptc1 heterozygous)	- Cloned blastocyst able to assist postimplantation progression, as the embryo looked normal and demonstrated estensive differentiation, even though not feasible once achieved E8.5.	(Li et al., 2003)
ii. Nuclear Transfer (two-step cloning)	Melanoma (RAS+/Ink4a/Arf$^{-/-}$	- NT ES cells can form teratoma as well as produce chimeras. Insertions into tetraploid blastocycts result in normal embryos viable until E9.5.	(Hochedlinger et al., 2004)
iii. Nuclear Transfer	Embryonal carcinoma	- Nuclei from EC capable to regulate preimplantation progression, producing normal blastocyst, higher proficiency of generating an ES cell line compared to the differentiated cells, even though the level of differentiation depended on the cell line used.	(Blelloch et al., 2004)
2. Embryonic stem cells (ESC) extract	Teratocarcinoma NCCIT cells	- Extract of undifferentiated carcinoma cells possibly elicit differentiation plasticity or else more developmentally constrained cell types. 293T and NIH3T3 cells were reprogrammed by extracts of undifferentiated NCCIT cells to acquire characteristics of pluripotency.	(Taranger et al., 2005)
3. H-1 parvovirus	Human leukemia and breast cancer cell lines (K562, U937, T47D,MDA-MB231, BT20 & 184B5)	- (tpt1/TCTP) had a robust differential expression after reprogramming, decreased in the reversion of U937- & SIAH-1 elevated expressing cells.	(Tuynder et al., 2002)
	Colon, lung and melanoma cancer cell lines	- TCTP was recognised as a target and drugs were identified that reduce its expression and destroy tumor cells.	(Tuynder et al., 2004)
	Multiple Myeloma (MM) Cells	- Isolated revertant cells demonstrated a significantly reduced malignancy, *in vitro* and *in vivo*.	(Ge et al., 2011)

4. iPSC i. Defined transcription factor (OKM)	Melanoma (R545)	- The chimera has shown tumor-free at 5-month old.	(Utikal et al., 2009)
ii. Defined transcription factor (OSKM)	Gastrointestinal cancer, DLD-1 entirely categorised	- After differentiation, iPS cells responded to chemotherapy, decreased invasion and tumorigenicity, elevated expression of p16 and p53.	(Miyoshi et al., 2010)
iii. Defined transcription factor (OSKM)	KBM7 cells derived from blast crisis stage chronic myeloid leukemia (CML)	- Imatinib resistance characteristic, loss of BCR-ABL- dependent signaling.	(Postovit et al., 2008)
iv. Defined transcription factor (OSLN)	A549 lung cancer	- Tumorigenic characteristics were improved as transplanted into NOD/SCID mouse, more aggressive also infiltrative, teratoma (-)	(Mathieu et al., 2011)
v. Defined transcription factor	Non-small cell lung cancer (NSCLC) cell lines	- Reprogramming proficiency was greater as compared to normal primary lung fibroblasts. DNA methylation changes describe the downstream transcriptional effects, which probably affected the decreased tumorigenicity in iPCs. Hence suggesting that reprogramming reverses the abnormally dysregulated genes in NSCLC both epigenetically and transcriptionally.	(Mahalingam et al., 2012)
vi. Defined Essential reprogramming factors (OCT3/4, SOX2, Klf4, Nanog, c-Myc	Human prostate cancer cell lines (DU145 and PC3) and primary prostate cancer tissue	- Prostate tumor cells expressing pluripotent stem cell transcription factors are particularly tumorigenic.	(Bae et al., 2010)
	Osteosarcoma cell lines		(Mahalingam et al., 2012)

vii. Defined transcription factor (OSKM) viii.Defined transcription factor (OSKM) ix. Defined transcription factor (OSKM)	Saos-2, HOSMG63,(osteosarcoma); SW872 (liposarcoma) H3581, H460 (lung)	- Osteosarcoma cell lines, Saos-2, MG-63, G-292 and U-2 OS revealed variable responses on reprogramming and extended culture effect of the reprogrammed cells. - Sarcoma cells were directly reprogrammed and allowed to recapitulate terminal differentiation with loss of tumorigenic characteristics. - Producing stimulated pluripotent somatic cells (iPS) follow Elite model in which a distinctive sub-category of cells has been selected from a heterogeneous primary cancer cell population. - By eliminating mutations of the parental cell lines, reprogramming reduces tumorigenicity.	(Choong et al., 2014) (Zhang et al., 2013) (Lai et al., 2013)
5. Embryonic Microenvironment	Metastatic melanoma, breast cancer	- Invasion and tumor growth were reduced. Down-regulation of Nodal signaling via Lefty activation.	(Postovit et al., 2008)
6. microRNA (miR-302a,b,c,d)	Melanoma (Colo), prostate cancer cell line (PC3)	- Decreased capacity of migration, decreased expression of cell cycle related genes, DNA methylation mediator, MeCP2 MECP1-p66, also several oncogenes related to melanoma.	(Lin et al., 2008)
7. Oocyte extract	Breast cancer (MCF7 and HCC1945 cell lines)	- Directly alter cancer cell chromatin and induce re-expression of silenced tumor suppressor genes. The long term effects of this treatment are revealed in tumor growth reduction *in vivo*.	(Allegrucci et al., 2011)

1.12 Aims and Objectives

Published result from our group (Allegrucci et al., 2011) revealed the ability of amphibian oocyte extracts to epigenetically reprogram breast cancer cells. These results were used as starting point of this study. Hence, this project aimed to understand molecular basis and mechanisms of the tumour reversed phenotype observed in the previously described mouse xenografts that showed a suppression of tumour growth (or tumour reversion) after treatment of cancer cells with axolotl oocyte extracts.

The objectives of this study were:

i. To analyse the gene expression profile of reverted tumours by microarray analysis (Chapter 3).
ii. To investigate the role of different cell signalling pathways that might be involved in the inhibition of malignant growth elicited by specific extract molecules (Chapter 4).
iii. To determine the contribution of epigenetic reprogramming to tumour reversion (Chapter 5).
iv. To discuss the strengths and limitations of the study and the potential of the oocyte extract treatment as a tool to explore the molecular mechanisms involved in carcinogenesis and to develop novel targeted treatments for breast cancer (Chapter 6).

2.0 Materials and methods

2.1 Microarray gene-expression profiling

The overall steps involved for microarray analysis beginning from RNA extraction from the tumour xenograft, followed with validation and subsequently hybridised for microarray analysis are summarised in Figure 2.1. Total of 8 RNA samples in triplicate were sent to Source BioScience for hybrisation to the Illumina Human HT-12 v4 Expression BeadChip array. The quality and quantity of extracted RNA have been measured using a NanoDrop spectrophotometer (NanoDrop; LSN-X-013) and (Agilent 2100 bioanalyser: RNA 6000pico assay II). Samples were then normalised to 100ng and were processed according to the Illumina TotalPrep™-96 RNA Amplification Kit (LSN-X-036).

Qualitative and quantitative QCs were performed on the labelled cRNA (LSN-X-013 and LSN-X-024) and 1.5µg of labelled cRNA was hybridised to a HumanHT-12.v4 Beadchip and scanned by the BeadArray Reader (LSN-X-036). The array intensity data was loaded into the Illumina GenomeStudio software v2010.2 (a software package that permits visualisation and normalisation of the data). All analyses reported were used the 'quantile' normalisation method with background correction within GenomeStudio (LSN-Bi-In-003). Gene Ontology (GO) analysis and Ingenuity Pathways Analysis (IPA) was applied to fit with the post-analyses of microarray data.

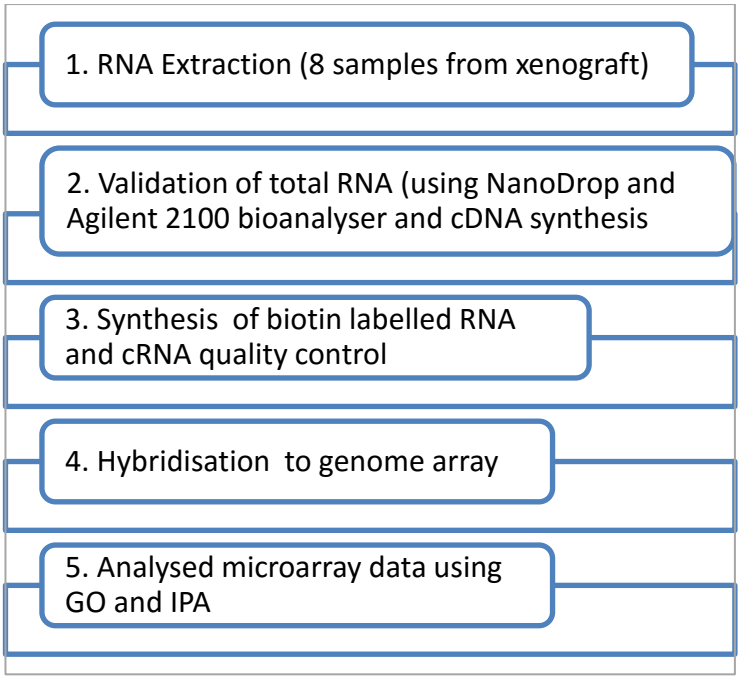


Figure 2.1. Steps involve in the process of analysing gene expression using microarray.

Gene Ontology analysis was conducted using DAVID bioinformatics resources to provides typical batch annotation and gene-GO term enrichment analysis to highlight the most relevant GO-terms connected with a given gene list (Huang da et al., 2009, Huang et al., 2008). Results from functional annotation analysis were considered applying a *p* value cut-off at 0.05, and further implemented as input for ReviGO, a web server that Reduces and Visualizes long lists of Gene Ontology terms and visualized in treemaps that cluster ontologies with high semantic similarity (http://revigo.irb.hr/) (Supek et al., 2011). IPA software provided the extensive curated data-base and tools for interpretation of signalling and metabolic pathways, molecular networks, and biological processes that are most relevance affected in a dataset of interest (http://www.ingenuity.com) (Gokmen-Polar et al., 2014).

2.2 Cell line and culture

2.2.1 Maintenance of cell line

The MCF-7 cell line (ATCC, HTB-22) was grown in RPMI medium 1640 (Life Technologies, Gibco®, 31870-025) containing 10% fetal calf serum (FCS) (Sigma-Aldrich F7524), 2 mM L-glutamine (Life Technologies, 21051040), 1% non-essential amino acids (Life Technologies, Gibco®, 11140035), 1% sodium pyruvate (Life Technologies, Gibco®, 11140039), and 1% penicillin/streptomycin (Life Technologies, Gibco®, 15070063). Culture medium was changed every two days until reach 70-80% confluency. Cells were passaged by incubation with 0.25% trypsin (Life Technologies, Gibco® 25200-072) for 2-3 minutes after washing with sterile PBS (Life Technologies, Gibco® 18912-014). The enzymatically activity of trypsin was deactivated with double volume of complete RPMI medium. The detached cells were then transferred into 15ml centrifuge tube (Greiner, Scientific Laboratory Supplies Ltd, G188271) for centrifugation at 400 x g for 4 minutes and the pellet were re-suspended in 3 ml of media. Cells suspension was divided into new tissue culture flasks at a splitting ratio of 1:5 supplemented with fresh culture medium. The cells were placed in a 37°C humidified incubator with 5% of CO_2.

2.2.2 Freezing of cell line

The trypsinised MCF-7 cells were collected by centrifugation at 400 x g for 4 minutes. The supernatant were aspirated off and resuspended the pellet in freezing medium (FCS + 20% dimethyl sulphoxide) (DMSO) (Fisher Scientific, D/4121/PB08). The suspensions were aseptically aliquot into sterile cryovials (Thermo-Scientific Nunc, 377267) and immediately freeze overnight at -80°C before transfer to the liquid nitrogen for the long term storage.

2.2.3 Thawing of cell line

The cryovials stored in liquid nitrogen were thawed in a 37°C water bath for 1 to 2 minutes. The cells were resuspended in culture medium and plate them in 80cm^2 culture flasks (Thermo Scientific Nunc, 178905) further placed them in the incubator. The media was changed as soon as the cells were attached.

2.3 Cell permeabilisation

The method described by Alberio *et al*. (2005) was used to permeabilise the cells. After trypsinisation, cells were washed in permeabilisation buffer (PB) (170 mM potassium gluconate (Sigma-Aldrich, P1847), 5 mM KCl (Sigma-Aldrich, P9333) 2 mM $MgCl_{2.6}H_2O$ (Fisher BioReagents, BP214-500, 1 mM KH_2PO_4 (Sigma-Aldrich, 30407, 1 mM EGTA (Sigma-Aldrich, E3889-28G), 20 mM Hepes (H3375), pH 7.25, freshly prepared prior to use) and subsequently incubated in 1 µl of digitonin (20mg/ml) (Sigma-Aldrich, D141) for 1.45 minutes on ice. To permeabilise cells, the cells use was 2 x 10^6 cells/ml of cold PB in Sterilin™ 7 ml Polystyrene Bijou Containers (Thermo Scientific-Sterilin 129A, 1696-228743). The permeabilisation reaction was stopped by adding 9 ml of PB containing 2 µl of protease inhibitor cocktail (Sigma-Aldrich, P8340) followed by centrifugation at 400 x g for 4 minutes at room temperature. Finally, the permeabilised cells were counted and assessed by staining with propidium iodide (PI) (Life Technologies, P3566) under fluorescence microscope. The percentage of cell recovery after permeabilisation was measured by counting the viable cells recovered in relative to initial cell numbers.

2.4 Preparation of Oocyte extracts

Axolotl oocyte extracts (AOE) were prepared from mature females according to method of (Alberio et al., 2005) and (Bian et al., 2009). In particular, the ovaries treated with collagenase II (Sigma, C6885) for 2-3 hours at room temperature. In every 15-20 minutes, free oocytes were removed and washed in abundant saline 0.9% NaCl (Fisher Scientific, S/3160/60). After complete digestion, the ovaries were

washed several times in saline. The necrotic eggs (white and swollen) were removed and washed three times in saline. Finally, the oocyte was washed with oocyte extraction buffer (20mM Hepes (Sigma-Aldrich, H3375), 100mM KCl (Sigma-Aldrich, P9333), 5mM $MgCl_2$ (BP214) and subsequently the protease inhibitors (0.1%) were added. The eggs were pack into centrifuge tubes and excess buffer was removed before centrifugation at 10,000g for 10 minutes at 4°C. The cytoplasmic layer (middle layer) formed was later removed and proceed with another centrifugation at 10,000 g for 10 minutes at 4°C to remove the debris. The extract was aliquot and stored at -80°C.

2.5 Treatment of cells in oocyte

Permeabilised cells were added to oocyte extracts (5000 cells/µl extract) supplemented with an energy regenerating system (150 µg/ml creatine phosphokinase (Sigma-Aldrich, C-3755), 60 mM phosphocreatine (Sigma-Aldrich, P7936), 1 mM ATP (Sigma-Aldrich, A6419) and incubated at 17°C for 1-6 hours in AOE. For epigenetic inhibitors experiment, cells were treated with the specific inhibitors during incubation with AOE. After 6 hour reprogramming, cells were pelleted and prepared for protein extraction, whereas for 12 hour reprogramming treated cells were cultured overnight and the recovered for protein extraction.

2..6 Matrigel culture (3D on-top assay)

Preparation of Matrigel (Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix, A1413202) culture was adapted from Lee *et al*. (2007). After incubation of permeabilised cells with AOE at 17°C, the cells were pelleted by centrifugation at ~115g followed by suspension in 250 µl medium. For the 4 wells plate (Thermo Scientific Nunc, 176740) used, 120 µl of matrigel was used for coating and the total amount of volume in each well was 500 µl. 40,000 of cells were used as recommended amount of malignant cells to be used for each wells were in the ranges of 0.175-0.20 x 10^5 cells/cm^2. The cells were then allowed to settle and attach to the Matrigel for about 10-30 minutes at 37°C. Agitation of the plate in the x-y plane at

intervals during incubation helped with preventing cell concentration in the center of the well. Then, the remaining medium (250 µl) was chilled on ice and 10% Matrigel was added to the medium and gently loaded to the plated culture. The culture was maintained for 4 days and the Matrigel-medium mixture was changed every 2 days.

2.7 3D Imaging Cell Proliferation

The monitoring of cell proliferation in 3D culture was employed using Click-iT® EdU Imaging Kits (Invitrogen, C10337). After pre-warm 2x working solution of EdU, half of the medium in matrigel culture were replaced to obtain 10 µM final concentration of EdU. Then, the cells were incubated for 1 hour to allow incorporation of EdU into living cells. By the end of incubation time, the medium was removed and further fixed the cells with 4% formaldehyde in PBS. The cells were then continued for 15 minutes of incubation in room temperature. The fixative solution was removed and cells were washed twice in each well utilising 0.5 mL of 3% BSA in PBS. Afterward, 0.5 mL of 0.5% Triton-X in PBS were added into each well and further incubated at room temperature for 20 minutes.

Before proceeding with the EdU detection step, the 0.5% Triton-X in PBS (permeabilisation buffer) were removed and continued with twice washing steps. To detect the EdU incorporation, 250 µL of Click-iT reaction cocktail (containing 1X Click-iT reaction buffer, $CuSO_4$, Alexa Flour Azide and reaction buffer additive) were used within 15 minutes preparation. Subsequently, the plate was briefly rocked to ensure the reaction cocktail distributed equally. The plate was further incubated for another 30 minutes at room temperature in light protected. Later after removing the cocktail solution, the cells were washed with 0.5 mL of 3% BSA in PBS. Then, to counterstain the DNA, Hoechst 33342 solution was used by incubating the cells for 30 minutes at room temperature in light protected condition. Finally, the cells were washed twice with 1 mL of PBS and further viewed under fluorescence microscope.

2.8 Cell Recovery from Matrigel

The isolation of cells grown in matrigel was adapted from a procedure described by manufacturer Cultrex CellSperse™ (Cultrex, 3450-048-05) with some modification. Medium from matrigel cell cultures were removed before proceeding with one PBS wash. Then, two volumes of CellSperse™ were added for every one volume of matrigel. Cells were then incubated with CellSperse™ for 2 hours at 37°C to assure complete dissolution of the Matrigel. Cells and melted gel was pipetted up and down several times to disperse cell suspension. The mixture was transferred to a sterile centrifuge tube followed by incubation with trypsin (10 to 15 minutes). Dissociation to single cells was achieved by repeated pipetting and several passages through a 23G 11/4 (0.6 mm x 30 mm) needle (BD Microlance™3, 1303). The action of CellSperse™ was stopped by diluting of Ca^{++} and Mg^{++} using 5-10 mM EDTA (Fisher BioReagents®). Finally, the cells were centrifuged and washed several times with PBS prior flowcytometry analysis.

2.9 EdU Staining and cell cycle analysis

For 5'ethylnyl-2'-deoxyuridine (EdU) incorporation analysis, the details method described from Click-iT® EdU Flow Cytometry Assay Kits (Invitrogen, C10418) were used. The cells grown in Matrigel were pulsed with 10 µM of EdU for 1 hour. Then cells were washed with PBS and the pellet dislodged in 1% BSA in PBS. Then, the pellet was dislodged with 100 µL of Click-iT® fixative. The cells were incubated for 15 minutes and washed with 3 mL of 1% BSA in PBS. Subsequently after washing with 1x Click-iT® saponin-based permeabilisation and washing reagent, the cells were preceded for labelling by adding 0.5 mL Click-iT® reaction cocktail. The reaction mixture was incubated for 40 minutes at room temperature and protected from light.

For the cell cycle analysis, after incubation with Click-iT® reaction cocktail, the cells were washed with 1X Click-iT® saponin-based permeabilisation and washing reagent. They were further stained with 1 ml µg/ml 50 µg/ml propidium iodide 0.1 mg/ml RNase A (Thermo Scientific, EN531) in PBS for 40 minutes at 37°C. Cells were analysed by gating on an area vs. width dot plot to exclude debris and doublets, and 10,000

singlet cells were examined with BD FACS CANTO™ II with Alexa Fluor® 488 azide, using 488 nm excitation with a green emission filter.

2.10 Immunohistochemistry

Histology sections (5 µm) were de-waxed and processed for antigen retrieval (citrate buffer pH 6 for 15 minutes in microwave). For BrdU staining, sections were treated with PBS containing 0.25% Triton X-100 and 0.1% goat serum for 10 minutes at room temperature (RT) and blocked with PBS containing 10% goat serum for 1hour at RT. After blocking, sections were incubated with 0.02U/ul DNAse in DNAse buffer (Qiagen) for 10 min at 37 °C and then washed with PBS 3 times for 10 min at RT. Incubation with the mouse anti-BrdU antibody (0.6 µg/ml, Roche 11170376001) in PBS containing 5% goat serum was performed for 1h at 37 °C. The FITC-goat anti-mouse secondary antibody (7.5 µg/ml, Jackson ImmunoResearch Laboratories, 115-095-068) was incubated 1h at RT. For p27 staining, after antigen retrieval slides were blocked with PBS containing 5% BSA and 0.1% Tween 20 for 1 hour at RT, followed by incubation with rabbit p27 antibody overnight at 4 °C (0.1 µg/ml, Cell Signalling Technology, 3686). The secondary antibody goat anti-rabbit Alexa 488 (5 µg/ml, ThermoFisher Scientific, A-11008) was incubated 1 hour at RT. Slides were washed after each antibody incubation 3 times with PBS containing 0.1% Tween 20 and mounted with Vectashield mounting medium containing DAPI (Vector Laboratories). Slides were observed under a Leica DM5000B microscope with the Leica Application Suite software.

2.11 Preparation of protein sample and quantification for cell signalling western blotting

Permeabilised MCF-7 cells were incubated with the AOE at 17°C for 6 hours. The cells were then pelleted either for protein extraction or kept in -80°C until analysis. In general, cells were harvested with RIPA Buffer (Cell Signalling Technology, 98065S) (150 mM NaCl, 50mM Tris.HCl, pH 7.5, 5 mM EGTA, 1% Triton, 0.5% DOC (Sodium desoxicolate), 0.1% SDS, supplemented with Protease Inhibitor Cocktail (P8340) and

Phosphatase Inhibitors cocktail (P0044) for 30 minutes on ice. The harvested cells were then lysed with lysis buffer for 30 minutes on ice and centrifuged at 12,000 x g for 10 minutes at 4°C. The protein content was quantified using a Qubit Protein Assay Kit (Life Technologies, Q3321) following the manufacturer's protocol. To generate a standard curve, 10µL of 3 different pre-diluted BSA (Qubit™ standard) was diluted into 190µL of Qubit™ working solution in three different tubes. For the sample measurement, the sample can be used anywhere between 1 µL and 20 µL with a volume of Qubit™ working solution in the range of 180 µL and 199 µL. The reactions were mixed properly by vortexing 2-3 seconds before proceed with incubation in 15 minutes a room temperature. The measurement was determined using Qubit® 2.0 Fluorometer (Life Technologies) following calibration guidelines of the machine. Upon completion, the standard curve will be generated and displayed on the screen. The concentration of each sample was measured based on standard curve generated in the machine following the below equation:

Concentration of sample = QF value x (200/x)
QF value = the value given by the Qubit® 2.0 Fluorometer
X = the number of microliters of sample added to the assay tube

2.12 Acid Histone Extraction for histone western blotting

The protocol, adapted from Abcam, Inc. was used to extract the histone from MCF-7 cells. The adherent cells were trypsinised, harvested and further washed twice with ice-cold PBS. The cells were resuspended in Triton Extraction Buffer (TEB: PBS containing 0.5% Triton™ X-100 (Fisher BioReagents™) at a cell density of 10^7 cells per ml. Then, the collected cells were lysed on ice for 10 min with gentle stirring before centrifugation at 6,500 x g for 10 minutes at 4°C to spin down the nuclei. The supernatant was removed and after that, the nuclei were washed in half the volume of TEB and centrifuged as before. Pellets were then resuspended in 0.2 N HCl at a density of 4 x 10^7 nuclei per ml. The histones were acid extracted overnight at 4°C. The samples were then centrifuged at 6,500 x g for 10 minutes at 4°C to pellet debris. Finally, the supernatant was removed for protein content determination.

2.13 SDS-PAGE and Western blot analysis

Equal amounts of protein from each sample were separated by gel electrophoresis on 12% SDS-PAGE gels (appendix 1) depending on the objective of experiments. For cell signalling approach, 30 µg proteins were loaded whereby for the histone modification, 2.5 µg of samples were used. To denature the proteins, each sample was mixed at a ratio of 1:1 with 2x SDS gel-loading buffer, which consisted of 0.5 M Tris-HCl pH 6.8, 10% SDS, 0.5% bromophenol blue, glycerol, and 50 µl β-mercaptoethanol (Sigma-Aldrich, M-3148). The mixture was then heated at 100°C in a heating block for 3 minutes. Next, proteins were separated on 4% acrylamide stacking gel by gel electrophoresis at 100 Volts, and on 10% acrylamide resolving gel at 150 Volts (Supplementary Table S4 in Appendix II). The ColorPlus Prestained Protein Ladder, Broad Range (10-230 kDa) (New England Biolabs, P7711S) was used as a molecular weight marker on every gel. The separated proteins were transferred to PVDF transfer membrane (GE Healthcare, RPN303F, 0.45 µm) using a semi-dry transfer blotting machine (Fisher Scientific, FB57793) at 80mAmps for 20 minutes. The 0.45 µm PVDF membrane (GR Healthcare, RPN303F) was pre-wet in methanol (Fisher Scientific, M/4056/17) for 1 to 2 minutes followed by soaking in transfer buffer prior used for this purpose. The membranes were blocked for one hour at a room temperature in blocking solution and incubated overnight at 4°C with the primary antibodies listed in Table 1 and Table 2. Then, the membrane was incubated with a dilution of HRP-conjugated secondary antibody (Table 1 and Table 2) with the ratio 1:10,000 for histone modification and 1:2000 for cell signalling for one hour on a shaker at RT. The signals were detected by chemiluminescence (GE Healthcare ECL Prime Western, 28980926) after 5 minutes of incubation with the solution. Subsequently, the excess ECL solution of the membrane was drained off and wrapped in Saran Wrap before placing in a cassette for film exposure between 5 seconds to 10 minutes (depending on protein expression) using a film developing machine (KONICA MINOLTA: SRX-101A).

Table 2.1. Primary and secondary antibodies used in western blotting for epigenetic modifications.

Primary antibody	Reactivity	Supplier and catalogue no	Dilution	Secondary antibody	Supplier and product code	Dilution
1. H3K9me2	Mouse monoclonal	Abcam (Abcam 1220)	1:2000	ECL Mouse IgG, HRP-linked whole Ab	GE Healthcare Life Sciences	1:10000
2. H3K9me3	Rabbit polyclonal	Active Motif (39765, 39766)	1:1000	ECL Rabbit IgG, HRP-Linked Whole Ab	GE Healthcare Life Sciences	1:10000
3. H3K4me3	Mouse Monoclonal	Abcam (ab12209)	1:1000	ECL Mouse IgG, HRP-linked whole Ab	GE Healthcare Life Sciences	1:10000
2. H3K27me3	Rabbit polyclonal	Active Motif (39155, 39157)	1:1000	ECL Rabbit IgG, HRP-Linked Whole Ab	GE Healthcare Life Sciences	1:10000
3. H3K9Ac	Rabbit polyclonal	Active Motif (39917, 39918)	1:1000	ECL Rabbit IgG, HRP-Linked Whole Ab	GE Healthcare Life Sciences	1:10000
4. H4K20me1	Mouse Monoclonal	Active motif (39727, 39728)	1:1000	ECL Mouse IgG, HRP-linked whole Ab	GE Healthcare Life Sciences	1:10000
5. H4K20me2	Rabbit polyclonal	Active motif (51533)	1:2000	ECL Rabbit IgG, HRP-Linked Whole Ab	GE Healthcare Life Sciences	1:10000
6. H4K20me3	Mouse Monoclonal	Active motif (39671, 39672)	1:1000	ECL Rabbit IgG, HRP-Linked Whole Ab	GE Healthcare Life Sciences	1:10000
7. Histone, H3	Rabbit polyclonal	Active Motif (61277, 61278)	1:10000	ECL Rabbit IgG, HRP-Linked Whole Ab	GE Healthcare Life Sciences	1:20000

Table 2.2. Primary and secondary antibodies used in western blotting for signalling pathways.

Primary antibody	Reactivity	Supplier and product code	Dilution	Secondary antibody	Supplier and product code	Dilution
1. Phospho-p44/42 MAPK (Thr202/Tyr204) (D13.14.4E)	Rabbit monoclonal	Cell Signaling Technology® (4370)	1:2000	Anti-rabbit IgG, HRP-linked antibody (7074)	Cell Signaling Technology® (7074)	1:2000
2. p44/42 MAP Kinase (137F5)	Rabbit monoclonal	Cell Signaling Technology (4695)	1:1000	Anti-rabbit IgG, HRP-linked antibody (7074)	Cell Signaling Technology® (7074)	1:2000
3. Phospho-p38 MAPK(Thr180/Tyr182) (D3F9)	Rabbit monoclonal	Cell Signaling Technology (4511)	1:1000	Anti-rabbit IgG, HRP-linked antibody (7074)	Cell Signaling Technology® (7074)	1:2000
8. P38 MAPK (D13E1)	Rabbit monoclonal	Cell Signaling Technology (4511)	1:1000	Anti-rabbit IgG, HRP-linked antibody (7074)	Cell Signaling Technology® (7074)	1:2000
9. Phospho-SAPK/JNK (Thr183/Tyr185) (81E11)	Rabbit monoclonal	Cell Signaling Technology (4511)	1:1000	Anti-rabbit IgG, HRP-linked antibody (7074)	Cell Signaling Technology® (7074)	1:2000
10. SAPK/JNK (56G8)	Rabbit monoclonal	Cell Signaling Technology (4511)	1:1000	Anti-rabbit IgG, HRP-linked antibody (7074)	Cell Signaling Technology® (7074)	1:2000

2.14 Membrane Stripping and Re-probing

The same membrane could be used to investigate more than one antibody, for instance a protein of interest and a loading control. To remove or strip off the previous antibody, the membrane was incubated in a stripping buffer 0.2 M glycine (Fisher Scientific UK,G/P460/53) pH 2.8 at 50°C for 30 minutes in a shaking water bath. Then, the membrane was incubated in neutralising stripping buffer (0.2 M glycine, pH 7.4) and was further rinsed 3 times with 1x TBST at room temperature on a shaker. Finally, the membrane was blocked again before re-probing with a different antibody.

2.15 RNA extraction

RNA for xenograft tumours was extracted by homogenisation of the tissue with gentleMACS M Tubes (Miltenyi Biotech) followed by NucleoSpin® RNA II (MACHEREY-NAGEL, 740955.50) kit. RNA from cells pellets were extracted using the NucleoSpin® RNA II kit. This procedure was performed as described in the manufacturer's protocol. Briefly, the cells pellets were mixed to 350 µL Buffer RA1 followed by addition 3.5 µL β-mercaptoethanol into the sample. The cell lysate was filtered through NucleoSpin® Filter by centrifugation for 1 minutes at 11,000x g. The NucleoSpin® Filter was discarded and 350 µL 70% ethanol (Fisher Scientific UK, E/066DF/17) was added to the homogenised lysate and mixed the sample homogenously. For RNA binding, the sample was filtered using NucleoSpin® (light blue ring) and centrifuged for 30s at 11,000x g. Then, the column was placed in the new collection tube and 350 µL MDB (Membrane Desalting Buffer) was added and proceeded with centrifugation 11,000x g for 1 min to dry the membrane. Then, 95 µL DNase reaction mixture was applied directly onto the centre of the silica membrane of the column and further incubated at room temperature for 15 minutes. Next, the columns were washed 3 times using Buffer RA2 and RA3 and then dried by further centrifugation. Finally, the RNA was eluted in 40 µL RNase-free H_2O to obtain higher RNA concentration. The concentration of RNA was measured using a NanoDrop spectrophotometer (NanoDrop; ND-8000). The ratio of UV light absorption at 260 nm

and at 280 nm (260/280) indicated good quality of prepared RNA. The acceptable range was 1.8-2.2.

2.16 cDNA preparation

The cDNA synthesis was performed using Superscript™ III Reverse Transcriptase (Invitrogen, 18080-044). 1 µg -5 µg of total RNA dissolved in RNase-free water with total 11 µl and added with 1 µl random hexamers primers (Invitrogen, N8080127) and 1 µl of 10 mM dNTP Mix (Invitrogen, 18427-013). Then, the mixture was heated at 65°C for 5 minutes and incubated on ice for at least 1 minutes. After that, the solution was mixed with 4 µl 5X of First-Strand Buffer, 1 µl 0.1 M DTT and 1 µl RNaseOUT™ Recombinant RNase Inhibitor (Invitrogen, 10777-019) and 1 µl of SuperScript™ Transcriptase (Invitrogen, 18080-044). Samples mixed by pipetting gently up and down and further incubated at 25°C for 5 minutes, 50°C for 30-60 minutes. Finally, the reaction was inactivated by heating at 70°C for 15 minutes. The preamplified samples were diluted down to 300 ng by the addition nuclease-free water and aliquot before kept in -20°C.

2.17 Real Time Polymerase Chain Reaction

The differences in expression of gene interest between control and AOE group were quantified using Taq Man® real time polymerase chain reaction (qPCR), Roche LightCycler® 480. The available assay used comprises of a pair of unlabeled PCR primers and a TaqMan® probe with a FAM™ or VIC® dye label on the 5' end and minor groove binder (MGB) and nonfluorescent quencher (NFQ) on the 3' end (Table 2.3). Starting material of cDNA with the concentration of 300ng/µl was used for each sample. 20 µl each reaction contained 10 µL TaqMan® gene expression assay master mix (Applied Biosystems, 4369016), 1 µL of TaqMan® probe assay, 1 µL of cDNA (300 ng) and nuclease-free water. The cycling protocols set up on a Roche LightCycler 480 were as followed: 1 cycle of enzyme activation (50°C for 2 minutes), 1 cycle of initial denaturation (95°C for 10 minutes), 45 cycles of denaturation (95°°C for 15 seconds)

and annealing (60°°C for 1 min). Three technical replicates were prepared for all samples and controls in each of experiment conducted.

Table 2.3. List of TaqMan® gene expression assays with a FAM™ dye label used.

Genes	Probes
1. CDKN1B	Hs01597588_m1
2. E2F3	Hs00605457_m1
3. E2F5	Hs00231092_m1
4. CDK4	Hs00175935_m1
5. TOB1	Hs03986111_s1
6. BTG2	Hs00198887_m1
7. CCND1	Hs00765553_m1
8. CDKN3	Hs00193192_m1
9. TGFB3	Hs01086000_m1
10. MAPK9	Hs00177102_m1
11. CDKN2B	Hs00793225_m1
12. WEE1	Hs00268721_m1
13. MLL5	Hs01096121_m1
14. RPLP0	Hs99999902_m1

2.17.1 Optimisation of Internal Control and Calibrator Genes

To identify the most stably expressed endogenous control genes, five candidates of commonly used housekeeping genes (18S, Actin, GAPDH, HPRT and RPLPO) were selected. The expressions of all selected genes were compared between control and AOE treatment groups. For statistical validation, the data obtained were analysed using BestKeeper Software version 1 (http://www.genequantification.de/bestkeeper.html).

2.17.2 Quantitative PCR Data Analysis

Relative gene expression presents the data of the gene interest relative to some calibrator or internal control gene. A widely used method to present relative gene expression is the comparative Ct (threshold cycle) or Cp (crossing point-PCR-cycle) method also referred to as the Delta Delta Ct method (ΔΔCt). The Cp value (threshold

cycle during amplification process) were provided and calculated from the Lightcycler 480 SW 1.5 software. This protocol allows an overview of the comparative Cp method for quantitative gene expression studies. Below are the general procedures of calculations applied of this method;

i. Initially, the ΔCp between the target gene and the reference gene was estimated (for the identified sample and calibrator sample)

   $\Delta Cp = Cp_{target} - Cp_{reference\ gene}$

ii. After that, the difference between the ΔCp of the identified sample and the ΔCp of the calibrator was calculated, gave the ΔΔCp value

   $\Delta\Delta Cp = (Cp_{target} - Cp_{reference\ calibrator}) - (Cp_{target} - Cp_{reference\ sample})$

iii. The normalised target in the sample is then equal to $2^{-\Delta\Delta Cp}$ and this value can be used to compare expression levels in samples.

iv. Standard deviation (SD) of ΔCp = $(SD\ target^2 - SD\ reference^2)^{1/2}$

   SD of fold enrichment = ln2 (SD ΔΔCT) $2^{-\Delta\Delta Cp}$

2.18 Statistical Analysis

Data were expresses as means ± SD. Data were considered significantly different when $p<0.05$. All statistical analyses were performed using GraphPad Prism 6. Data from permeabilisation experiments, real time PCR and western blotting were analysed by unpaired t-test. Cell cycle analysis was analysed by two-way ANOVA followed by Bonferroni test. Epigenetic inhibitor experiments were analysed by one way ANOVA followed by Tukey test.

Chapter 3.0 Gene expression profiling of breast cancer cells after treatment with axolotl oocyte extract: Microarray study of tumour xenografts

3.1 Introduction

In a published study by Allegrucci et al. (2011) that revealed the effect of AOE on breast cancer cells *in vivo*, the histopathological analysis of tumour xenografts demonstrated a significant reduction in the AOE-treated tumour size as compared to the untreated group. Hence, this chapter aimed to analyse the gene expression profile of the tumour xenografts to dissect the specific mechanisms involved in the growth inhibition and tumour reversion in response to AOE treatment using Illumina microarray analysis. The objectives of this chapter were:

i. To identify specific gene networks that might be involved in the tumour reverted phenotype
ii. To determine gene-associated signalling pathways
iii. To identify the relationship between the biological functions affected by the AOE treatment

Microarrays have been used extensively to analyse differentially expressed genes of cancer samples compared to normal tissue or following treatment (Tarca et al., 2006). They have also been used to elucidate the molecular mechanisms involved in cancer progression (Dhanasekaran et al., 2001) or response to treatments in *in vivo* models (Toyoda et al., 2014). Besides, the use of microarray to analyse gene expression of xenograft-derived cell lines has been reported by several studies. Zou et al. (2015) revealed the anticancer activity of Docosahexaenoic acid (DHA) on colon cancer cells that was demonstrated in a nude mouse HCT-15 xenograft model. Gene expression profiles were analysed and indicated a reprogramming pattern and transcription factors related with the action of DHA on colon cancer cells. Moreover, a number of genes associated to tumour growth and metastasis were recognised.

Analysis of gene expression using microarray technology has provided important knowledge on disease pathology, development, resistance to treatment as well as response to cellular microenvironments (Tan et al., 2003). Therefore, it has been used to advance early diagnosis and novel therapeutic strategies in cancer management (Macgregor and Squire, 2002). The application of microarray technology in cancer research has also allowed the simultaneous evaluation of all cellular processes by integrating clinical and molecular biological data for improved methods of diagnosis and prognosis (Grant et al., 2004). Cancer originates as a consequence of genetic and epigenetic defects causing an altered expression of oncogenes and tumour suppressor genes that regulate cell proliferation, apoptosis, adhesion and angiogenesis (Papagiannouli and Mechler, 2013). Therefore microarray technology allows an extensive combinatorial analysis of thousands of genes in tumour and healthy cells providing insights into those alterations in gene expression that are related to tumour cellular dysfunction and any associated regulatory pathways (Macgregor and Squire, 2002).

For breast cancer, work done by Bergamaschi et al. (2009) described a multifunctional breast cancer *in vivo* model system whereby hormone-dependent and independent tumour growth, progression and invasion as well as experimental treatments could be investigated. Engrafted primary tumour tissues over several passages in SCID mice have been analysed for morphological and molecular characterization. Gene expression profiling by microarray established that these xenograft models presented the histopathological characteristics of luminal and basal tumours which retained the same molecular characteristics as the tumour of origin. In addition, microarray analysis of an orthotopic model of breast cancer with MDA-MB-435 cells implanted into the mouse mammary fat pad, demonstrated that cultured metastatic explants presented a unique expression profile for tumours that metastasise to lymph node and identified the gene CD73 as a marker of the metastatic process (Lee et al., 2003). More recently, microarray of patient-derived tumour xenografts (PDTX) analysis has provided valuable insight into the mechanisms

of tumour progression in different patients, thus contributing to the development of personalised medicine (Tentler et al., 2012, Mattie et al., 2013).

In microarray technology, thousands of specific single-stranded sequences which are complementary to DNA or RNA sequences function as probes to form complementary hybrids. These are synthesized and spotted onto a glass support that has the same size as a microscope slide (Trevino et al., 2007). Generally, the DNA arrays are classified based on the types of spotted probes. They can either use single stranded oligonucleotides with approximately 22 nucleotides synthesized in situ or cDNA (Russo et al., 2003). A summary of the steps involved in microarray analysis is illustrated in Figure 3.1. Firstly, the extracted mRNA (optionally amplified) is converted to cDNA which is then labelled with fluorescent dyes before analysis. Then, the DNA array is hybridized with the labelled samples by overnight incubation followed by washing to eliminate unspecific hybrids. A confocal or scanner is able to then detect the light produced by exciting the hybridised samples labelled with a fluorescent dye using a laser. Digital images are generated from the excited microarray and further processed using specialized software. The image of each spots is then converted into a numerical reading proportional to the extent of hybridisation (Trevino et al., 2007).

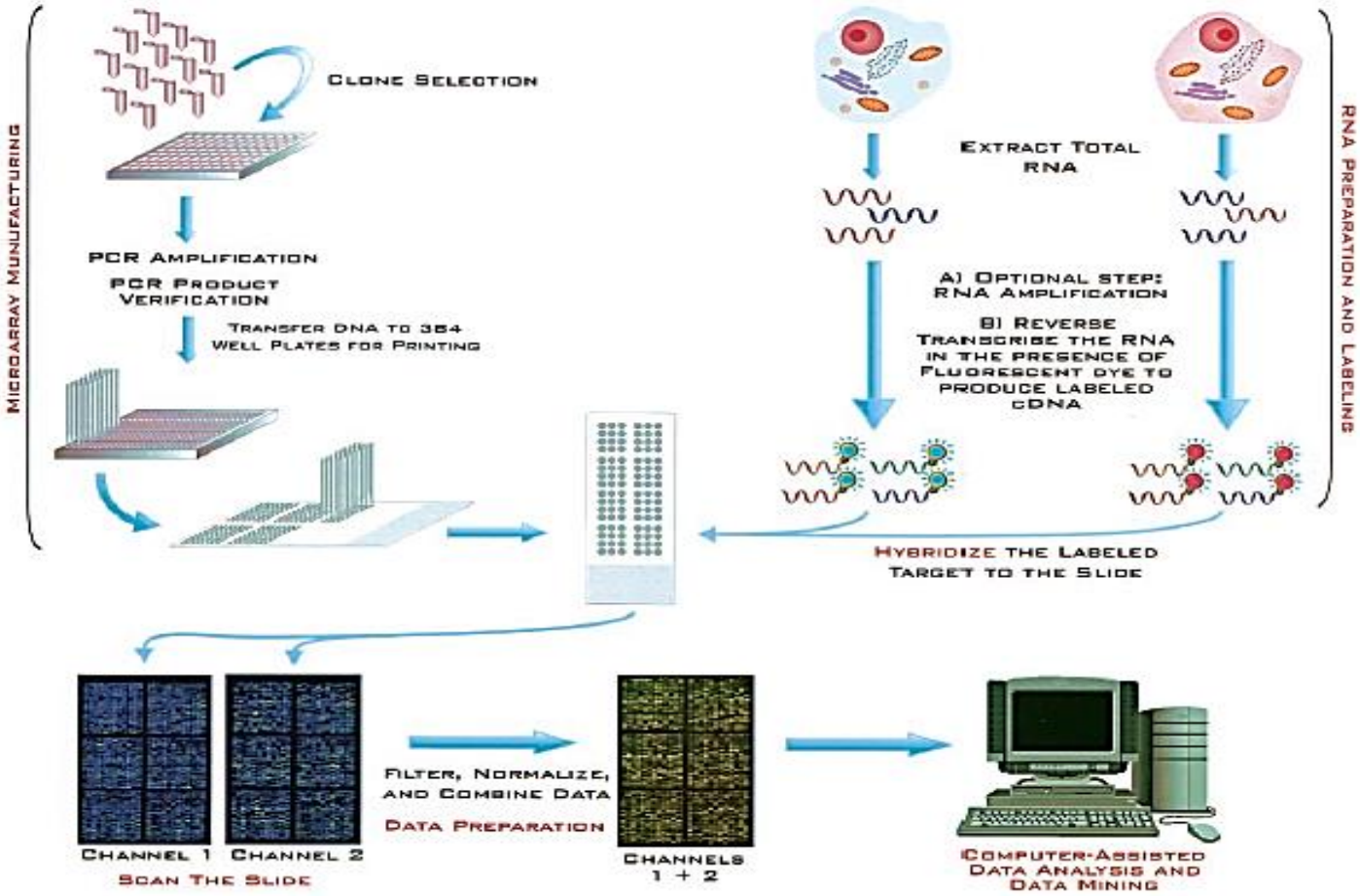


Figure 3.1. Steps involved in microarray experiments. Figure from (Macgregor and Squire, 2002).

The Illumina Whole-Genome gene expression microarray overcomes these limitations by using BeadArray technology that incorporates gene-specific oligonucleotide probes with beads in high density onto the arrays. Quality of data and reproducibility are maintained via great level of hybridisation coverage. The BeadChips contain oligonucleotides immobilised to beads fixed into microwells on top of the array surface. Using this design, the HumanHT-12 v4 Expression BeadChip offers genome-wide transcriptional analysis of highly-identified genes, potential genes candidates, splice variants and non-coding RNAs. Each array contains more than 47,000 probes that are available from National Center for Biotechnology Information Reference Sequence (NCBI) RefSeq Release 38 (November 7, 2009) and additional sources (http://www.illumina.com/documents/products/datasheets/datasheet_gene_exp_analysis.pdf).

Although microarrays have been used in wide-ranging applications, some limitations have been acknowledged. The arrays give an *indirect* measure of relative concentration. The signal level of arrays has a restricted limit of concentration in solution as it becoming saturated at high concentrations, whereas at low concentrations, equilibrium favours no binding. In addition, microarrays may not cover all sequences in the genome, especially splicing variants (Bumgarner, 2013). Finally, DNA arrays can have limited detection ability and only identify sequences that are specifically designed for detection. This is especially relevant for non-coding RNAs for which special arrays need to be designed and used (Bumgarner, 2013).

3.2 Results

3.2.1 Validation of total RNA quality

RNA integrity of the samples was validated using a NanoDrop spectrophotometer (NanoDrop; LSN-X-013) and Agilent 2100 bioanalyser. Only samples that gave readings of NanoDrop spectrophotometer with a ratio of absorbance 260 nm and 280 nm ≥ 1.8 were utilised. Electrophoretic data obtained from the Agilent Bioanalyser 2100 showed a good quality of RNA (Figure 3.2). Intact total RNA was indicated by two strong ribosomal peaks with a 28S/18S ratio close to 2.0. Conversely, RNA degradation was characterised by a reduced proportion of ribosomal bands, additional peaks below the ribosomal bands, reduced overall RNA signal, and decreased overall RNA signal and presence of shorter fragments. The RNA integrity number (RIN) was also provided by the Agilent Bioanalyser software to evaluate the integrity of total RNA samples (Table 3.1). A RIN number of 10 indicate 'perfect' RNA with the RIN number of 5 partially degraded RNA and a RIN of 1 totally degraded RNA. In these experiments, only RNA that gave the RIN > 7.0 was used for further analysis.

Table 3.1. Analysis of RNA integrity using Agilent RNA *pico* labchip

Tube ID	RIN Number	Pass/Fail/Questionable
Untreated 1	8.9	Pass
Untreated 2	7.3	Pass
AOE 1	7.8	Pass
AOE 2	8.6	Pass

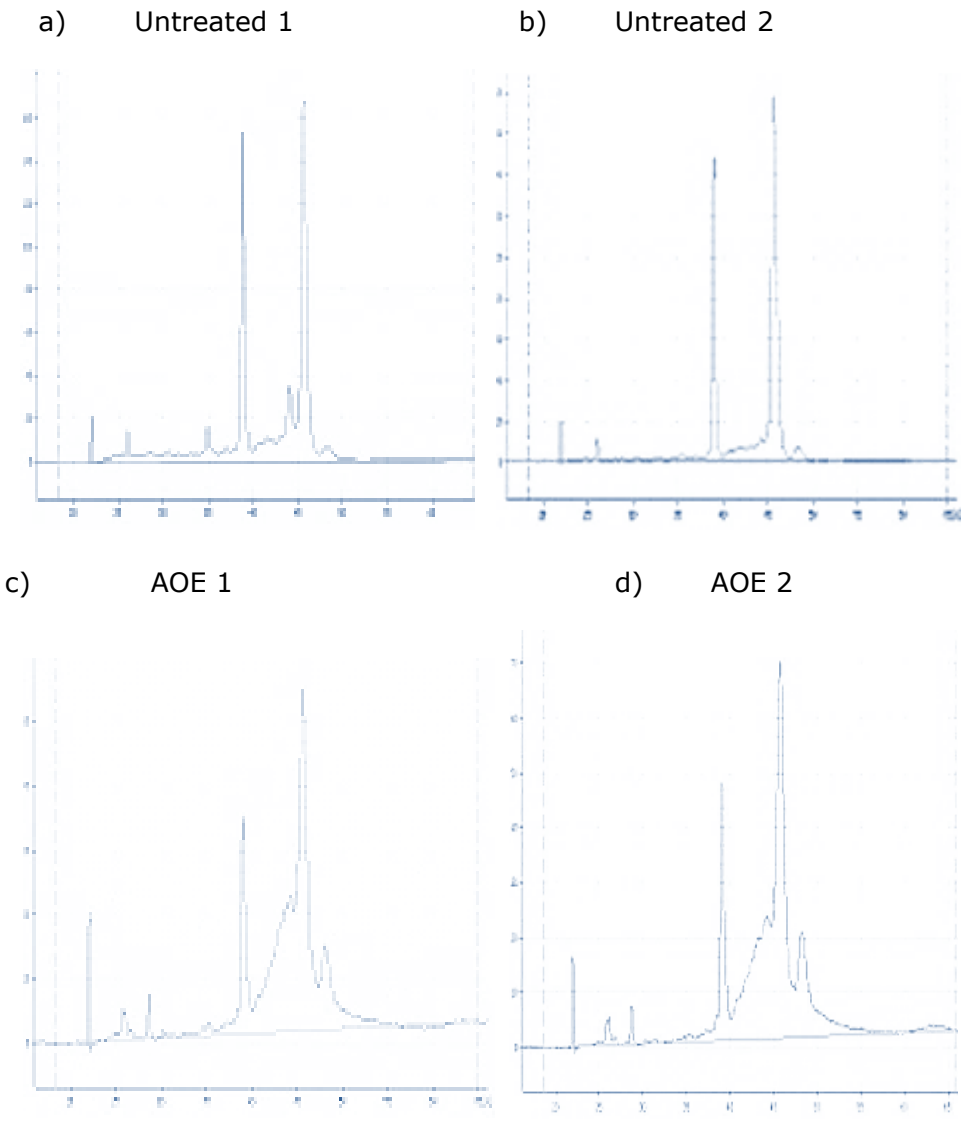


Figure 3.2. Electropherograms of RNA integrity for four samples with two untreated (a, b) and treated xenografts (c, d) analysed on the Agilent Bioanalyzer 2100 using RNA 6000 Nano or Pico Assay. The 18S and 28S peaks were clearly visible indicating intact rRNA respectively with the (18/28S) ratio close to 2.0 and RIN > 7.5.

3.2.2 Gene expression profiling

The differently throughout expressed genes between control and AOE treated tumours were determined by bioinformatics analysis (performed by Prof. Richard Emes, ADAC, University of Nottingham). The analysis revealed 1976 differentially expressed genes in AOE treated tumours, with a cut-off for adjusted *p*-value established at 0.05 and fold change value at ± 2, as shown in the volcano plot in Figure 3.3. Red dots of the plot show genes which are differentially expressed above a 2-fold threshold. These represent nearly 2000 genes with the part of the graph on the left of the zero representing genes which are down-regulated (1254 genes) and the one on the right those gene which are up-regulated (741 genes).

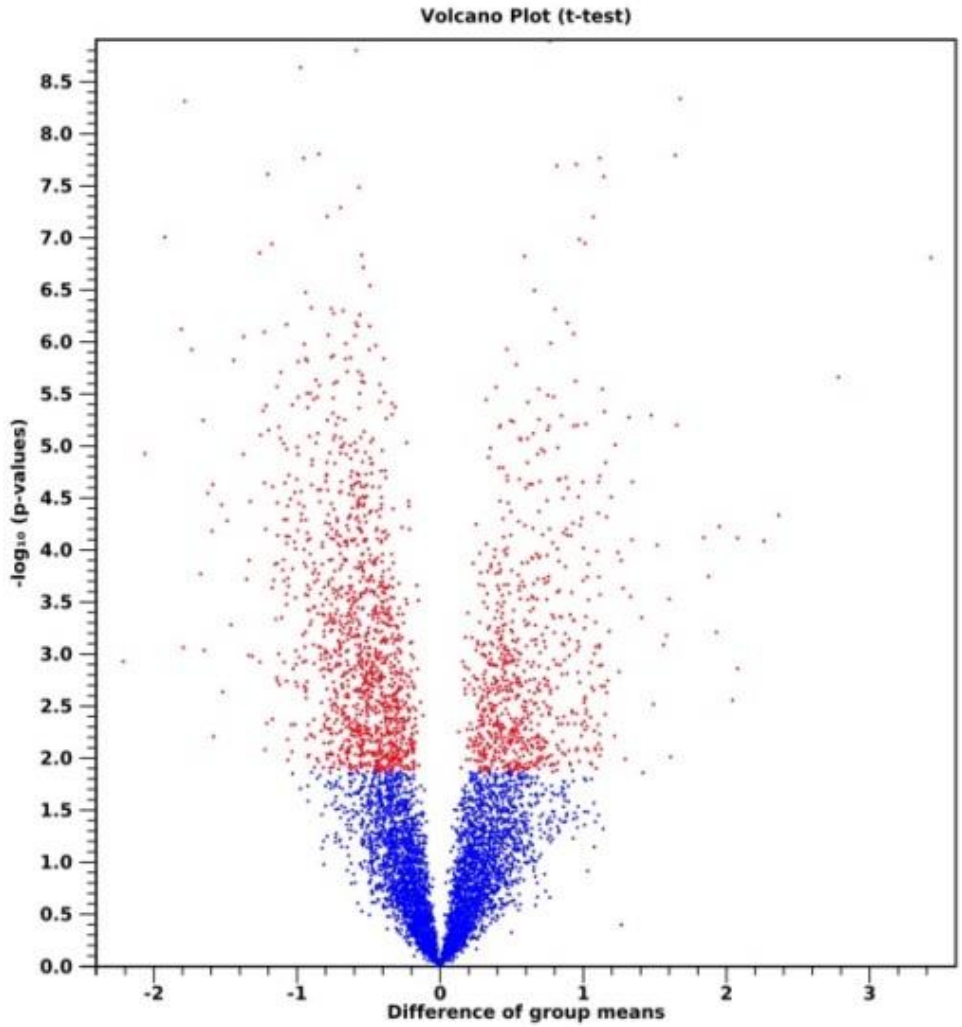


Figure 3.3. **Volcano plot illustrating differential gene expression between control and AOE treated groups**.

3.2.3 Gene Ontology analysis

Gene Ontology (GO) analysis was first performed to highlight the biological processes involved in the reprogramming process. The software DAVID was first used to obtain the GO terms associated with the differentially expressed genes obtained by microarray analysis (Huang et al., 2008). This was followed by further analysis with REVIGO software which simplified GO terms using clustering algorithms that rely on semantic similarities (Supek et al., 2011). GO analysis of all differentially expressed genes revealed Translation, RNA processing, Cell cycle, Protein transport and Cellular metabolisms as the most significant biological processes involved in the reprogramming of tumour cells by AOE (Table 3.2, Figure 3.4, Figure 3.5).

Table 3.2. List of biological processes enriched in all differentially expressed genes obtained by GO analysis using REVIGO. Only processes with p <0.05 are listed.

GO term ID	Description	Frequency	log10 p-value
GO:0006412	translation	1.98%	-14.9747
GO:0006396	RNA processing	1.63%	-9.1035
GO:0006414	translational elongation	0.31%	-7.5591
GO:0016071	mRNA metabolic process	1.10%	-5.5918
GO:0015031	protein transport	3.60%	-5.433
GO:0006397	mRNA processing	0.82%	-5.2411
GO:0034660	ncRNA metabolic process	0.92%	-4.9957
GO:0046907	intracellular transport	3.54%	-4.8356
GO:0006259	DNA metabolic process	3.16%	-4.7721
GO:0007049	cell cycle	3.70%	-4.6383
GO:0006091	generation of precursor metabolites and energy	6.57%	-4.5817
GO:0006261	DNA-dependent DNA replication	0.27%	-4.5575
GO:0033554	cellular response to stress	3.48%	-4.1096
GO:0019318	hexose metabolic process	0.69%	-3.8268
GO:0006457	protein folding	0.72%	-3.7825
GO:0016192	vesicle-mediated transport	3.27%	-3.0209
GO:0046394	carboxylic acid biosynthetic process	0.80%	-2.9811
GO:0000278	mitotic cell cycle	1.73%	-2.8138
GO:0051301	cell division	1.71%	-2.631
GO:0010605	negative regulation of macromolecule metabolic process	3.67%	-2.5946
GO:0048548	regulation of pinocytosis	0.01%	-2.5913

GO:0051276	chromosome organization	2.33%	-2.5322
GO:0009303	rRNA transcription	0.07%	-2.4657
GO:0007040	lysosome organization	0.06%	-2.322
GO:0022403	cell cycle phase	0.01%	-2.234
GO:0010608	posttranscriptional regulation of gene expression	0.84%	-2.2117
GO:0030514	negative regulation of BMP signalling pathway	0.07%	-2.0994
GO:0009451	RNA modification	0.30%	-2.0905
GO:0051130	positive regulation of cellular component organization	1.70%	-2.0454
GO:0009057	macromolecule catabolic process	2.26%	-1.8843
GO:0010324	membrane invagination	0.06%	-1.7259
GO:0032268	regulation of cellular protein metabolic process	3.40%	-1.683
GO:0006106	fumarate metabolic process	0.00%	-1.658
GO:0061024	membrane organization	1.63%	-1.5749
GO:0007030	Golgi organization	0.10%	-1.4849
GO:0006354	DNA-templated transcription, elongation	0.20%	-1.4847

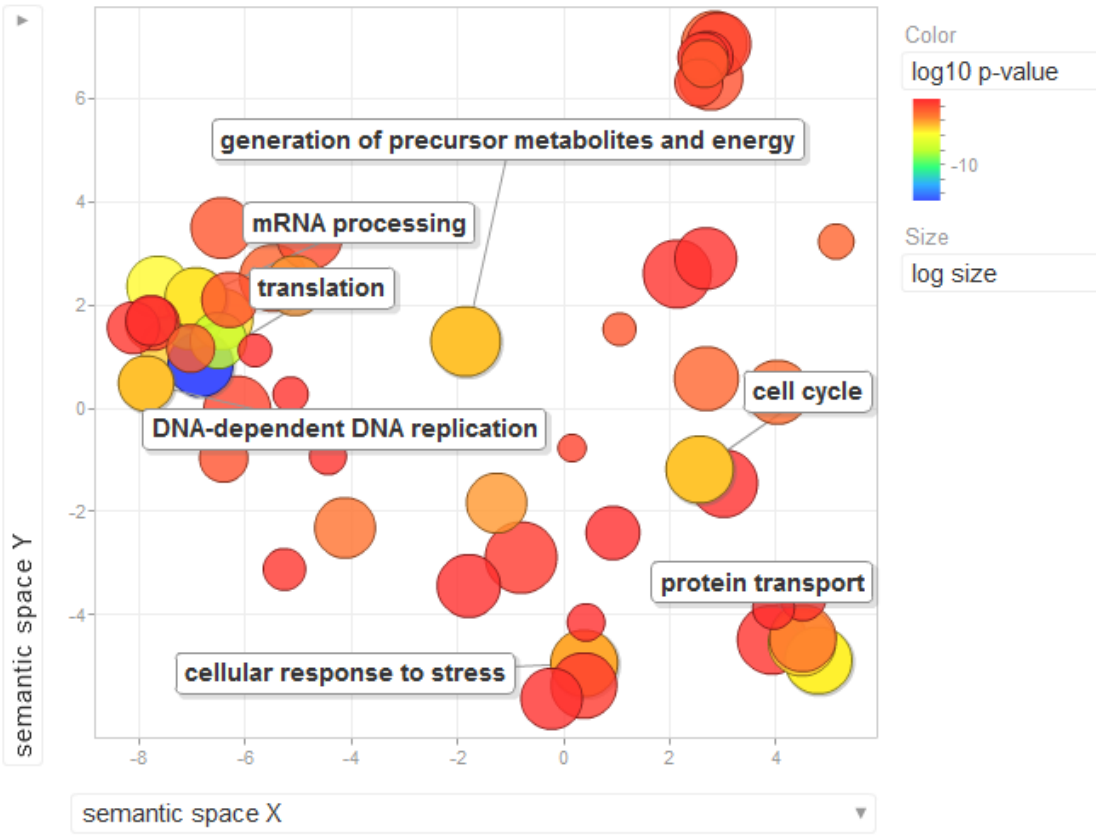


Figure 3.4. GO enrichment analysis of all differentially expressed genes visualised as a scatter plot using REVIGO. GO terms represented by circles are plotted according to similarities to other GO terms (close circles are most related). Circle size is proportional to GO term frequency and colour indicates the log10 p-value (red higher, blue lower).

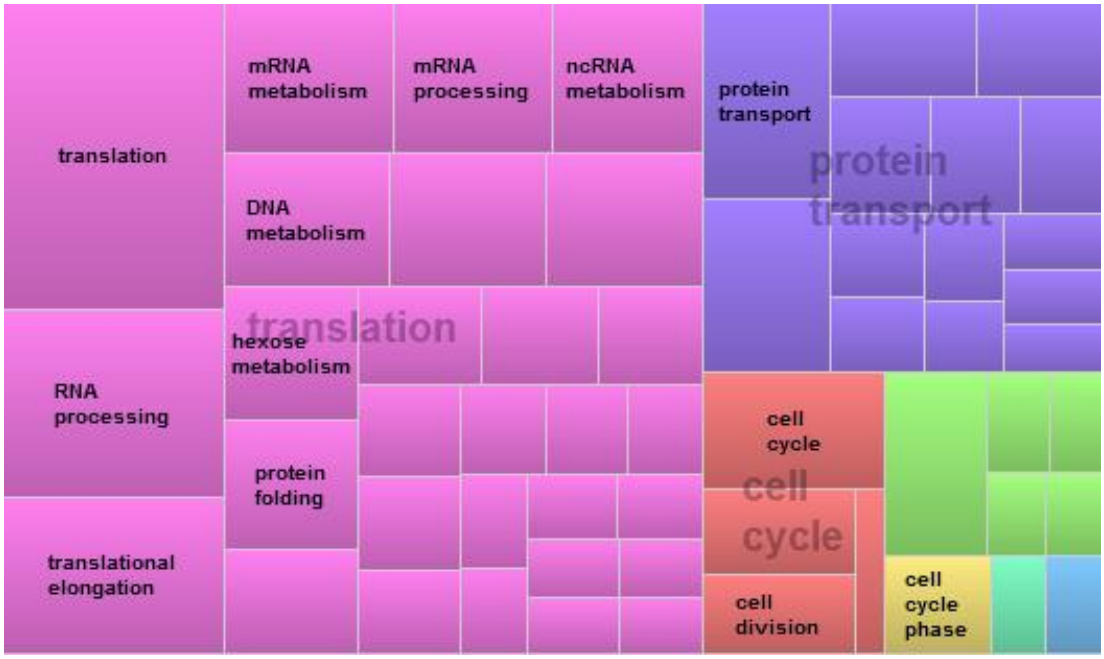


Figure 3.5. Treemap distribution of all differentially expressed genes among biological processes generated by REVIGO. The size of the rectangle is proportional to the p-value of functional groups.

Among all the differential expressed genes, a GO analysis either the upregulated or downregulated genes was next performed. The analysis of the upregulated genes highlighted that the RNA processing, Protein transport, Cell cycle, Chromatin assembly, Negative regulation of metabolic processes were the most significant biological processes involved in the reprogramming with AOE (Figure 3.6, Figure 3.7, Table S3.1 in Appendix).

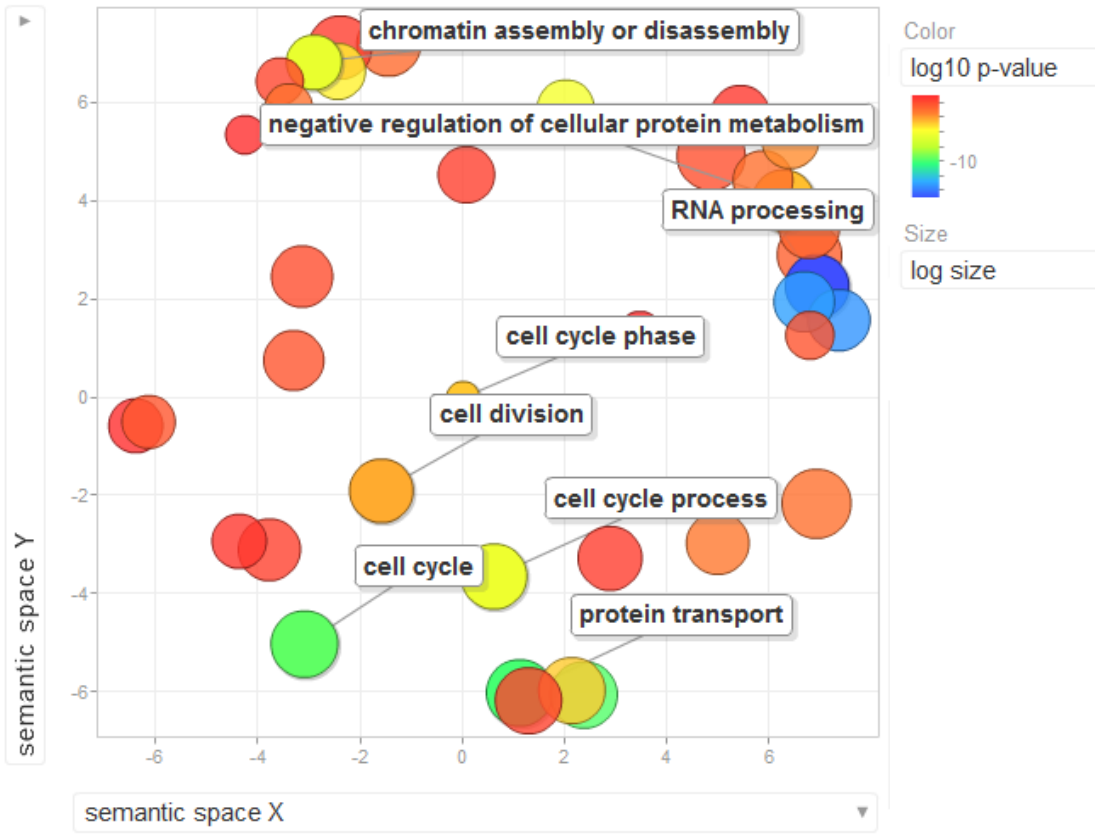


Figure 3.6. GO enrichment analysis of upregulated genes visualised as a scatter plot using REVIGO. GO terms represented by circles are plotted according to similarities to other GO terms (close circles are most related). Circle size is proportional to GO term frequency and colour indicates the log10 p-value (red higher, blue lower).

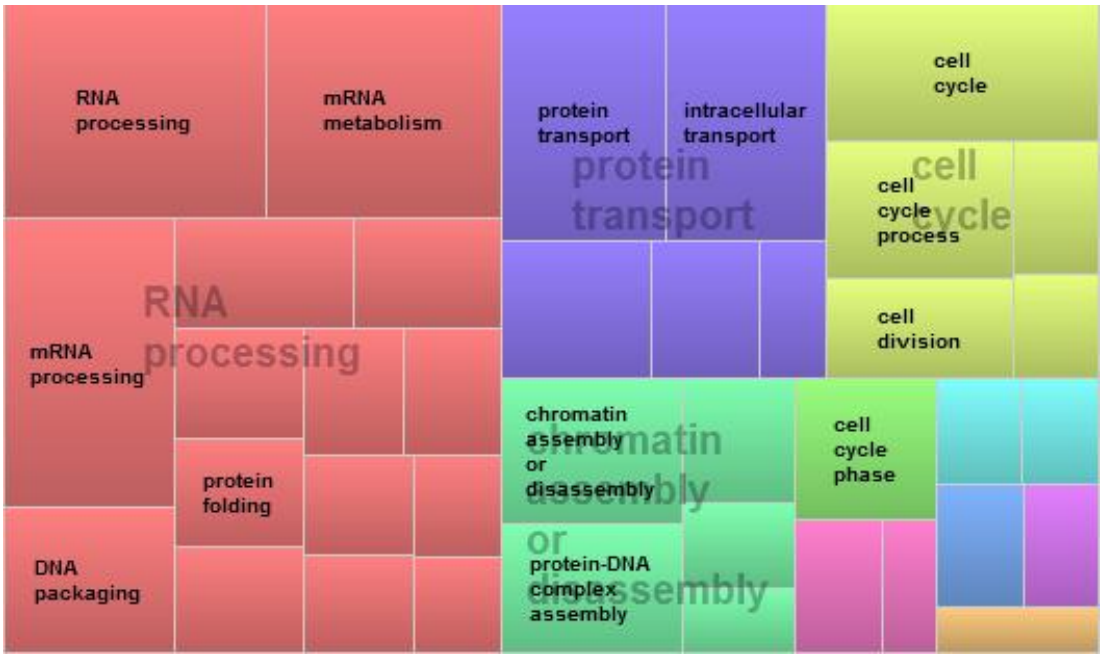


Figure 3.7. Treemap distribution of all upregulated genes among biological processes generated by REVIGO. The size of the rectangle is proportional to the p-value of functional groups.

In contrast, Translation, Mitochondrial function, RNA processing, Cellular metabolism, Cell death, Cellular response to stress were the most significant biological processes enriched in the downregulated genes (Figure 3.8, Figure 3.9, Table S3.2 in Appendix)

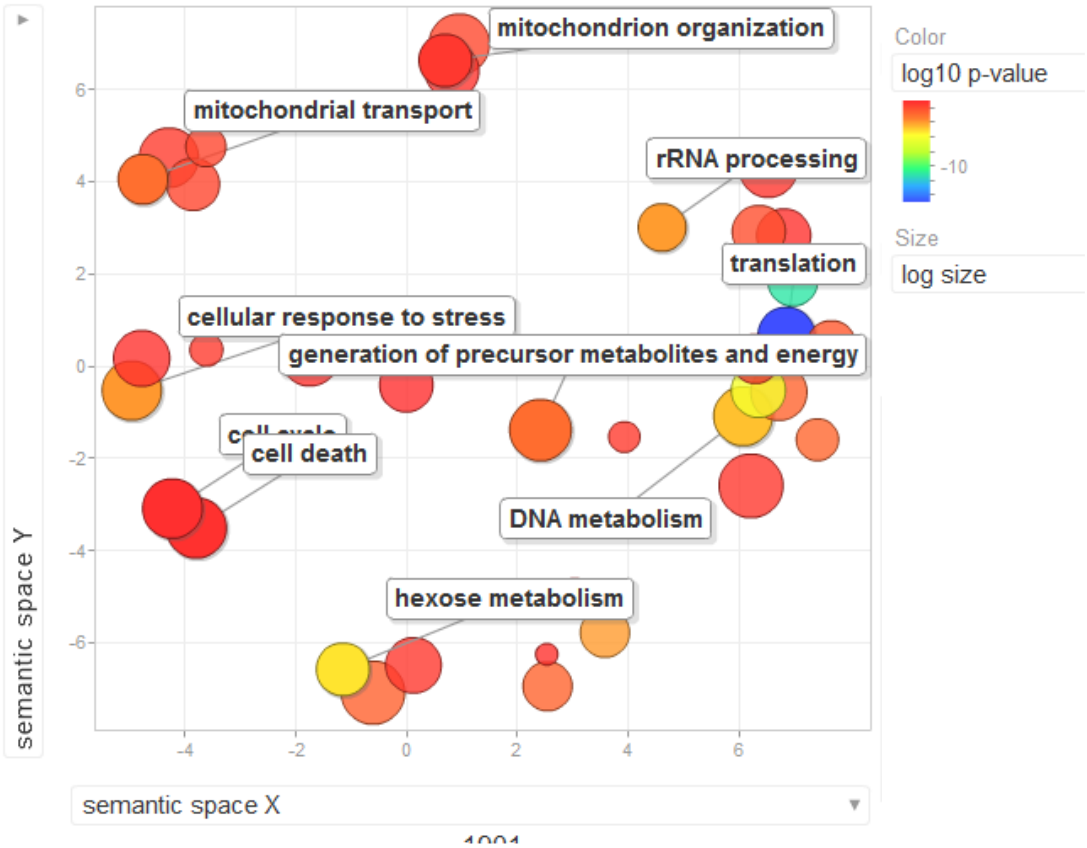


Figure 3.8. GO enrichment analysis of downregulated genes visualised as a scatter plot using REVIGO. GO terms represented by circles are plotted according to similarities to other GO terms (close circles are most related). Circle size is proportional to GO term frequency and colour indicates the log10 p-value (red higher, blue lower).

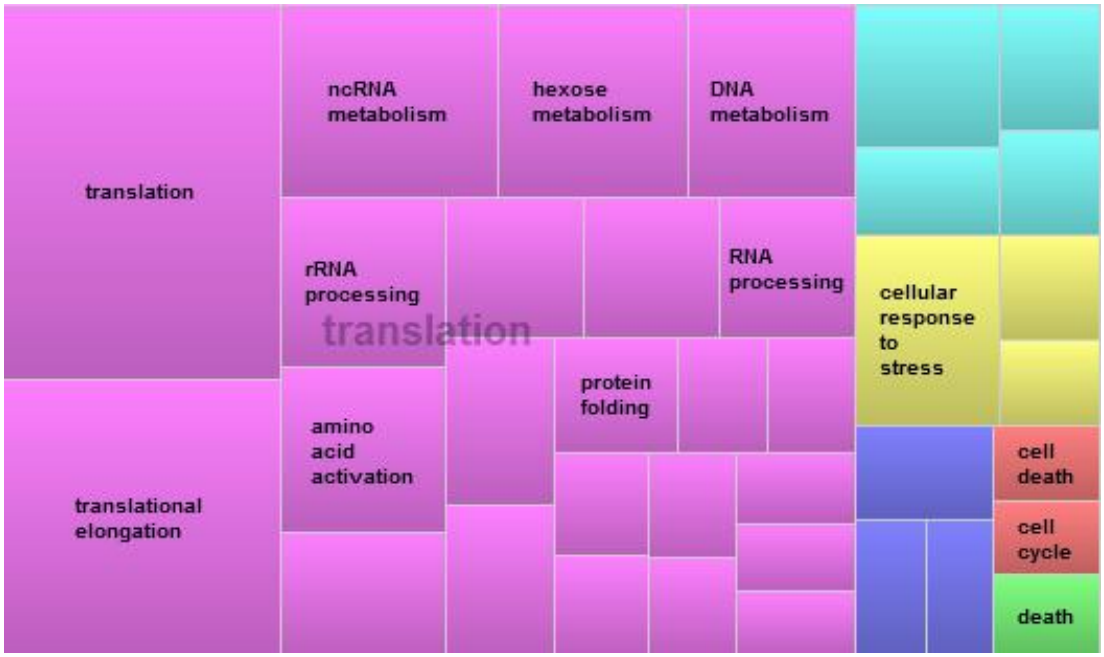


Figure 3.9. Treemap d**istribution of all downregulated genes among biological processes** generated by REVIGO. The size of the rectangle is proportional to the p-value of functional groups.

3.2.4 Canonical pathway analysis

The Ingenuity Pathways Analysis (IPA) software program was next utilised to analyse the differentially expressed genes in order to identify the statistically significant biological functions, signalling and metabolic pathways and molecular networks modulated and well-matched by the genes differently expressed. Right-tailed Fischer Exact Test was used to calculate a *p* value which characterises the significance of gene association to a given pathway. In this analysis, a *p* value less than 0.05 indicated a statistically significant, non-random association.

All canonical pathways from the IPA library that were most significant to the data set were categorised. Canonical pathways were ranked by referring to the ratio value, i.e. number of molecules in a given pathway that met the cut-off criteria divided by the total number of molecules that signified that pathway (Jimenez-Marin et al., 2009). Figure 3.10 illustrates the canonical pathways that were significantly modulated by AOE treatment. The y-axis shows the -log of *p*-value which is evaluated by Fisher's exact test right-tailed, with taller bars associated to increased significance.

The orange and blue coloured bars indicate predicted pathway activation, or predicted inhibition, respectively (z-score). White bars are those with a z-score at or very close to 0. Grey bars indicate pathways where no predictions could be currently made.

Table 3.3 highlights the 5 most represented pathways with the associated genes involved. The most enriched pathways (with highest ratios) were the EIF2 Signaling, Mitochondrial Dysfunction, Germ Cell-Sertoli Cell Junction Signaling, mTOR Signaling and Protein Ubiquitination Pathway. Of note, among the top canonical pathways, the "Antiproliferative role of TOB in T cell signalling has one with the highest gene ratio, indicating a significant mechanistic association. Indeed, the antiproliferative factors TOB1 and BTG2 were upregulated in the AOE treated xenografts (see Figure 3.15 below).

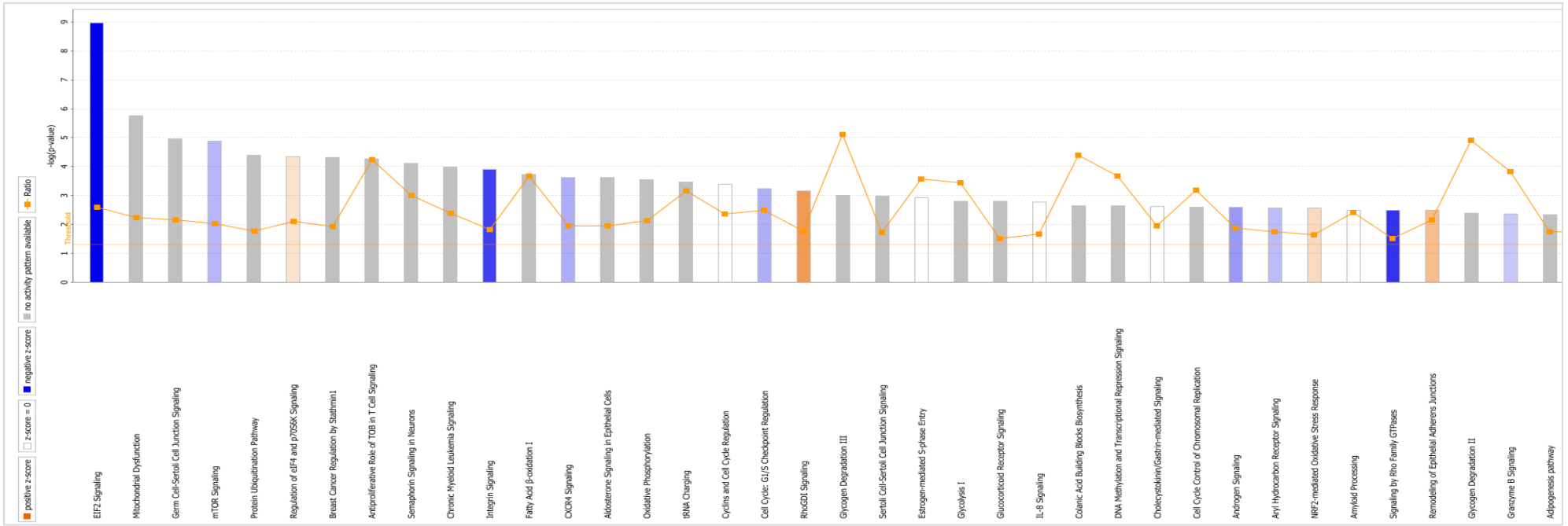


Figure 3.10. **Canonical pathway analysis of genes regulated by AOE treatment**. The high level functional groups that are involved in this analysis are presented along the *x* axis in decreasing order of significance. The y axis shows the –(log) sigificance. The significance is expressed as a *p* value, which was calculated using the right-tailed Fisher's exact test. Threshold: $p<0.05$.

Table 3.3. Summary of top 5 canonical pathways.

Name	***p*-value**	**Molecules**
1. EIF2 Signaling	1.07E-09	RAF1,RPL27A,RPS3A,RPS18,RPS23,RPS11,RPS28,RPL14,MAP2K2,RPL18A,RPS20,EIF3D,RPL23A,MAPK3,RPS9,EIF5,GSK3B,RPL36,RPS3,PPP1CA,RPL18,RPL13,PABPC1,RPL4,NRAS,RPL3,RPS2,EIF3J,RPL12,EIF3E,EIF3G,FAU,RPS16,RPL5,EIF3L,RPL13A,EIF3K,RPS14,RPL38
2. Mitochondrial Dysfunction	1.77E-06	HSD17B10,ATP5D,COX6C,RHOT2,TRAK1,CYB5R3,ATP5E,NDUFA2,NDUFB9,GPX4,ATP5J,NDUFV1,COX17,CPT1A,UCP2,SURF1,COX7C,MAPK9,APP,FIS1,NDUFB11,ATP5B,NDUFS8,NDUFA11,COX7A2,APH1B,BACE2,ATPAF2,UQCRC1,UQCRQ,NDUFB2
3. Germ Cell-Sertoli Cell Junction Signaling	1.10E-05	MAP3K11,RHOT2,BCAR1,PAK1,AGGF1,RHOG,MAP2K2,RHOB,RHOT1,MAPK3,RHOF,EPN1,NRAS,CFL1,TJP1,TUBB4B,TNFRSF1A,TUBB2A,MAPK9,CDH1,TUBB6,ACTA2,RHOA,TGFB3,ZYX,ACTN4,CLINT1,PVRL2
4. mTOR Signaling	1.33E-05	PRKAB1,RPS3A,RPS18,RHOT2,RPS23,PRKAG1,RPS11,RPS28,RHOG,RPS20,RHOB,RHOT1,EIF3D,MAPK3,RPS9,RHOF,RPS3,EIF4B,NRAS,RPS2,EIF3J,EIF3E,MLST8,EIF3G,PPP2R1A,FAU,RPS16,RHOA,EIF3L,EIF3K,RPS14
5. Protein Ubiquitination Pathway	3.92E-05	CRYAB,HSPA14,PSMD7,HLAA,USP5,USP11,DNAJC12,HSPA1A/HSPA1B,DNAJA1,TCEB2,UBE4A,DNAJC4,DNAJC30,DNAJC22,AMFR,PSMA6,UBB,DNAJC9,HSPH1,PSMD5,THOP1,PSMD3,HSPA8,PSMC1,DNAJC5,HSCB,PSMD2,DNAJB11,BAP1,HSP90AA1,PSMD1,CDC34,UBA1,USP34,BIRC2,HSPB1,UBE2I

The best match of differentially expressed genes to canonical pathways is presented in Figure 3.11. Among these pathways, the Cell Cycle: G1/S Checkpoint Regulation and the Anti-Apoptosis pathways showed the highest ratio (yellow line), indicating a high number of genes from the dataset represented in the total number of genes involved in those pathways.

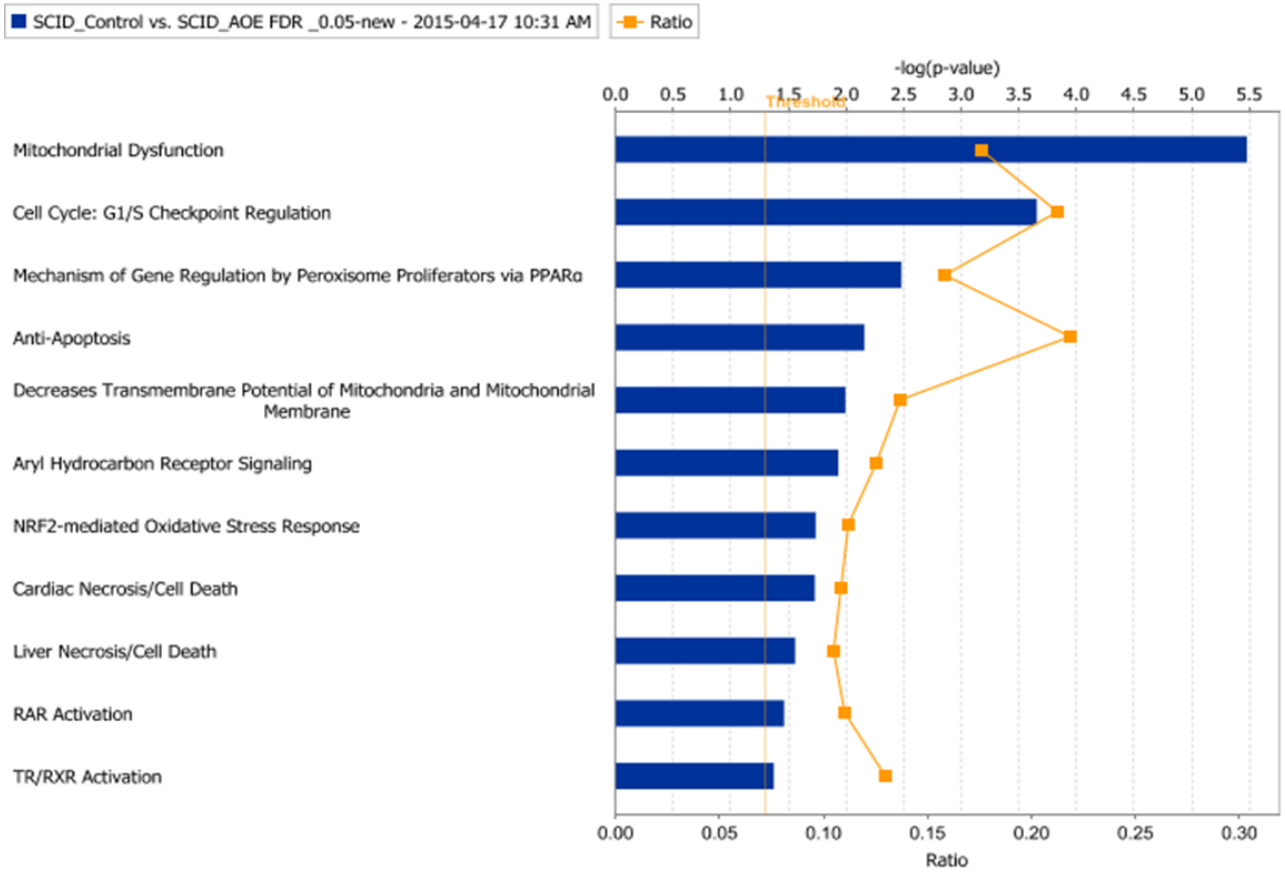


Figure 3.11. **Best match canonical pathways**. The high level functional groups that are involved in this analysis are presented along the x axis in increasing order of significance. The y axis displays the –(log) significance. The horizontal line denoted the cut off for significance (*p* value of<0.05).

Several genes that could be related to G1 to S cell cycle phase regulation are shown in Table 3.4 and Figure 3.12. The key components involved in this checkpoint are the cell cycle cyclin-kinases complexes CDK4/6-cyclin D and CDK2-cyclin E, and the transcription complex that includes the retinoblastoma protein (Rb) as well as the transcription factor E2Fs. CDK inhibitors that function as a negative regulator on the activity of CDK complexes are p21, p27, p19, p18, p16 and p15, with up-regulation of expression observed for the p27 gene in the dataset. Several stimuli exert check point control including transforming growth factor β (TGFβ), DNA damage, UV stress, replicative senescence, growth factor removal and contact inhibition.

Table 3.4. List of associated genes in Cell Cycle: G1/S Checkpoint Regulation pathway.

No.	Gene symbol	Gene description	Type/function	Fold change	p value
1.	RAF1	Raf-1 proto-oncogene, serine/threonine kinase	kinase	1.030	6.36E-03
2.	TFDP1	transcription factor Dp-1	transcription regulator	1.066	7.65E-04
3.	SUV39H1	suppressor of variegation 3-9 homolog 1 (Drosophila)	enzyme	-1.054	3.25E-03
4.	WEE1	WEE1 G2 checkpoint kinase	kinase	1.078	5.45E-03
5.	CDK4	cyclin-dependent kinase 4	kinase	-1.071	1.04E-06
6.	E2F3	E2F transcription factor 3	transcription regulator	-1.045	2.44E-03
7.	SIN3A	SIN3 transcription regulator family member A	transcription regulator	-1.040	2.29E-04
8.	CCNA2	cyclin A2	other	1.070	1.20E-02
9.	PPP2R1A	protein phosphatase 2, regulatory subunit A, alpha	phosphatase	-1.030	5.19E-03
10.	HDAC11	histone deacetylase 11	transcription regulator	-1.147	1.16E-03
11.	TGFB3	transforming growth factor, beta 3	growth factor	1.090	6.88E-03
12.	E2F5	E2F transcription factor 5, p130-binding	transcription regulator	1.116	1.30E-02
13.	GSK3B	glycogen synthase kinase 3 beta	kinase	-1.052	3.80E-04
14.	CDKN1B	cyclin-dependent kinase inhibitor 1B (p27, Kip1)	kinase	1.056	1.62E-05
15.	CDK2	cyclin-dependent kinase 2	kinase	1.043	1.04E-05
16.	SIN3A	SIN3 transcription regulator family member A	transcription regulator	-1.040	2.29E-04
17.	TFDP1	transcription factor Dp-1	transcription regulator	1.066	7.65E-04
18.	FOXO1	forkhead box O1	transcription regulator	-1.158	9.30E-03
19.	RPL5	ribosomal protein L5	other	-1.050	4.40E-05
20.	CDC34	cell division cycle 34	enzyme	-1.097	6.85E-04

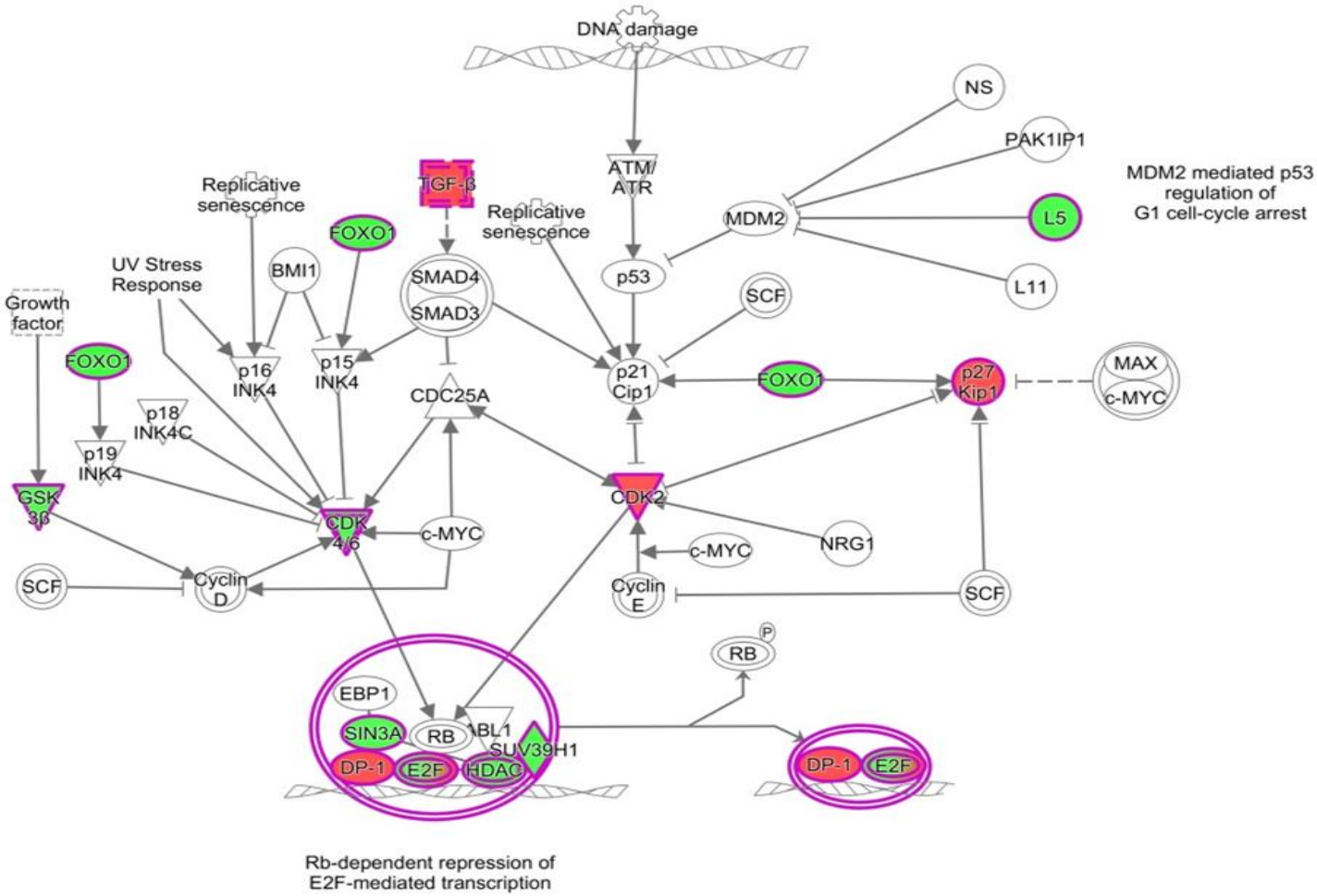


Figure 3.12. **Graphical representations of network of key genes associated in Cell Cycle: G1/S Checkpoint Regulation pathway**. Red and green colours indicate up- and down- regulation, relative to untreated tumour sample respectively.

3.2.5 Top diseases and functions analysis

Table 3.5 summarises the top 10 most significant diseases and functions regulated by AOE. The most significant disease and functions are also illustrated in Figure 3.13. Functions involving cell proliferation, cell death, cell survival and cell cycle were found to be significantly affected. Cancer was recognised as a top disease involved.

Table 3.5. The top 10 diseases and functions affected by AOE treatment

Name of diseases and functions	***p* value**	**Number of differently expressed genes**
1. Protein Synthesis	7.17E-11-1.17E-02	197
2. RNA Post-Transcriptional Modification	2.05E-10-9.3E-03	67
3. Gene Expression	5.53E-10-1.49E-02	315
4. Infectious Disease	9.27E-10-1.22E-02	233
5. Cellular Growth and Proliferation	7.39E-09-1.49E-02	463
6. Cell Death and Survival	7.5E-09-1.49E-02	926
7. Cell Cycle	9.72E-08-1.49E-02	234
8. Cancer	3.34E-07-1.49E-02	1245
9. Organismal Injury and Abnormalities	3.34E-07-1.49E-02	1252
10. DNA Replication, Recombination and Repair	5.7E-07-1.35E-02	143

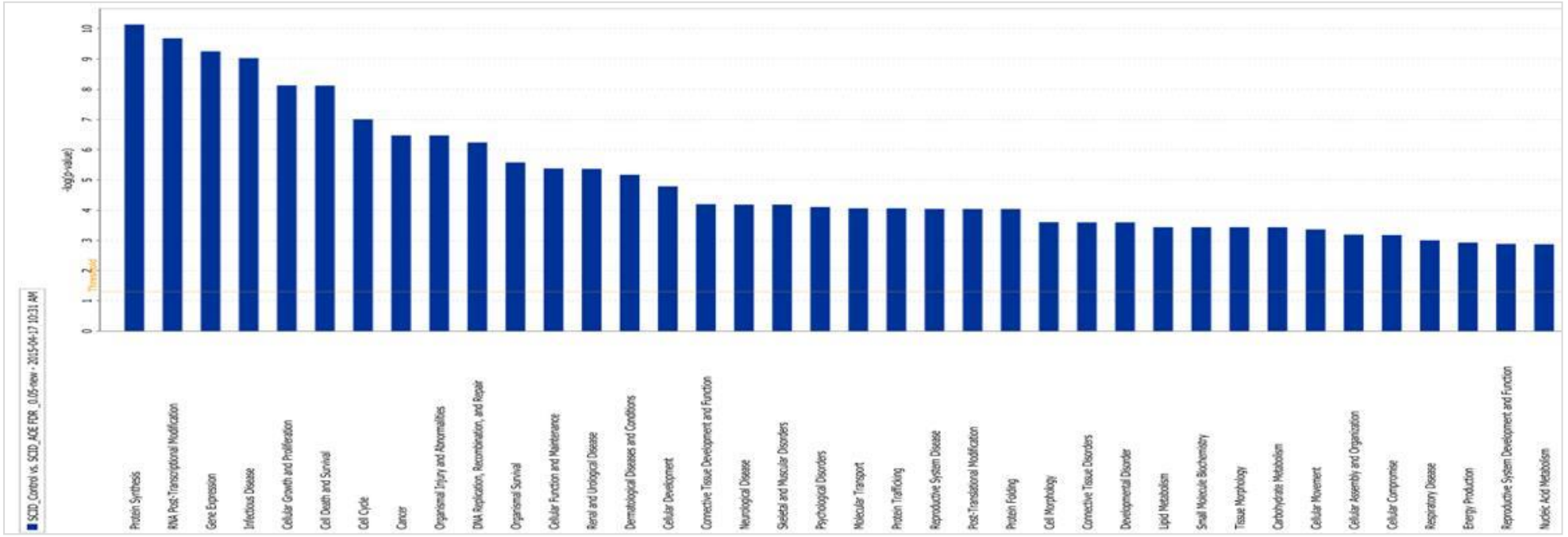


Figure 3.13. Illustration of diseases and functions affected by AOE treatment. The higher the log *p* value (taller bars), the more significant the function is. The horizontal line represents the cut-off for significance (*p* value of 0.05). The y axis exhibits the –(log) significance.

3.2.6 Gene networks analysis

The interaction between genes that were differentially expressed in AOE-treated tumours was also explored by mapping these genes to gene networks identified by IPA (Table 3.6). Most significant networks presented a highest score. Figure 3.14 shows the interaction among the different gene networks. Two main network hubs were identified: the hub of the network 15, with genes involved in Lipid metabolism, Small molecule biochemistry, Cellular morphology and organisation; and the hub of the network 36, with genes involved in Developmental disorder, DNA replication, Cell cycle, and RNA post-translational modification. These data therefore indicate the importance of cell metabolism and cell proliferation in the AOE-treated xenograft phenotype.

Table 3.6. Networks of genes identified differentially expressed in AOE-treated tumours by microarray analysis.

ID	Score	Molecules	Top Diseases and Functions
1	44	34	Developmental Disorder, Hereditary Disorder, Metabolic Disease
2	44	34	Post-Translational Modification, Protein Folding, Cellular Assembly and Organization
3	44	34	Hereditary Disorder, Metabolic Disease, Lipid Metabolism
4	41	33	DNA Replication, Recombination, and Repair, Hereditary Disorder, Neurological Disease
5	41	33	Cell Death and Survival, Cellular Growth and Proliferation, Cell-To-Cell Signalling and Interaction
6	39	32	Cellular Movement, Cardiovascular System Development and Function, Molecular Transport
7	39	32	Amino Acid Metabolism, Small Molecule Biochemistry, Digestive System Development and Function
8	37	31	Gene Expression, Protein Synthesis, Embryonic Development
9	37	31	RNA Post-Transcriptional Modification, Carbohydrate Metabolism, Nucleic Acid Metabolism
10	37	31	RNA Post-Transcriptional Modification, Post-Translational Modification, Molecular Transport
11	36	33	Hereditary Disorder, Ophthalmic Disease, Organismal Injury and Abnormalities
12	34	30	Protein Synthesis, Cell Cycle, RNA Post-Transcriptional Modification
13	34	30	Lipid Metabolism, Small Molecule Biochemistry, Endocrine System Disorders
14	34	30	RNA Post-Transcriptional Modification, Infectious Disease, Organismal Injury and Abnormalities
15	34	30	Lipid Metabolism, Small Molecule Biochemistry, Vitamin and Mineral Metabolism
16	32	29	Infectious Disease, Carbohydrate Metabolism, Small Molecule Biochemistry
17	32	29	Cellular Assembly and Organization, Cellular Function and Maintenance, Cardiovascular Disease
18	32	29	Energy Production, Nucleic Acid Metabolism, Small Molecule Biochemistry
19	31	30	Neurological Disease, Hereditary Disorder, Psychological Disorders
20	30	28	Developmental Disorder, Hereditary Disorder, Metabolic Disease
21	30	28	Cellular Compromise, DNA Replication, Recombination, and Repair, Cell Death and Survival
22	29	27	Cellular Assembly and Organization, Cellular Function and Maintenance, Tissue Development
23	29	27	Lipid Metabolism, Small Molecule Biochemistry, Hereditary Disorder
24	28	27	Post-Translational Modification, Protein Folding, Cell Death and Survival
25	28	28	Cellular Assembly and Organization, Cellular Function and Maintenance, Hematological Disease
26	27	27	Cellular Assembly and Organization, Cellular Function and Maintenance, Cell Morphology

27	27	27	Cell Cycle, Connective Tissue Development and Function, Hematological System Development and Function
28	26	26	Amino Acid Metabolism, Small Molecule Biochemistry, Drug Metabolism
29	26	27	DNA Replication, Recombination, and Repair, Gene Expression, Cell Morphology
30	25	26	Cellular Assembly and Organization, DNA Replication, Recombination, and Repair, Cell Cycle
31	23	24	Gene Expression, DNA Replication, Recombination, and Repair, Cancer
32	23	24	Post-Translational Modification, Cell Morphology, Cellular Assembly and Organization
33	23	24	Molecular Transport, Organ Morphology, Tissue Morphology
34	22	23	Cellular Assembly and Organization, Cellular Function and Maintenance, Dermatological Diseases and Conditions
35	22	23	Cellular Assembly and Organization, Cellular Function and Maintenance, Hereditary Disorder
36	22	23	Cancer, Endocrine System Disorders, Organismal Injury and Abnormalities
37	21	23	Cancer, Organismal Injury and Abnormalities, Cellular Response to Therapeutics
38	21	23	Gene Expression, Nervous System Development and Function, Cellular Development
39	19	21	Metabolic Disease, Organismal Injury and Abnormalities, Renal and Urological Disease
40	18	22	Developmental Disorder, Hereditary Disorder, Organismal Injury and Abnormalities
41	17	20	Developmental Disorder, Hereditary Disorder, Metabolic Disease
42	17	20	Small Molecule Biochemistry, Cellular Assembly and Organization, DNA Replication, Recombination, and Repair
43	17	20	RNA Post-Transcriptional Modification, Developmental Disorder, Hereditary Disorder
44	16	20	Cell Morphology, Connective Tissue Development and Function, Tissue Morphology
45	16	19	Cellular Compromise, Developmental Disorder, Hereditary Disorder
46	15	18	Dermatological Diseases and Conditions, Immunological Disease, Inflammatory Disease
47	15	18	Molecular Transport, RNA Trafficking, Embryonic Development
48	15	18	Cellular Development, Cell Signalling, Nucleic Acid Metabolism
49	15	18	Lipid Metabolism, Nucleic Acid Metabolism, Small Molecule Biochemistry
50	15	18	Cell Morphology, Cellular Compromise, Connective Tissue Development and Function

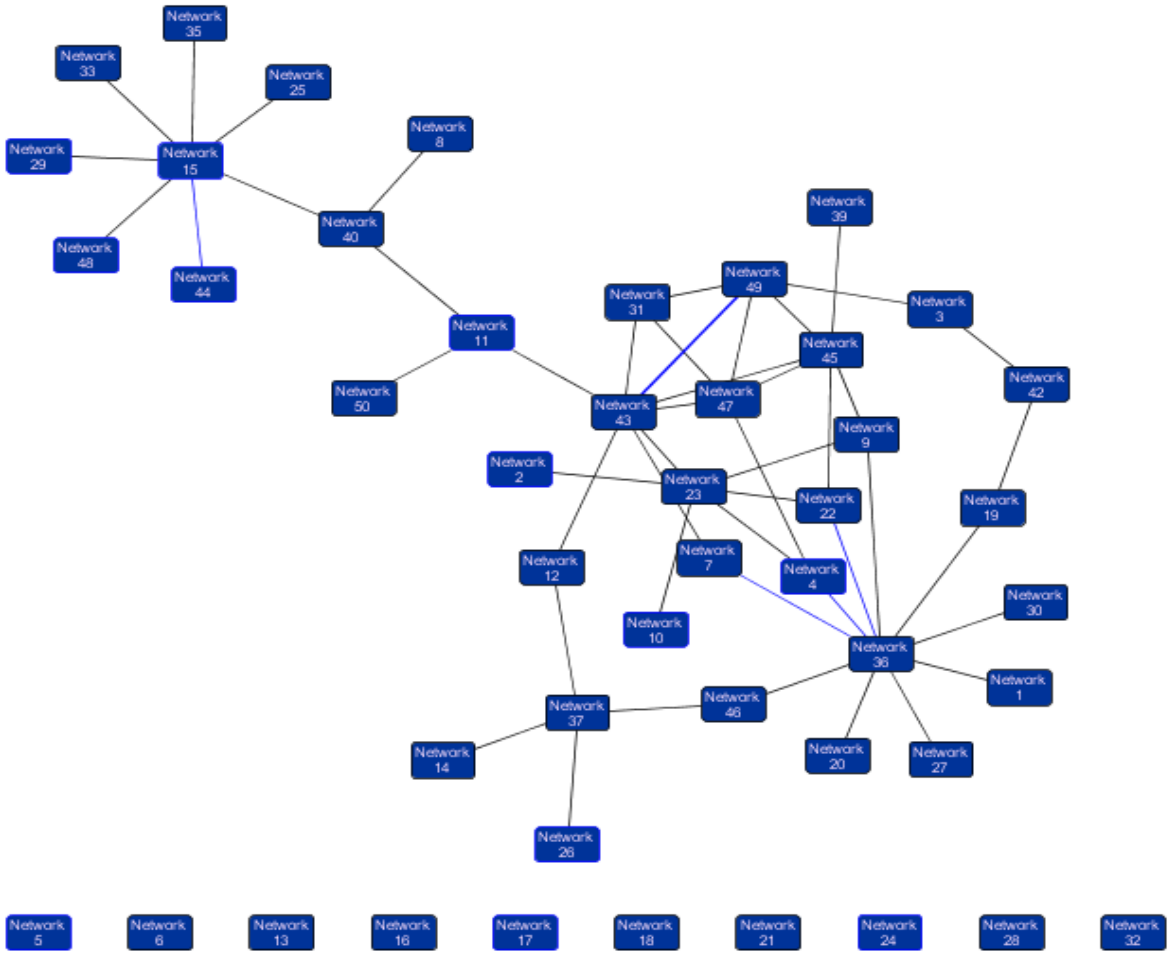


Figure 3.14. Interaction between networks for identified molecules with specific functions.

3.2.7 IPA upstream analysis

The upstream analysis of differentially expressed genes with IPA showed important cell cycle-related molecules that could be potentially involved in the regulation of the analysed targets. TP53, MYC, MYCN, EIF4E, HRAS, were all predicted to be associated with a negative Z score. Although these genes were not listed as differently expressed by microarray analysis, their down-regulation could act upstream of the cell cycle arrest (Table 3.7).

Table 3.7. Most significant upstream regulators of differentially expressed genes.

Upstream regulator	Molecular Type	Predicted activation	Activation Z score	*p*-value gene target overlap
TP53	transcription regulator	Inhibited	-2.282	5.83E-21
HNF4A	transcription regulator		-0.194	1.43E-19
MYC	transcription regulator		-0.974	1.15E-11
MYCN	transcription regulator	Inhibited	-3.235	5.08E-11
EIF4E	translation regulator		-1.428	4.09E-10
PDGF BB	complex		0.548	7.75E-09
HRAS	enzyme		-0.312	1.78E-08
MAPT	other			2.52E-08
INSR	kinase		-0.740	4.79E-08
TGFB1	growth factor		0.531	6.19E-08
ELAVL1	other			6.55E-08
PSEN1	peptidase		1.525	1.23E-07
MAP4K4	kinase	Activated	2.769	5.62E-07
ERBB2	kinase		-0.658	6.27E-07
E2F1	transcription regulator		-0.931	9.81E-07
RICTOR	other		1.480	1.60E-06
APP	other		-0.730	3.08E-06
FN1	enzyme		0.302	3.71E-06
TCOF1	transporter			3.92E-06
CDKN1A	kinase		-0.911	4.72E-06

3.2.8 Validation of differentially expressed genes related to cell cycle arrest

To validate the differentially expressed genes related to cell cycle regulation, quantitative PCR (qRT-PCR) was performed on the same samples used for microarray analysis. Figure 3.15 (b) shows the validated differential expression in AOE-treated

compared to untreated xenografts. The qRT-PCR confirmed the same results obtained by microarray analysis. p15 was also analysed as downstream of TGFβ signalling, although this gene not found among differentially expressed genes as did not pass the quality control of hybridisation of the microarray (Fig 3.15 (a)(b))

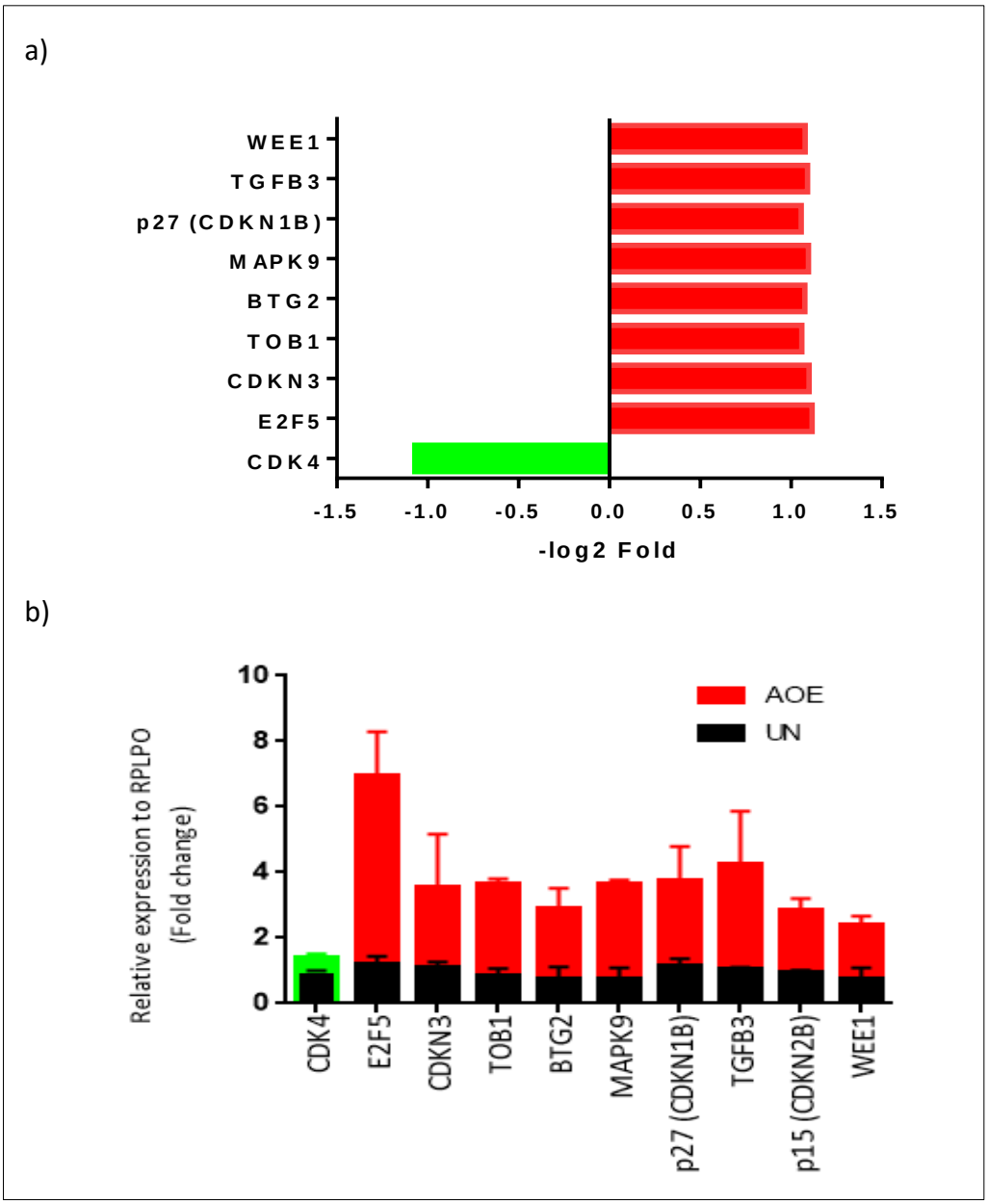


Figure 3.15. Expression of cell cycle related genes in AOE-treated tumours compared untreated controls. Data are shown as fold increase compare to the control group. In all cases, relative quantification to the expression of RPLPO was performed for each gene (n=2).

3.2.9 Validation of cell cycle block *in vivo*

In order to demonstrate the cell cycle block and inhibition of DNA replication *in vivo*, another xenograft experiment was conducted whereby BrdU was injected into mice at the end of the study (8 weeks after AOE treatment) and one hour before sacrifice. Immunohistochemistry of labelled xenografts showed a significant reduction in BrdU staining in the AOE-treated xenografts, demonstrating a block of cell proliferation and BrdU incorporation during S phase of the cell cycle (Figure 3.16). In addition, the same tumours were stained for p27 expression to further validate the observations made at the gene expression level. The intensity of the nuclear staining was much increased in the AOE xenografts, confirming the gene expression data (Figure 3.17).

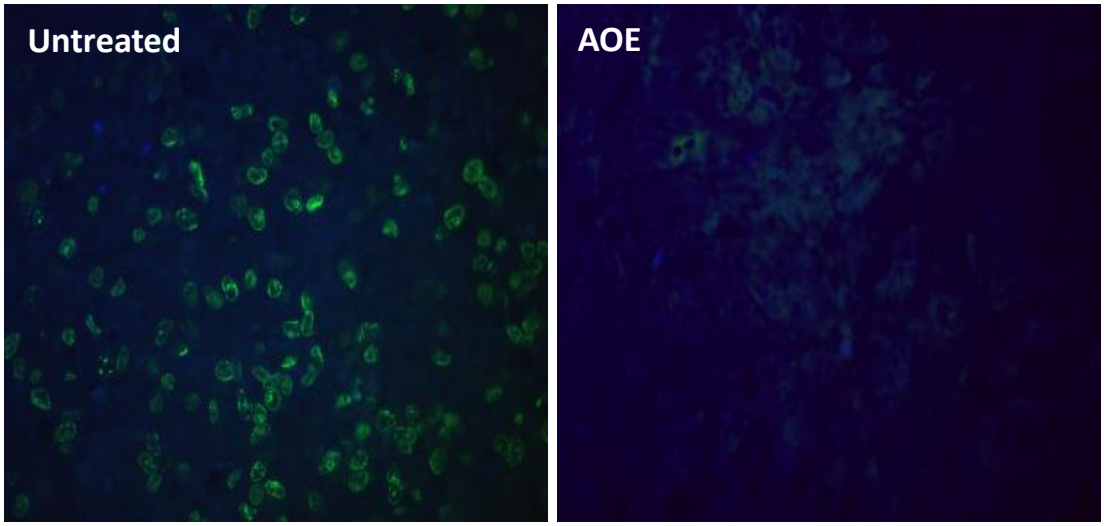


Figure 3.16. BrdU staining of nuclei in AOE-treated and untreated tumour xenografts (10X magnification).

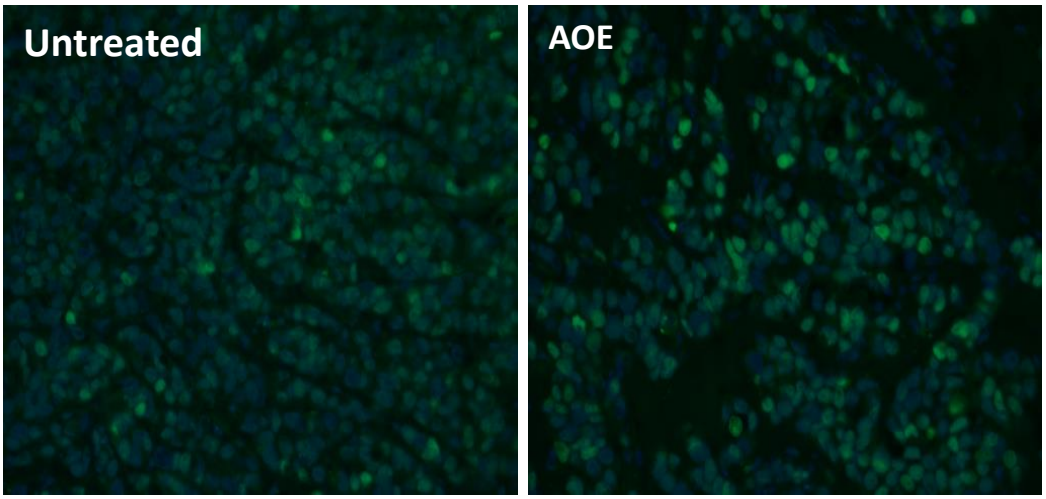


Figure 3.17. Immunoflorescence of p27 expression in AOE-treated and untreated tumour xenografts (10X magnification).

3.3 Discussion

In this chapter, analysis of the gene expression profile of AOE treated xenografts was began by with making sure the RNA used met the quality standards required to obtain meaningful and reproducible expression data. Successful quality checks of initial RNA samples are crucial for synthesis of cDNA before microarray hybridisation. Several criteria of RNA quality were considered, including having a ratio of A260/A280 ≥ 1.8 and A260/A230 ≥ 1.1 meaning that the RNA sample was free from protein and/or solvent contamination, respectively. Moreover, for the peaks of electropherograms generated by the bioanalyser representing the 18S and 28S rRNAs, the ratio 28S/18S should be >1.8, with the total of both rRNA peak areas higher than 30% of all RNA. The QC standards were also incorporated for sample handling.

Microarray analysis of two untreated and two AOE-treated xenografts revealed a significant number of genes differentially expressed, suggesting a major effect of the oocyte extract on the reprogrammed tumours. Bioinformatics analysis of these genes identified a number of biological processes and functions that were affected after treatment with oocyte molecules. Cell cycle was among the biological processes identified as enriched by the GO analysis of the upregulated genes after AOE

treatment. Among the biological functions, Cell Cycle: G1/S Checkpoint Regulation was found clearly related to inhibition of cancer growth. In addition, the best match pathway analysis also suggested cyclins and cell cycle regulation were strongly affected by AOE treatment. Therefore, the genes in this pathway were scrutinised. These included RAF1, TFDP1, SUV39H1, WEE1, CDK4, E2F3, SIN3A, CCNA2, PPP2R1A, HDAC11, TGFB3, E2F5, GSK3β, CDK1B, CDK2, SIN3A, TFDP1, FOXO1, RPL5 and CDC34. All the listed genes are proteins involved in important functions such as transcriptional regulatory proteins that are involved in cell cycle, cell proliferation, cell differentiation, apoptosis, embryogenesis and regulators of CDK kinases. The accurate sequential transition from G1 phase of the cell cycle to S phase is critical for the regulation of eukaryotic cell proliferation, and uncontrolled cell division in involved in tumourigenesis (Bertoli et al., 2013a).

Figure 3.12 shows the key components and regulators involved in G1/S checkpoint. Several regulators drive the G1/S checkpoint control. These include TGF-beta, DNA damage, contact inhibition, replicative senescence, and removal of growth factor. The transition from G1 to S phase in mammalian cells is controlled by the expression of cyclin D, E and A that bind to as well as trigger various Cdk catalytic subunits. Stimulation of Cdk4-cyclin D and/or Cdk6-cyclin D complex is required for cycling from beginning to mid G1 phase (Pan et al., 2002). In eukaryotes, cell cycle is controlled via the serial activation and inactivation of cyclin-dependent kinases (Cdks) to coordinate cell cycle transition via phosphorylation and dephosphorylation of multiple regulatory proteins (Pan et al., 2002). Stimulation of Cdk4-cyclin D and/or Cdk6-cyclin D complex is required for progression from beginning to mid G1 phase (Pan et al., 2002). CDK inhibitors instead function as cell cycle brakes to control the progression and induce cell cycle arrest.

After AOE treatment, the expression of the CDK inhibitor p27 was up-regulated. This was an important result as increased expression of p27 could be responsible for the inhibition of tumour growth exerted by the oocyte extract. Indeed, the increased expression of p27 inhibits cyclin E-Cdk2 complexes directing the block of entry into S phase (Ahmad et al., 2012). In addition to increased expression of p27, a reduced

expression of CDK4 and the S phase transcriptional activator E2F3 was observed after treatment. As the activation of cyclin D-CDK4/6 dimer is required for phosphorylation of retinoblastoma (RB) to release transcription factor E2F to modulate the progression from G1 to S phase, decreased levels of CDK4 potentially retard the G1 to S transition. As a consequence, the expression of the transcriptional activation E2F3 could be decreased due to the inactivation of cyclin D-CDK4/6 complex (Baker and Reddy, 2012).

Conversely, the transcriptional repressor protein E2F5 was instead upregulated as well as the Cdk2 phosphatase CDKN3 gene that can function as tumour suppressor gene. However, the exact role of CDKN3 in the growth arrest is difficult to dissect because of its dual function as tumor-suppressor or oncogene depending on the different molecular contexts in diverse cancer types (Zang et al., 2015). WEE1, a tyrosine kinase that functions as a G2/M checkpoint regulatory protein (Ghiasi et al., 2013), was also upregulated. Once activated, WEE1 triggers an inhibitory phosphorylation of CDC2/CDK1 on Tyr15, which blocks cells undergoing mitosis to allow repair of damaged DNA (Igarashi et al., 1991; Wang et al., 2004). Therefore, elevated level of WEE1 after AOE treatment could lead to G2/M arrest and further accelerate cells to stop proliferating.

Differential expression of other genes connected to cell cycle regulation was also observed. Several genes involved in DNA replication and repair were downregulated. These included members of the origin recognition complex assembly (MCM2, MCM5, CDT1), DNA polymerases (POLA1, POLD2, POLD4) and DNA repair genes (RPA2, RAD23A, ERCC1, ERCC2, APEX, XRCC1, NTHL1, PARP1). Consistent with this, several genes regulating amino sugar and nucleotide sugar metabolisms were downregulated, including NANS, UGDH, AMDHD2, CYB5R3, GALK1, GPI, MPI and the thymidine kinase enzyme TK1.

In addition, important cell cycle modulators were found upregulated and their expression is consistent with cell cycle arrest. TOB1 and BTG2 are part of the TOB/BTG antiproliferative family of proteins, comprising TOB2, BTG1, PC3/TIS21/BTG2,

ANA/BTG3 and PC3/PC3K. The proteins have an essential function in suppressing tumour progression (Baranzini, 2014). TOB1 has been demonstrated to restrict cyclin D1 expression by recruiting the histone deacetylase HDAC1 to the cyclin D1 promoter (Yoshida et al., 2003). Moreover, TOB1 plays a pivotal role in maintaining cells in a quiescent state, thereby inhibiting cellular proliferation. TOB1 and BTG function upstream of RB in regulating the cell cycle and induce RB-dependent G1/S phase cell cycle arrest (Yoshida et al., 2003).

In the IPA analysis, TOB1 was found associated with T cell function. In the context of breast cancer, decrease or loss of TOB expression were correlated to breast cancer progression in breast carcinoma. Moreover, low or absence level of TOB expression was identified in aggressive breast cancer cell lines. The biological consequences of exogenous expression of TOB and shRNA knockdown of TOB in the human breast cancer MCF-7 cell line demonstrated the function of TOB as a tumour suppressor. TOB exerts the anticancer properties in breast cancer cells via regulation of the cell cycle and modulation of multiple downstream signalling pathways that crosstalk with other oncogenic pathways (O'Malley et al., 2009).

Another gene affected by AOE treatment was TGFB3. TGFB3 is one of TGFβ isoform that was also up-regulated in the AOE treated xenografts as well as p15 (CDKN2B). Primary loss of TGFβ growth control in breast cancer is associated with disease progression and deregulation of cell interactions that lead to severe disease (Moses and Barcellos-Hoff, 2011). TGFβ dependent SMAD signalling induces suppression of c-myc and up-regulation of cyclin dependent kinase inhibitors (p15, p16, p19, p21 and p57) (Massague´ 2008; Vijayachandra et al. 2009). Although p15 was not included in the list of differentially expressed genes by microarray analysis due to failure of hybridisation quality, p15 was found upregulated in the AOE treated xenografts by subsequent PCR analysis. Upregulation of p15 downstream of TGFβ signalling could also be responsible for the cell cycle arrest. Furthermore, after stimulation of p15 expression by TGFβ signalling, inactive p27 from CyclinD/CDK4 complexes can be released thus allowing p27 dependent inhibition of CyclinE/CDK2 and CyclinA/CDK2 complexes (Moses and Barcellos-Hoff, 2011, Massague, 2004). Therefore the

interplay of these two cell cycle inhibitors could play an important role in the growth-arrested phenotype of the AOE treated xenografts.

The GO process of negative regulation of protein metabolism and the protein ubiquitination pathway were also suggested to be implicated in the reduction of tumour growth in response to AOE treatment. This particular pathway plays a significant role in the regulation of important cellular functions such as cell cycle control, antigen processing, transcription and signal transduction. In cell cycle control, both positive regulators of CDKs, the cyclins, and negative regulators, the CDK inhibitors (CKIs), are regulated for degradation by the ubiquitin proteolytic pathway which controls their removal in response to mitogenic and anti-mitogenic stimuli. Besides, several studies recognised the association of the ubiquitin proteasome pathway (UPP) in degradation and reuse of the protein regulators, important for cancer initiation and development (Liu et al., 2005). Most of the proteasome pathway enzymes catalyse the proteolysis of tumour suppressor proteins, i.e p53, p21 and p27. Rapid proteasomal degradation of CKIs in successive cell cycle phases correlates with deregulated cell division in cancer cells. Due to their established role in cancer progression, several compounds with proteasome inhibition activity has been assessed and accepted as possible anticancer drugs against haematological diseases and some solid cancers (Rastogi and Mishra, 2012).

The other pathway affected after AOE treatment was Germ Cell-Sertoli Cell Junction Signalling. Several adhesion molecules were observed to be down-regulated in this pathway that could be related to a block of proliferation. The identified molecules include F-actin, Epsin, Zxy, RHO GTPase, RAK and p130 GAS. Several genes of the RHO family were found differentially expressed. RHOA, RHOF, RHOG were downregulated, whereas RHOB was upregulated. RHOA has been shown to promote G1/S transition and also negatively regulating p21 and p27 expression (Mammoto et al., 2004). In the other hand, RHOB is considered a tumour suppressor gene being stimulated by TGFβ and negatively regulating integrin and growth factor signalling (Vasilaki et al., 2010, Vega et al., 2012). Integrin Signalling and the Signalling by Rho Family GTPases pathways were also identified with a negative Z scores, suggesting that

downregulation of adhesion molecules and related signalling could be directly linked to reduced proliferation and function as tumour suppressors.

In addition to the Cell Cycle regulation pathway, the GO and IPA analyses showed Translation and EIF2 Signalling as the most significant canonical pathway affected by AOE treatment. EIF2 (eukaryotic initiation factor-2) is an initiator factor involved in protein synthesis by engaging the initiator methionyl tRNA to ribosomes to initiate translation (Marciniak and Ron, 2006, Beilsten-Edmands et al., 2015). Translational regulation is a critical factor in cancer progression, being responsible for directing not only a control of protein synthesis but also translation of specific mRNAs that stimulate tumour cell survival, angiogenesis, transformation, invasion and metastasis (Silvera et al., 2010). The fact that this signalling pathway was associated with downregulated genes by GO and a negative Z score (-2.065) indicative of negative regulation, suggests that downregulation of protein synthesis could be associated with cell cycle arrest.

Several genes were identified as downregulated in the EIF2 pathway, including GSK3β, MEK1/2, ERK1/2, RPIc, 40s, EIF3, EIF4B, EIF4E2, PABP, and 60S. In addition, a negative regulation of EIF4E was also found among the most significant upstream events by IPA analysis. Targeting the translation machinery is a very important therapeutic goal in cancer research as components of the translation machinery, e.g EIF4E are found upregulated in many cancer types and these are associated with increased expression of oncogenes and cell cycle regulators. Inhibition of such components has a dramatic effect on cancer growth and minimum interference with the expression of housekeeping genes, making this therapeutic approach quite selective for the specific targeting of cancer cells (Bhat et al., 2015).

The other potential pathway that is very much related to tumour growth is the mammalian target of rapamycin (mTOR) kinase signalling pathway. mTOR is a vital pathway regulating translational of mRNA, protein synthesis, glucose metabolism, lipid synthesis and autophagy as well as malignant transformation (Vicier et al., 2014). The negative value of z score (-0.577) demonstrate the ability of AOE to stop cells

proliferation with several genes downregulated; ERK 1/2, AMPK, mTORC1, mTORC2, PP2A, RHO, EIF4B, 40s ribosome, EIF3 and EIF4E. A key control of the mTOR pathway is the PI3K/AKT kinase cascade. Stimulation of the PI3K/AKT cascade by several growth factors proceeds via TSC1/TSC2 (tuberous sclerosis complex) and Rheb (Ras homolog enriched in brain) activates mTOR. The signals converge on mTOR complexes to stimulate protein synthesis and cell-cycle transition by phosphorylating substrates such as S6K1 (p70 ribosomal protein S6 kinase 1) and the EIF4E (eukaryotic initiation factor 4E)-binding proteins 4EBP1 and 4EBP2 (Rathbone et al., 2013, Fingar et al., 2004). Hence, the reduced expression of mTOR complexes potentially suggests a negative modulation of cell cycle and protein translation through this pathway.

Related to translation, RNA processing was also among the biological processes found significantly represented in the downregulated genes after AOE treatment. Indeed, RNA processing was affected with the RNA polymerase III genes being downregulated, thus affecting the synthesis of rRNA and tRNA. Consistently, several genes in the Aminoacyl tRNA biosynthesis and ribosomal genes were downregulated. Importantly, other genes involved in RNA splicing were also found downregulated. Among these, the genes SF3A2 and SF3B2 which are two important slicing factors. Targeting the spliceosome is an important new drug discovery approach as alternative splicing contributes to expression of oncogenic gene variants and drugs targeting splicing factors are being developed as novel antitumour therapies (Bonnal et al., 2012).

To complement a tumour arrested phenotype so far characterised by growth inhibition due to cell cycle arrest, inhibition of protein synthesis and RNA processing, another interesting observation was that the AOE treatment was associated with an inhibition of cellular metabolisms. Indeed, another pathway suggested by the IPA and GO analyses was Mitochondrial Dysfunction. In general, theories of cancer cells undergoing metabolic re-programming and the function of mitochondria in carcinogenesis continues to be a debateable subject (Sanchez-Alvarez et al., 2013). However, several arguments for the targeting of mitochondrial ATP production makes this therapeutic approach convincing. As a nutrient-poor environment with

lack of glucose and oxygen, the cellular core of most of solid tumours is poorly perfused but still able to survive. Thus, poorly diffused tumours have restricted glucose supply but only sufficient oxygen to produce mitochondrial ATP. Therefore, preventing production of mitochondrial ATP might induce apoptosis in poorly perfused tumours (Weinberg and Chandel, 2015).

Certain types of tumours are also highly dependent on oxidative phosphorylation of ATP. These tumour cells tend to be responsive to drugs that constraint mitochondrial ATP production due to a malfunction of glycolytic compensation (Vazquez et al., 2010). Finally, preventing mitochondrial ATP production could give synergistic effect to treatments that eliminate glycolysis, such as the use of inhibitors for PI3K signalling pathway. In addition, important genes regulating glucose metabolisms (glycolysis and gluconeogenesis) were downregulated, including TPI1, ALDH9A1, ENO1, ENO2, GALM, GPI, GAPHD, PFKP, PGM1, PDHB, PKM2.Therefore, the effect of AOE on tumour metabolism could represent an additional important element in cancer progression control.

The best match pathway analysis suggested the Anti-Apoptosis pathway involvement in tumour inhibition after AOE treatment. Cell death was also found enriched in the GO analysis on the downregulated genes. The steps in the progression of cell cycle and programmed cell death (apoptosis) consist of a complex interaction of several families of proteins in an organised and coordinated regulation. Several genes were identified to be affected in response to AOE treatment with an anti-apoptotic effect such as BCL2L1, BNIP3, BNIP1, BFAR, BAG3, BCL2L2 and BIRC2. Even if at first this result seemed counterintuitive as data of cell cycle regulation would suggest an arrest of proliferation which is generally associated with increased apoptosis, an interesting hypothesis emerges when the cell cycle regulation is evaluated in the context of the reduced metabolism and inhibition of apoptosis after the AOE treatment. These features seem to fit the description of cellular quiescence, a reversible non-dividing state that cells can enter in response to absence of growth factor stimulation, lack of angiogenesis and immune response (Yeh and Ramaswamy, 2015).

The quiescent state crucially important for development and tissue maintenance. During quiescence, cells activate a program that retains them from apoptosis, differentiation, and oxidative stress (Lemons et al., 2010, Sang et al., 2010). In cancer, the acquisition of quiescence enables cells to survive owing to escape from apoptosis or differentiation (Legesse-Miller et al., 2012). Therefore, the relationship between apoptosis and quiescence has been proposed to be closely related. Upregulation of the inhibitor of apoptosis NFKB2 was observed in an *in vitro* model of quiescence and cells arrested by mitogen withdrawal upregulate MET and downregulate BAK, inhibitor and inducer of apoptosis, respectively (Coller et al., 2006). Therefore, a close link between apoptosis, metabolisms and quiescence could be at the basis of the cell cycle arrest and growth inhibition observed after AOE treatment in the xenograft experiments.

Cellular response to stress was also found a significant biological process by GO analysis. Quiescence can be induced by stress signals and this can ultimately lead to downregulation of the RAS/MAPK and PI3K/Akt pathways, regulating cell proliferation and survival respectively. The MAPK kinase pathway was found downregulated after AOE treatment (MEK2, ERK1, ELK1) and stress-related gene JNK2A (MAPK9) was instead upregulated. In addition, GADD45B was upregulated and this has been found directly related stressful growth conditions (Liebermann et al., 2011). It is also upregulated by terminal differentiation and quiescence (Glynne et al., 2000). Importantly, GADD45B inhibits G2/M transition but can also act on the G1/S checkpoint (Liebermann et al., 2011, Zhou et al., 2006).

In summary, the bioinformatics analysis seems to point to a cell cycle arrest and a state of quiescence at the basis of the AOE growth inhibition in the cancer tumour xenografts. Although is not clear whether the quiescence phenotype including the reduced metabolism and angiogenesis could be a cause and consequences of the growth arrest caused by the AOE treatment, the molecular profile is consistent with a quiescent and not a senescent state. Quiescence is generally associated with low mTOR activity, which is what the bioinformatics analysis pointed out. In addition, lysosome biosynthesis was also downregulated (the majority of genes in this

pathways are downregulated) whereas increased lysosome biosynthesis is a feature of senescence (Cho and Hwang, 2012).

So what is at the basis of this induction of quiescence? The interplay of growth factor and cytokine signalling together with epigenetic regulation of the chromatin could contribute to this phenotype. Oocytes are rich in TGFβ and reprogramming with oocytes is associated with modifications in DNA methylation and histone modifications (Jackowska et al., 2013, Allegrucci et al., 2011). Chromatin assembly and disassembly was found among the biological processes enriched in the upregulated genes by GO analysis. This finding together with the ones discussed above will form the basis of the experiments described in the following chapters, whereby the relationship between signalling and epigenetic reprogramming will be addressed.

3.4 Conclusions

In the present study, the microarray results provided a molecular mechanistic insight to the functional effect of AOE exposure of breast cancer cells. A number of molecular pathways have been found affected in response to AOE treatment. One of the best predicted pathways affected was Cyclins and Cell Cycle Regulation. This finding suggests that oocyte extracts may exert a growth-inhibitory effect via modulation of the activities of several key regulators in G1 to S phase of the cell cycle. To further validate the microarray results, the specific pathways involved from G1 to S transition will need to be determined and this will be the focus of the following experimental chapters. The effect of specific pathways which drive cell cycle progression will be therefore measured together with genes involved in the related pathways as suggested by the bioinformatics analysis. In conclusion, the use of microarrays for global expression analysis allowed the interrogation of the expression for thousands of genes in a high-throughput manner. It is also allowed a unique chance to attain the molecular signature associated with a specific phenotype. For those validated genes, microarrays proved to be accurate and reliable. This feature, together with powerful bioinformatics analysis assisted the elucidation of the

intricacy of the great amount of differently expressed genes resulted from the reprogramming experiment.

Chapter 4: Modulation of cell signalling by AOE extracts and effect on growth inhibition

4.1 Introduction

As revealed by the microarray data, cell cycle regulation was selected as the most relevant pathway that could function as main regulator of carcinogenesis. The data analysis suggested that oocyte extract-mediated reprogramming of breast cancer cells may involve a block of cell cycle in G_1/S transition. This chapter aimed to determine the expression of cell cycle genes and function of signalling pathways during the reprogramming window in which MCF-7 cells are treated with axolotl oocyte extracts and incubated, before injection into inmmunocompromised mice in an attempt to ascertain the mechanisms that could initiate and contribute to the cell cycle arrest observed in the tumour-reverted xenografts. Molecules and cell signalling pathways involved during this transition were selected based on established studies conducted on breast cancer (Caldon et al., 2006, Ahmad et al., 2012) as well as suggested pathways from microarray data analysis. Predominantly, the selected pathways are involved in cell proliferation/growth and cell survival including MAPK family (p44/42 MAPK, p38 MAPK and JNK), PI3K/Akt, mTOR and TGFβ pathways. The study of these signalling pathways was carried out by western blotting, based on the quantification of the phosphorylation state of these pathways relative to the total protein fraction.

During the G1 to S transition cells are stimulated by various signals to proliferate and also checked for any DNA damage to be repaired. Cells can therefore decide to proliferate by entering S phase or arrest after integration and interpretation of diverse metabolic stresses and environmental cues (Massague, 2004). As the accurate interpretation of these inputs is a highly regulated process following a tightly-ordered sequence, its deregulation can lead to tumour development (Park and Lee, 2003). Several mechanisms and molecules are involved in the regulation of G1 to S phase transition, including expression and phosphorylation of cyclins, cyclin dependent

kinases (CDKs), CDK inhibitors (CDKIs) and members of the retinoblastoma gene family (Neganova and Lako, 2008). During G1/S transition, this expression is governed by the E2F family of transcription factors and by dimerization of partner proteins. Deregulated E2F function is commonly detected in cancer. Two families of E2F family exist and they are associated with either transcriptional activation (E2F1, E2F2 and E2F3A) or transcriptional repression (E2F3B, E2F4, E2F5, E2F6, E2F7 and E2F8) (Bertoli et al., 2013b). For each phase of the cell cycle, expressions of genes that encode cell cycle regulatory proteins is required. Therefore, expression of genes related to growth mechanisms are functionally linked to proliferation and cell cycle progression (Stein et al., 2001).

In breast cancer, several cyclins and CDK inhibitors have been identified that are involved in cell-cycle regulation and potentially function as oncogenes or tumour suppressor genes, such as cyclins D1 and E1 and the CDK inhibitors p27 (Kip1), p21 (WAF1/Cip1) and p16 (INK4A). Another cell-cycle modulator particularly involved in breast cancer is the proto-oncogene c-Myc (Caldon et al., 2006). After receiving signals from growth factors and hormones (estrogen or progesterone), breast epithelial cells react by inducing phosphorylation of Rb and progression of the cell cycle by influencing the levels and response of cyclins and CDK inhibitors to regulate the G1/S transition (Caldon et al., 2006). Various extracellular inputs transduced by signalling pathways affect DNA replication and division during the G1 phase (Massague, 2004).

In breast cancer deregulation of signalling by Receptor Tyrosine Kinases (RTK), particularly by epidermal growth factor receptor (EGFR) (i.e HER1 (EGFR, ErbB1), HER2 (Neu, ErbB2), HER3 (ErbB3), and HER4 (ErbB4)) affects key signalling pathways which leads to stimulation of a low molecular weight G protein Ras as well as of PI3K (Figure 5.1)(Regad, 2015). These pathways are involved in regulating cell survival, proliferation, growth and motility. Phosphorylation of the RTK upon ligand binding activates Ras and the induced serine/threonine kinase Raf. This then leads to phosphorylation of MEK1/2 which further activates ERK1/2 to the influence

phosphorylation of different transcription factors and a number of final outcomes such as enhanced transcription of cyclin D1. Several transcription factors are associated with this signalling, including the Fos and Jun family members (Caldon et al., 2006). Raf also triggers activation of other MAPK family members, such as MAP3 kinases that then further stimulate MKK4/7, MKKK3/6 and MEK5, followed by JNK1/2, p38 and ERK5 activation sequentially. Moreover, MAP3Ks are activated by inflammatory cytokines, oxidative stress and UV radiation. Thus far, the MAPK family is classified into three members: extracellular-signal-regulated kinase 1/2 (ERK1/2), c-Jun-amino-terminal kinase (JNK) and p38 as well as the ERK5 MAPK pathways that is less well-characterised (Junttila et al., 2008).

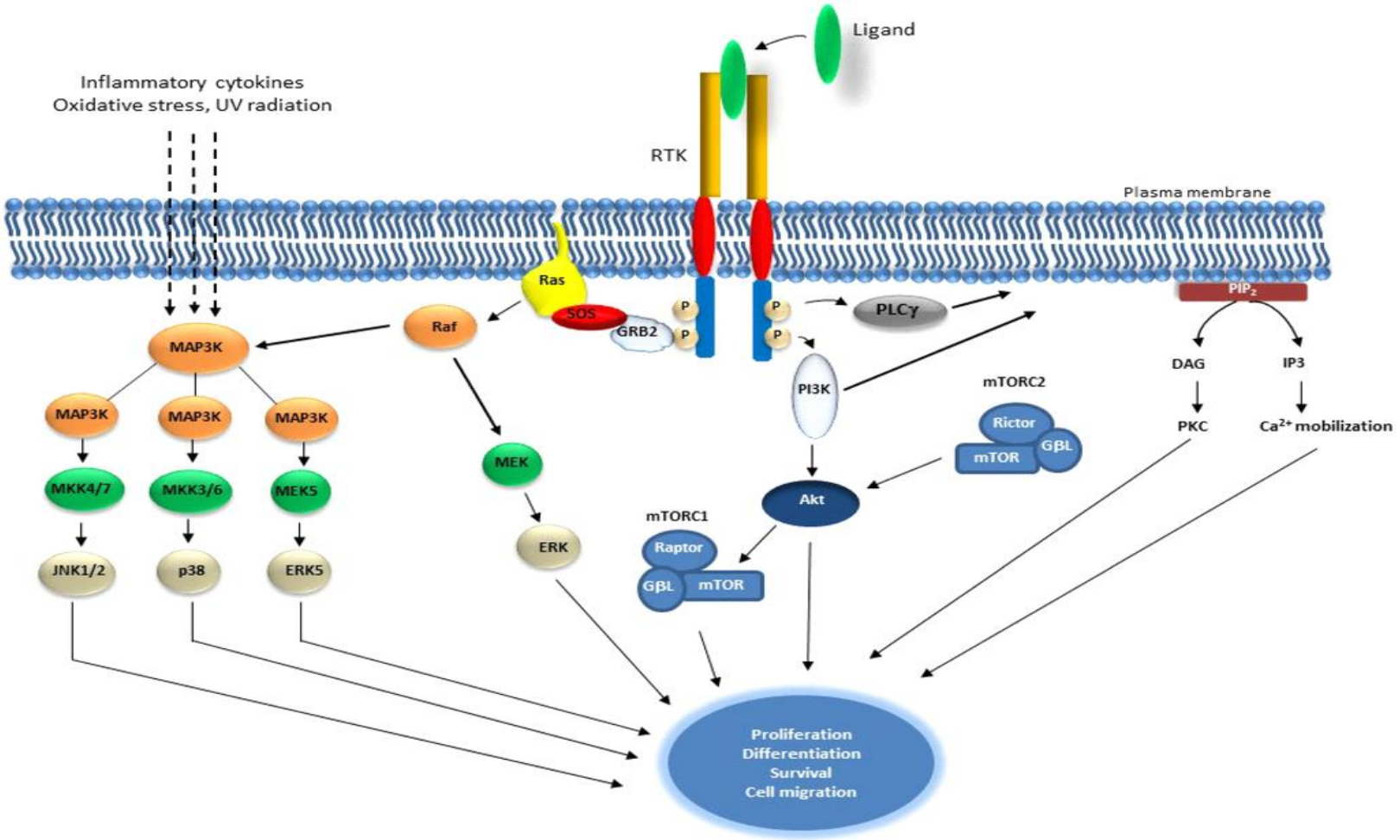


Figure 4.1. Illustration of interacting signalling pathways comprising the MAPK family, PI3K/Akt and mTOR and downstream signalling pathways in response to phosphorylation of the receptor tyrosine kinase (RTK). Figure from (Regad, 2015).

Another signalling pathway that is activated by RTK in breast cancer is PI3K which represents a most important pathway involved in cell survival. In breast cancer, the activation of PI3K occurs via amplification/overexpression of HER2 and somatic mutation (Cidado and Park, 2012). When activated, PI3K dynamically produce PIP3 at the plasma membrane and this process is negatively regulated by lipid phosphatases PTEN and SHIP. Yet, in most of human cancer types PTEN activity is usually deactivated due to deletion, mutation, or silencing, leading to an enhanced effect of Akt. Indeed, the accumulation of PIP3 activates serine/threonine kinase Akt/PKB thus directing to the G1/S cell-cycle transition via phosphorylation (Arcaro and Guerreiro, 2007).

As a central mediator of the PI3K pathway, Akt also enhances protein synthesis and cell growth by triggering activation of mTOR (as part of the mTOR-raptor or mTORC1 complex). mTOR functions downstream of the PI3K/Akt pathway constitutively activated in several types of human cancer. Indeed, the activation of mTOR has long been related to malignant transformation and apoptotic resistance (Sun et al., 2005). It modifies cellular proliferation through deactivation of cell cycle inhibitors (p27 and p21) and activation of cell cycle proteins (c-Myc and cyclin D1), causing an increase in cellular proliferation (Azab, 2013).

Another signalling pathway playing an important role in the regulation of the G1 to S transition is the transforming growth factor (TGF)-β. During mammary development, TGF-β is crucially important as it regulates epithelial cell proliferation, apoptosis, and remodelling of extracellular matrix. Differences in TGF-β response in different cell types are associated with regulation at different levels and cellular context (Moses and Barcellos-Hoff, 2011). TGF-β signals through transmembrane serine/threonine kinase receptors which phosphorylate SMAD proteins, subsequently promoting the formation of SMAD heterocomplexes. The complexes formed then coordinate transcription of several genes, including regulators for cell cycle such as p21, p15 and/or p27 (Caldon et al., 2006, Dennler et al., 2002, Nagaraj and Datta, 2010) and up-regulate the activity of p15, direct loss of cyclin D1-Cdk4 activity and p27

redistribution to cyclin E-Cdk2 complexes. In addition, the cyclin E-Cdk2 activity is also repressed due to binding of p21 and loss of Cdc25A activation (Caldon et al., 2006).

Upon TGF-β ligand binding to the type II transmembrane receptor serine/threonine kinases (TβRII), phosphorylation stimulates the type I receptor (TβRI; ALK5). After activation, TβRI phosphorylates downstream effectors SMAD2 and SMAD3, which then associate with SMAD4 (Fig 5.2). The newly formed complexes translocate into the nucleus and regulate transcription of several target genes. SMAD7 function as an inhibition of SMAD to restrict TGF-β signalling via combining to TβRI, which prevent SMAD2/3 phosphorylation and activation. Besides, it can acts through recruiting the protein phosphatase PP1/GADD34 or the E3 ubiquitin ligases (SMURF1/2, WW1) or phosphatase GADD34/PP1 to the receptor complexes, activating proteasomal degradation or dephosphorylation of the latter, respectively. TGF-β pathway also potentially triggers activation of mitogen-activated protein kinases (MAPKs) and PI3K/AKT pathways (Perrot et al., 2013).

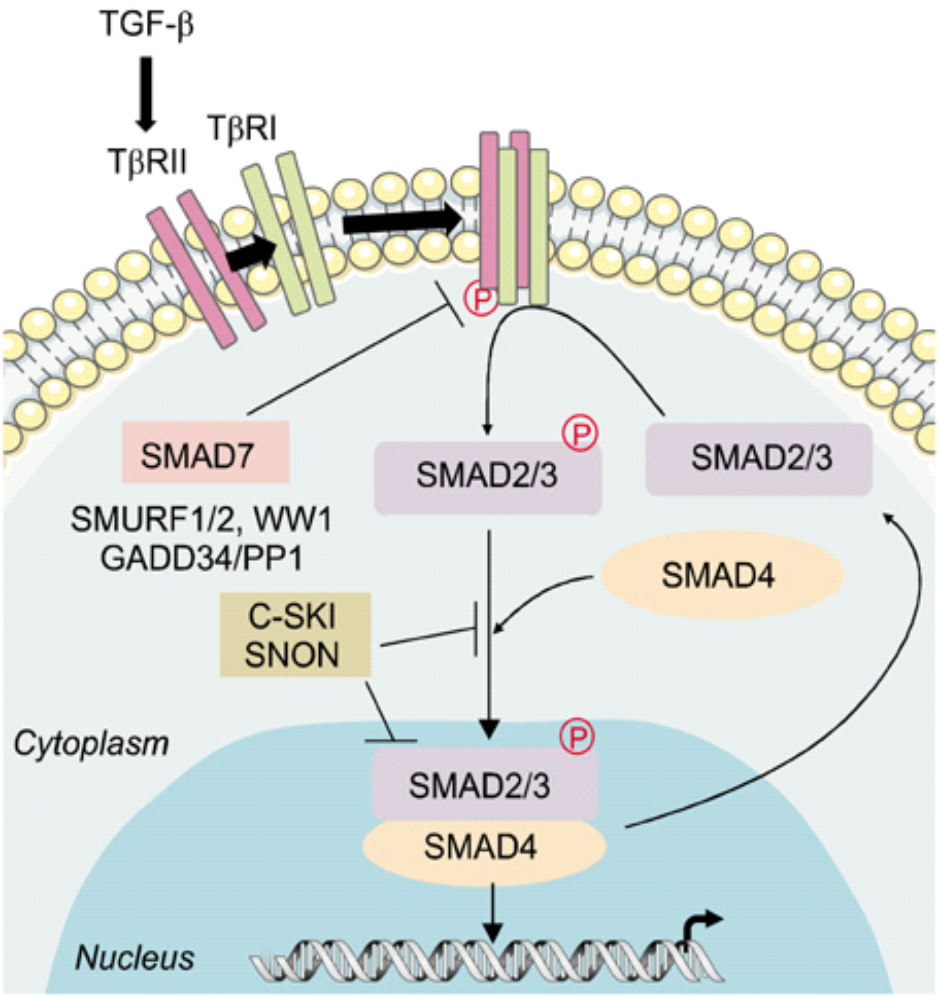


Figure 4.2. TGF-β signaling cascade. After binding to type II receptor, phosphorylation activates type I receptor (TβRI) which further phosphorylates SMAD2/3. Association with co-SMAD, SMAD4 establishes a heterocomplex which then accumulate in the nucleus to regulate gene transcription. Figure from (Perrot et al., 2013).

The initial loss of TGF-β growth control in breast cancer is involved in disease progression and deregulation of mechanisms regulating cell interaction and phenotype, thus leading to aggressive disease (Moses and Barcellos-Hoff, 2011). Its role as pleiotropic growth factor that may affect multiple functions, TGF-β plays a complex role in embryonic development, tissue homeostasis and cancer (Perrot et al., 2013, Gordon and Blobe, 2008). The ability to drive cytostatic activity on normal epithelial cells has implicated TGF-β as a tumour suppressor pathway (Siegel and Massague, 2003). On the other hand, TGF-β has been demonstrated to be a potent pro-tumorigenic mediator, acting through autocrine and paracrine signalling to stimulate peri-tumoral angiogenesis, tumour cell migration, and escape from immune

surveillance (Perrot et al., 2013).

Thus far, a very limited number of studies have been conducted on the signalling pathways involved during cellular reprogramming induced by oocytes extracts. These mainly focus on the use of oocytes to reprogram somatic cells to pluripotent stem cells for regenerative medicine. A study done by Rathbone et al. (2013) identified proteins and pathways associated with pluripotency markers and proteins known to be repressed in pluripotent cells and induced during differentiation of embryonic stem cells. Some studies reported on the suitability of *Xenopus* oocytes as an experimental model system to assess cell cycle mechanisms and regulation of cell cycle and differentiation through development and neoplastic transformation (Hardwick and Philpott, 2015). Large volumes of extracts can be prepared from Xenopus oocytes and in these are contained many proteins that are involved in cell cycle regulation. These proteins can be immune-depleted from the extracts and therefore loss of function studies can elucidate their role in cell cycle regulation.

Preparation of large quantity of extract from eggs and oocytes by centrifugation enables the study of a large array of proteins which could modulate cell cycle events, nuclear transportation, microtubule polymerisation and apoptosis. Amphibian oocyte extracts can be also prepared from different phases of the cell cycle, and further used to dissect alterations in protein stability during the cell cycle (McDowell et al., 2014 and Vosper et al., 2009). Several experimental *in vitro* systems demonstrated the advantage of using cytosolic fractions of prophase-arrested oocytes instead of intact oocytes. Extracts also avoid the asynchrony demonstrated by individual oocytes which is a limitation when investigating signalling events. Moreover, they can be biochemically manipulated, for instance by proteins immunodepletion and reconstitution with recombinant proteins. Also, pharmacological agents, such as inhibitors, can easily be included into the oocyte extract fractions (Crane and Ruderman, 2006).

Amphibian oocytes can be easily injected with RNA and DNA plasmids and many studies have been conducted to study DNA replication, DNA repair and checkpoint control (Hardwick and Philpott, 2015)**.** Therefore amphibian oocytes can be used to model signalling during cancer progression. For instance a study reported by Cailliau et al. (2005) described the role of FGF in the proliferation of triple negative breast cancer cells. Amphibian oocytes do not express FGF receptors, and their overexpression after cloning from malignant breast cancer cell lines triggers progression of the oocytes G2/M phase of the cell cycle following exogenous FGF stimulation. Browaeys-Poly et al. (2009) further utilised the oocyte experimental system to describe the downstream factors responsible for transition into M phase. This project is based on the hypothesis that the molecules contained inside the axolotl extracts are able to modulate cancer cells activities for cancer inhibition. This chapter will investigate the role of different signalling pathways involved in cell regulation during the process of reprogramming of cancer cells after exposure to axolotl prophase-arrested oocyte extracts.

To this end, an in vitro system was first developed to optimise the reprogramming process and to study the cell cycle regulation response immediately after the AOE treatment. Therefore two time points were selected to optimise the reprogramming process, 6 hours and 12 hours. These represent the end of the AOE treatment and an additional overnight culture to allow cell recovery after treatment, respectively. The 12 hour time point also corresponded to the time of injection of reprogrammed cells into immunocompromised mice that was performed in the previous study by our group (Allegrucci et al., 2011), from which the xenograft tumours analysed in this project were obtained. Therefore, the analysis of events occurring at those time points would allow the study of early reprogramming processes that may be involved in the initiation of the tumour growth arrest associated with the treated xenografts. Listed below were the objectives in this chapter:

i. To investigate the immediate effect of reprogramming on cell cycle regulators

ii. To elucidate the role of MAPK family (p44/42 MAPK, p38 MAPK and JNK), PI3K/Akt, mTOR and TGFβ pathways to initiate cell cycle arrest.

4.2 Results

4.2.1 Development of the reprogramming process *in vitro*

4.2.1.1 Optimisation of permeabilisation and viability of MCF-7 cells

The reprogramming of somatic and cancer cells with AOE requires an exchange of molecules from the extract that can only be achieved by permeabilising the cells before the treatment (Alberio et al., 2005, Allegrucci et al., 2011). The degree of permeabilisation was considered to be optimal when almost complete (~80-90% of cells), but still compatible with cell viability. In these experiments, 1 minute 45 seconds was selected based on previous findings demonstrating that digitonin works effectively up to two minutes at a concentration of 30µg/ml (Alberio et al., 2005, Allegrucci et al., 2011). Results of permeabilisation are summarized in Tables 4.1 and 4.2. The incubation of MCF-7 cells with digitonin (20µg/ml) for 1 minute 45 seconds gave a good rate of cell permeabilisation (Table 4.1). Following incubation, the uptake of propidium iodide indicated efficient permeabilisation of cellular and nuclear membranes with retention of cytoplasm in approximately 80-90% of the cells (Figure 4.3 (a), (b) and Table 4.1). The percentage of permeabilisation was calculated by dividing total number of cells with red colour after PI staining (permeabilised and therefore taking up the dye) divided total number of cells observed under microscope under phase light. For the recovery of cells after permeabilisation, cells were counted after recovery post-permeabilisation and then divided by the number of starting cells before permeabilisation.

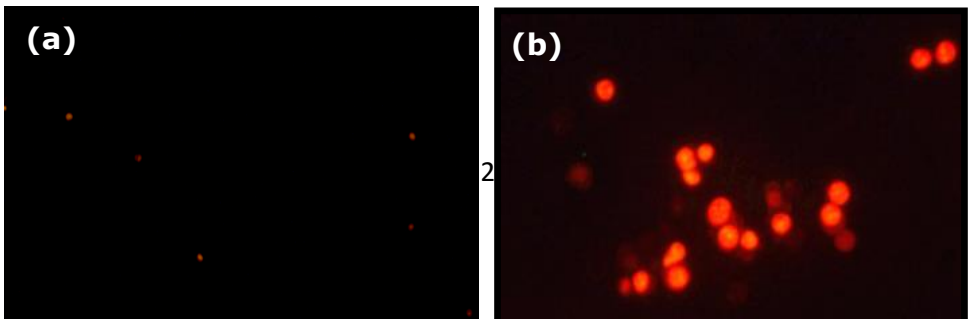


Figure 4.3. Unpermeabilised cells and digitonin permeabilised cells (a) No PI staining observed in unpermeabilised MCF-7 cells (b) PI (red) staining of the nucleus of MCF-7 cells after digitonin treatment (20µg/ml) for 1.45 min.

Table 4.1. Percentage of permeabilisation of MCF-7 cells treated with digitonin (20µg/ml) for 1.45 minutes (n=4). Data represent mean ± SD.

Time of Incubation	Permeabilisation (%) after digitonin treatment				
	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Mean
1.45 min	89	82.3	90.4	75	84.17±7.06

This incubation time also allowed a good recovery of cells as shown in Table 4.2.

Table 4.2. Percentage of MCF-7 cells recovered after treatment with digitonin as measured by counting number the cells that survived the permeabilisation (n=4). Data represent mean ± SD.

Time of Incubation	Cells recovery after permeabilisation (%)				
	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Mean
1.45 min	82	41.44	62.00	42.60	57.01±19.14

Therefore, based on these data, 1 minute 45 seconds provided adequate and consistent permeabilisation for the reprogramming experiments.

4.2.1.2 Cell cycle analysis after reprogramming

After optimisation of cell permeabilisation, the effect of AOE treatment on the proliferation of cancer cells was evaluated by analysis of the cell cycle in 2D cultures. This was done by incubation with EdU for 1 hour after treatment with AOE for 6 hours and further culture extract-free overnight (12 hours after reprogramming) (see Chapter 2, section 2.3). These two selected time points were chosen to see the immediate response after 6-hour incubation with AOE and also after plating the cells overnight which was the same time point when the cells were injected into the mice. The EdU (5-ethynyl-2'-deoxyuridine) is a modified thymidine analogue that can be used to detect DNA synthesis (Salic and Mitchison, 2008), similar to BrdU that was instead used for the assessment of DNA replication *in vivo* (see Chapter 3). In addition, cells were also stained with PI to evaluate their distribution across the different phases of the cell cycle, not only the S phase that was concomitantly evaluated by EdU staining (McGowan et al. 2011).

Figure 4.4 shows the proliferation of cells after incubation with AOE (for 6 hours) and overnight plating (12 hour after reprogramming) compared to the untreated group. The number of cells recovered after 12 hour reprogramming was counted and the result demonstrated that cells treated with AOE were significantly lower than the untreated group (Figure 4.4 (a)). Besides, the percentage of G0/G1 and S phase was slightly lower as compared to the control, but not significantly different. However, there was a significant reduction in cells in G2/M in the AOE group at this time point (Figure 4.4 (a) (b) (c)).

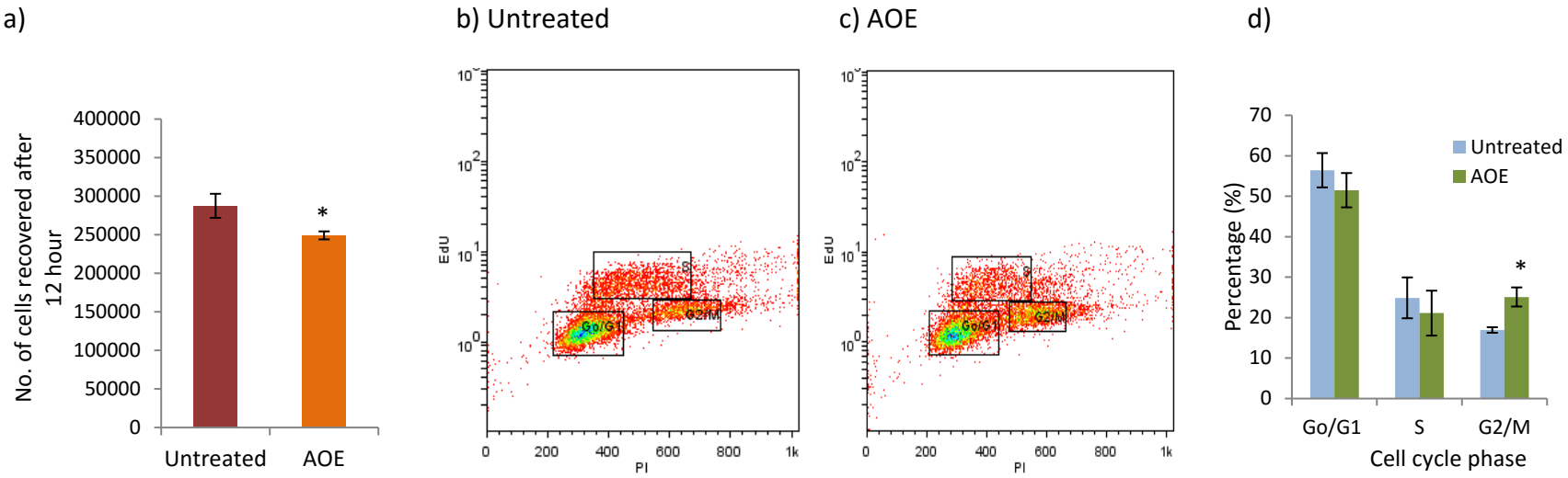


Figure 4.4. Number of cells recovered and flowcytometric analysis of EdU staining and cell cycle phases in untreated and AOE treated cells for 6 hours and extract-free culture overnight (12 hour after reprogramming). (a) The number of cells recovered after 12 hours before EdU and PI staining (mean ± SD). (b,c,d) Go/G1, S and G2/M phases cell populations were gated and the percentage of cells (mean ± SD) in each cell cycle phase are indicated by the bar graph. Data represent three biological replicates (n=3). * = $p<0.05$ as analysed by Unpaired t-Test (a) or Two-way ANOVA (d).

In order to assess the long-term effects of AOE in cell proliferation, cells were allowed to grow in 2D culture for four days after treatment. After four days of reprogramming, no major difference in cell morphology between control and treated cells was observed. In two-dimensional cultures, MCF-7 cells formed monolayers and showed a typical epithelial phenotype (Figure 4.5 (a) and (b)).

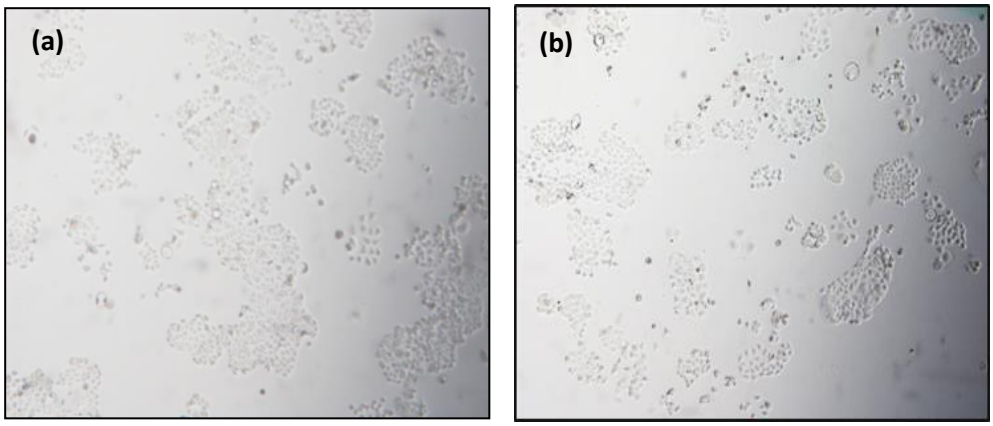


Figure 4.5. Morphology of permeabilised MCF-7 cells cultured on plastic (2D) after 4 days (a) MCF-7 cells in untreated conditions (b) MCF-7 cells treated with AOE (10X magnification).

At this time point, the cells number of AOE treated group recovered were not significantly reduced after four days of culture Figure 4.6 (a), however. The AOE treatment reduced the percentage of cells in S-phase, albeit with a modest effect. No differences were seen in the G2/M phase (Figure 4.6 (b) (c) (d)).

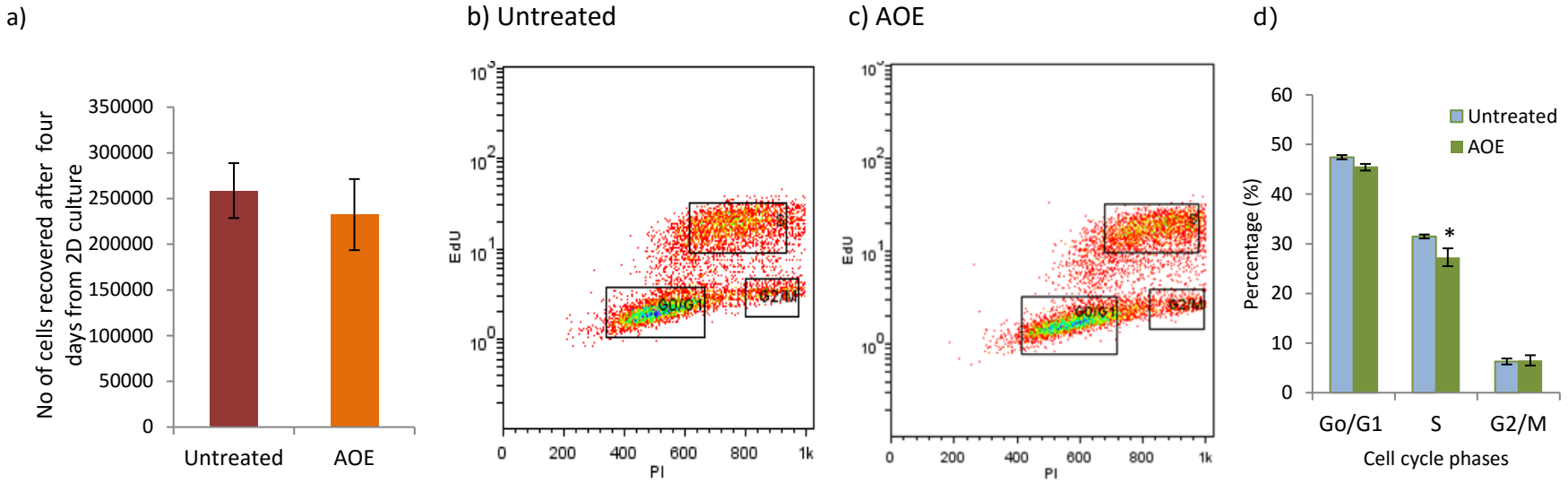


Figure 4.6. Number of cells recovered and flowcytometry analysis of EdU staining and cell cycle phases in untreated and AOE treated cells after four days in 2D culture. The number of cells recovered after four days before EdU and PI staining (a). Go/G1, S and G2/M phases cell populations were gated (b, c) and the percentage of cells (mean ± SD) in each cell cycle phase are indicated by the bar graph (d). Data represent three biological replicates (n=2). * = p<0.05 as analysed by Unpaired t-Test (a) or Two-way ANOVA (d).

4.2.2 Expression of genes involved in cell cycle regulation

4.2.2.1 Optimisation of reference genes

To study the modulation of cell cycle regulatory proteins during the G1/S transition, several key genes that could be used as a read out to monitor the reprogramming process after AOE treatment were selected and the expression measured by real-time PCR. For quantification of relative gene expression, stably–expressed internal reference genes were needed for the measurement of simultaneous expression along with target genes. In this analysis, five candidate internal reference genes were selected and compared between control and AOE treatment groups (Figure 4.7).

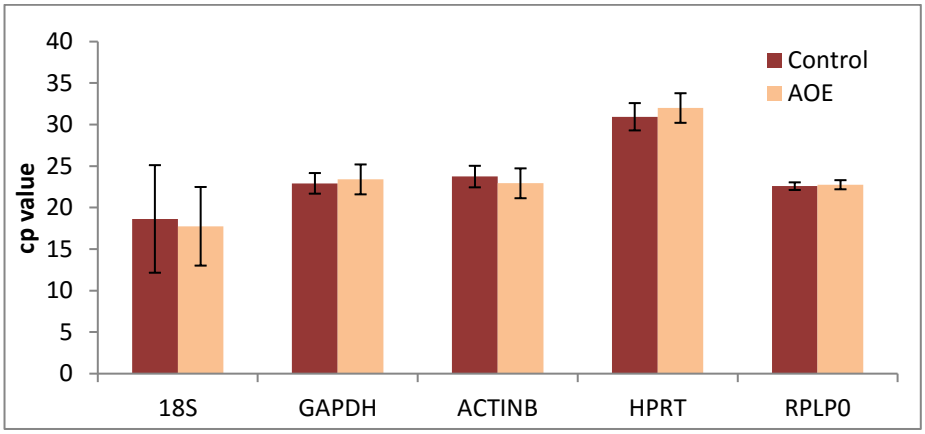


Figure 4.7. **Crossing point (CP) value for five selected reference genes in control and AOE groups**. Data represent two biological replicates in technical triplicates (n=2).

In general, all internal reference genes demonstrated similar CP values between control and AOE for each reference gene. ACTB (β-actin), GAPDH and RPLP0 showed the most similar values. Statistical analysis by the software BestKeeper which consider that the SD value of any studied gene should not be higher than 1, demonstrated that RPLP0 was the most stable reference gene (Pfaffl et al., 2004). RPLP0 presented the lowest crossing point standard deviation (SD [±CP]) (Table 4.3). Therefore, RPLP0 were used as internal reference gene for internal standardisation of target gene expression in all the gene expression gene studies.

Table 4.3: Descriptive analysis of five selected housekeeping using BestKeeper Software

Result - Genes: Repeated Pair-wise Correlation Analysis & Regression Analysis of ***BestKeeper*** **vs. Housekeeping Genes**

CP data of housekeeping Genes:

	18S	ACTIN	GAPDH	HPRT	RPLPO
	HKG 1	HKG 2	HKG 3	HKG 4	HKG 5
n	2	2	2	2	2
geo Mean [CP]	22.13	22.13	22.13	22.13	25.82
ar Mean [CP]	22.16	22.16	22.16	22.16	25.82
min [CP]	21.10	21.10	21.10	21.10	25.70
max [CP]	23.22	23.22	23.22	23.22	25.93
std dev [± CP]	1.06	1.06	1.06	1.06	0.12
CV [% CP]	4.78	4.78	4.78	4.78	0.45
min [x-fold]	-2.05	-2.05	-2.05	-2.05	-1.08
max [x-fold]	2.12	2.12	2.12	2.12	1.08
std dev [± x-fold]	2.08	2.08	2.08	2.08	1.08

4.2.2.2 Expression of cell cycle genes in MCF-7 cells after AOE treatment

A set of differentially-expressed genes involving key regulatory genes of cell cycle and cell cycle-related genes, which could contribute to growth inhibition, were found by bioinformatics analysis of microarrays of AOE treated xenografts, as described in Chapter 3. These included CDKN1B, CDK4, E2F3, E2F5, CDKN2B, CCND1, WEE1, TOB1, BTG2, TGFβ3, CDKN3, MAPK9, MLL5 and JUN. The expression of genes was measured at two time points, 6 hours and 12 hours after reprogramming reprogramming, by real time PCR. These time points represent the reprogramming treatment (6 hours) and the time of injection into immunocompromised mice (12 hours), respectively. Reprogramming experiments during the 6 hours incubation were also performed in the absence of serum to avoid the confounding effect of growth factors contained in it. However, the serum-free conditions could not be used for the time point 12 hours after reprogramming as cells could not survive starvation of serum after reprogramming.

The expression of key cell cycle genes after 6 hour incubation with AOE and also the extract-free overnight time point (12 hour after reprogramming) are presented in Figure 4.8. The only statistically significant difference in expression between the

untreated and AOE groups was found for the CDKN1B gene at 12 hours (p <0.05). Indeed, a 2-3 fold increase in expression was observed at 12 hour after reprogramming with AOE. This increase is consistent with the microarray data and suggests a possible effect in the induction of cell cycle arrest.

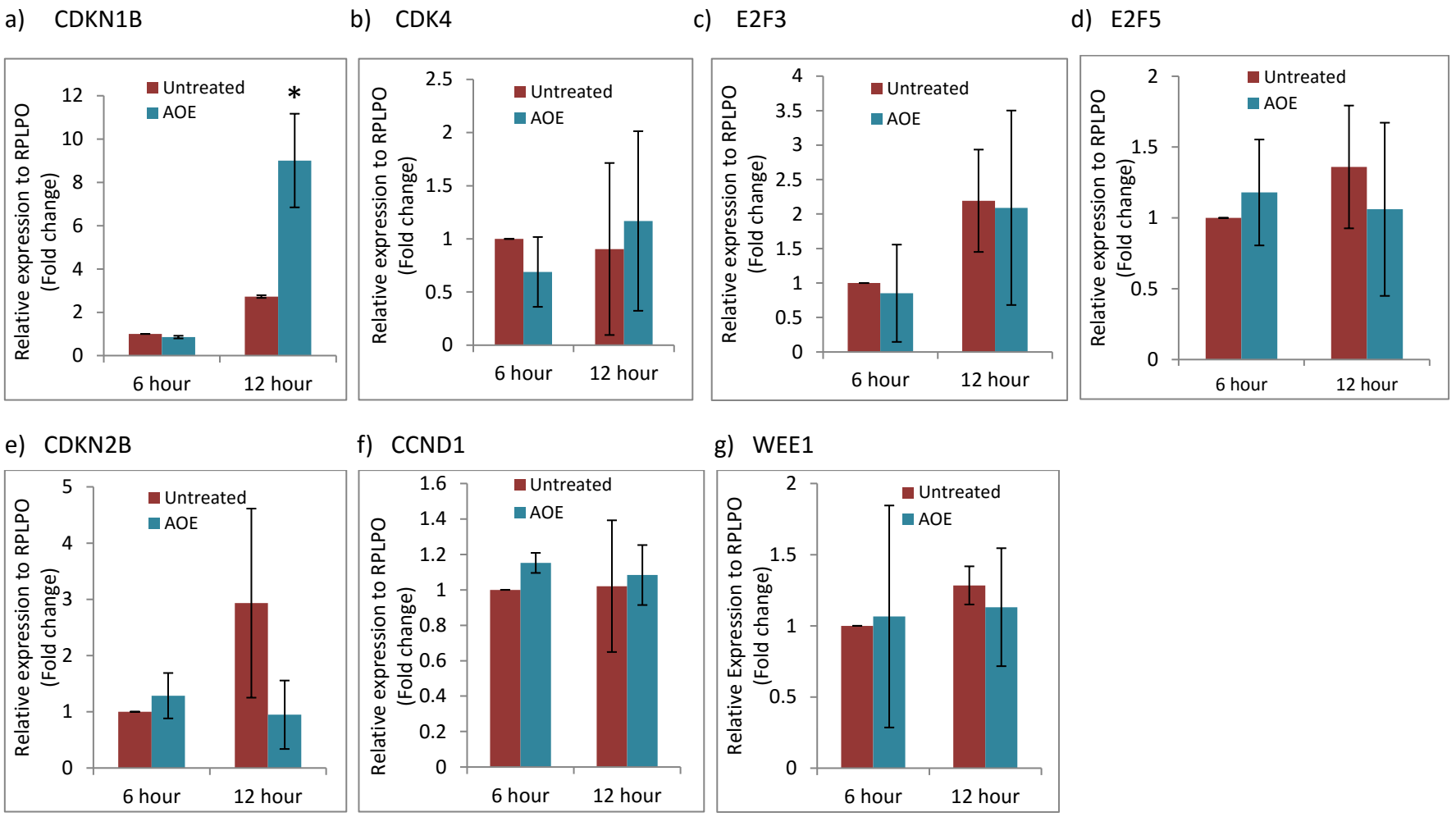


Figure 4.8: Expression of key regulatory genes of cell cycle after reprogramming of MCF-7 cells with AOE at 6 and 12 hours using real-time PCR. Data are shown as fold increase (mean ± SD) compared to the untreated group (6 hours). Relative quantification to the expression of RPLP0 was performed for each gene (n=2 of independent biological replicates). *= $p<0.05$ as analysed by Unpaired t- test.

The expression of genes modulating the cell cycle was analysed next. These included genes that were found upregulated (CDKN3, BTG2, TOB1, MLL5, MAPK9, TGFβ3) and downreguated (JUN) in the microarray data. For these genes, a significant difference in expression was found only for JUN, with the gene being less expressed after 6 hours of reprogramming (Figure 4.9). The decreased expression of this early response gene may also be associated with cell cycle arrest.

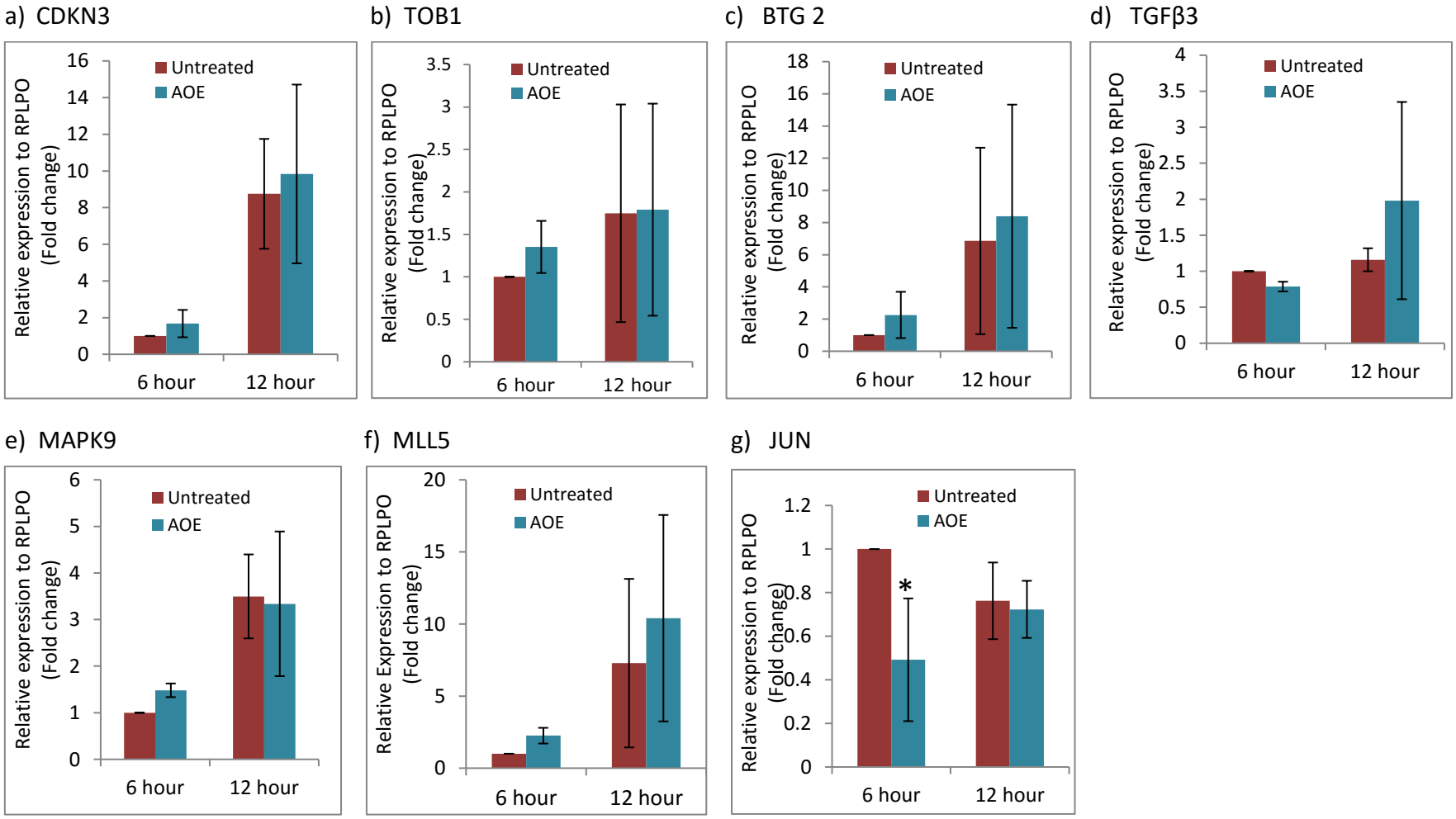


Figure 4.9. Expression of cell cycle modulating genes after reprogramming of MCF-7 cell with AOE at 6 and 12 hours using real-time PCR. Data are shown as fold increase compared to the untreated group (6 hours). Relative quantification to the expression of RPLP0 was performed for each gene (n=2- of independent biological replicates). *= p<0.05 as analysed by Unpaired t- test.

4.2.3 Cell signalling pathways involved in the inhibition of G1 to S transition

Because activation of signalling pathways by phosphorylation is a rapid response, the effect of signalling mediated by the MAPK family, Akt/mTOR and TGFβ on permeabilised MCF-7 cells treated with AOE was studied. Relative phosphorylation levels were assessed by western blotting by measuring phosphorylation level over total protein at four different time points ranging from 30 minutes (early response) to 6 hours (end of reprogramming experiment) of incubation with AOE. Phosphorylation could be detected at all time-points and therefore the quantification was only performed for the 6 hours' time point as this is the optimal time point for AOE incubation and the actual time point used for all reprogramming experiments, as previously established (Allegrucci et al., 2011).

4.2.3.1 Effect on phosphorylation levels of the MAPK family (p44/42 MAPK, p38 MAPK and JNK)

For the MAPK family, treatment of AOE produced a significant reduction in the levels of phosphorylation for p44/42 MAPK and p38 MAPK. For p44/42 MAPK, the reduction of phosphorylation level was clearly seen beginning after 3 hours of incubation whereas for p38 MAPK, the reduction started after 1 hour of incubation with AOE. Conversely, the phosphorylated levels of JNK were not very different from the control group, except for 3h incubation with AOE, when a reduction in phosphorylation level was observed (Figure 4.10 a, b, c). At 6 hours, significant reductions in phosphorylation of p44/42 MAPK and p38MAPK were observed, whereas no difference was found for JNK (Figure 4.11 a, b).

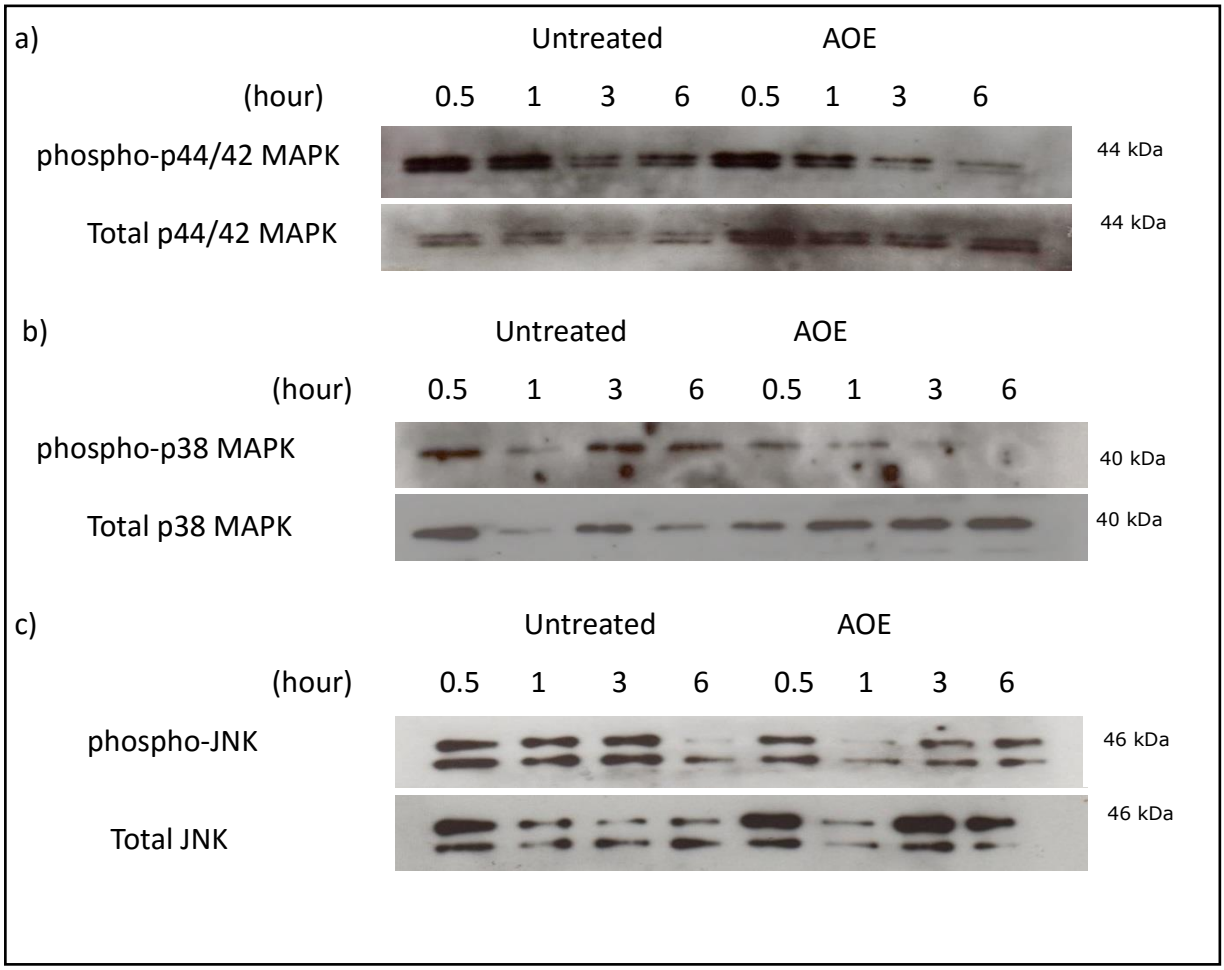


Figure 4.10. Phosphorylation of p44/42 MAPK, p38 MAPK and JNK pathways after incubation of permeabilised MCF-7 cells with AOE at four different time points (30 minutes, 1 hour, 3 hour and 6 hour). Figure show representative images of a replicate experiment (p44/42 MAPK n= 4; p38 MAPK n=4; JNK n=2). a) phosphorylation of p44/42 MAPK; b) phosphorylation of p38 MAPK; c) phosphorylation of JNK.

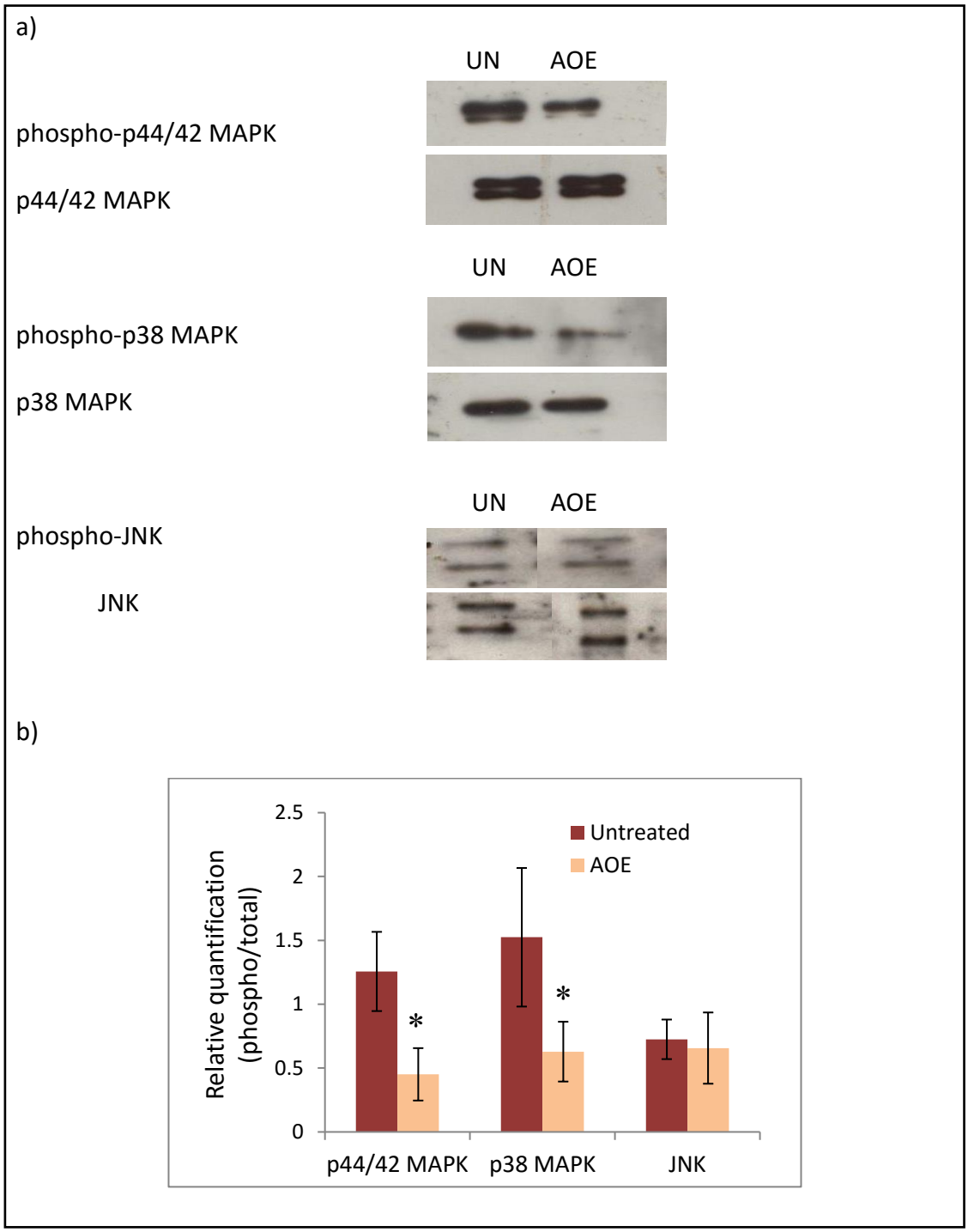


Figure 4.11. Level of phosphorylation of p44/42 MAPK, p38 MAPK and JNK pathways after AOE treatment of MCF-7 cells for 6 hours. (a. (a) shows representative images of a replicate experiment (p44/42 MAPK n= 4; p38 MAPK n=4; JNK n=2 independent biological replicates). (b) data represent mean ± SD with *=p<0.05 by Unpaired t-test.

4.2.3.2 Effect on phosphorylation levels of Akt and mTOR

For the PI3K pathway, phosphorylations levels of Akt were demonstrated stable up to 3 hours incubation, but an increase was observed at 6 hours. mTOR phosphorylation was instead constant across incubation times (Figure 4.12 A, B). Treatment with AOE significantly increased the level of phosphorylation after 6 hour of treatment. However, for the mTOR no significant change was seen in the level of phosphorylation at the end of incubation time (Figure 4.13).

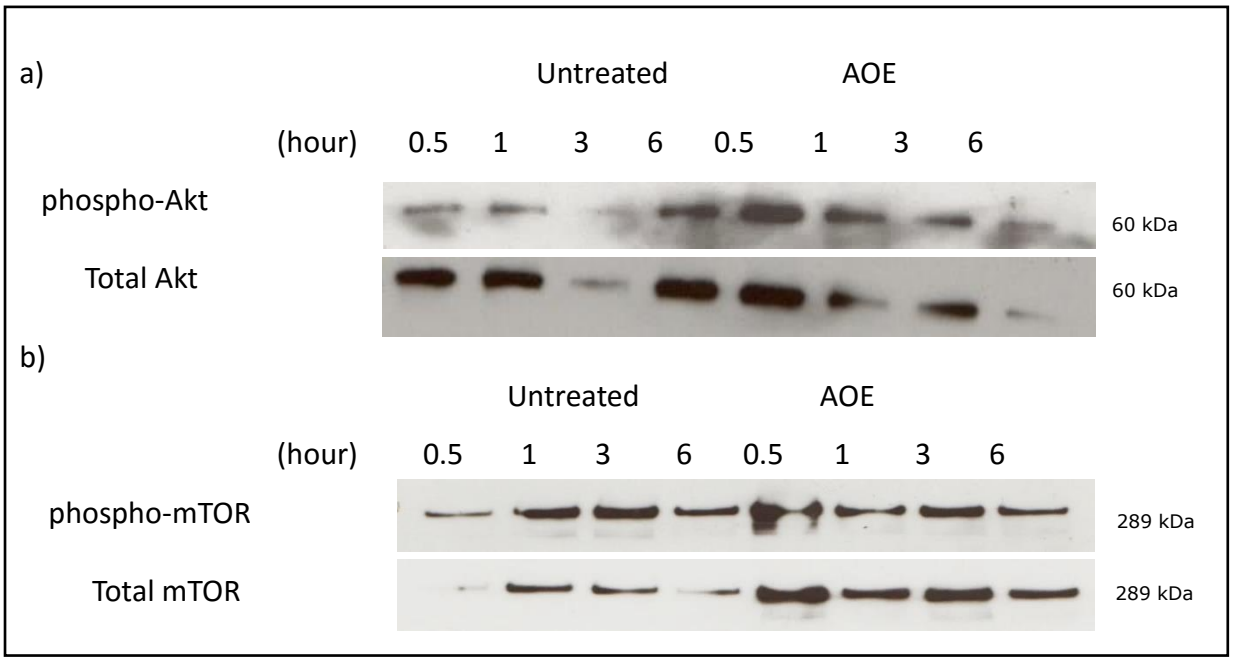


Figure 4.12. Phosphorylation of Akt and mTOR pathways after AOE treatment of MCF-7 Cells at four different time points (30 minutes, 1 hour, 3 hour and 6 hour). Figure show representative images of a replicate experiment (n= 3). a) phosphorylation of Akt; b) phosphorylation of mTOR.

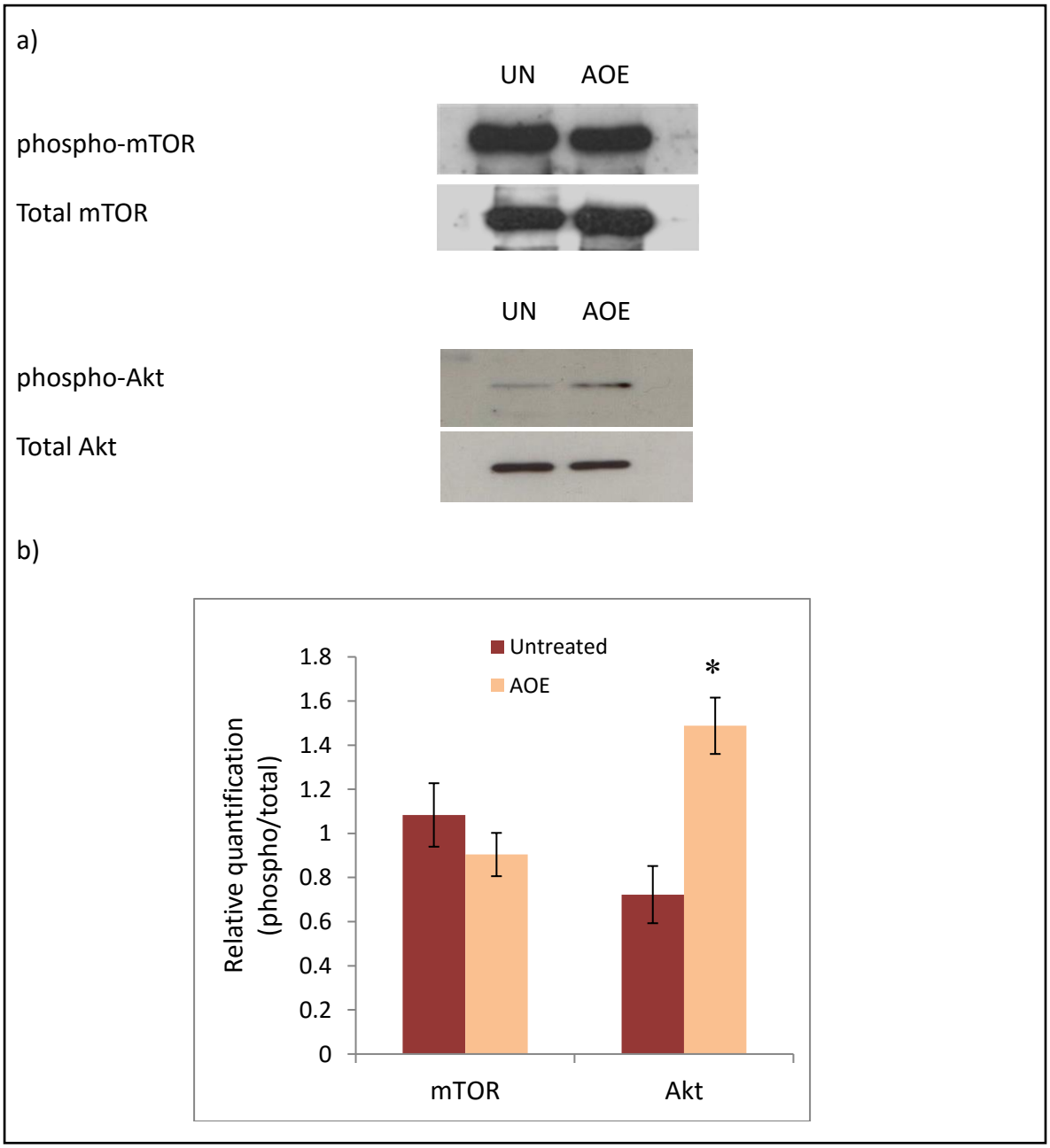


Figure 4.13. Level of phosphorylation of Akt and mTOR pathways after AOE treatment of MCF-7 for 6 hours. Figure show representative images of a replicate experiment (n=3 independent biological replicates). (b) data represent mean ± SD with *=p<0.05 by Unpaired t-test.

4.2.3.3 Effect on phosphorylation levels of TGFβ pathway

Next, the ability of AOE to modify the TGFβ signalling pathway was assessed. The phosphorylation level of Smad2 was assessed after incubation with AOE compared to MCF-7 cells and Hela cells induced with 10 ng/ml TGFβ as positive control. The western blotting results showed no activation of Smad2 in either MCF-7 incubated with AOE or after induction with 10 ng/ml TGFβ (Figure 4.14). This finding demonstrated no active TGFβ signalling in MCF-7 cells in the experiment as window of exposure and, similarly, no activation after 6 hours reprogramming. On the other hand, Hela cells responded to TGFβ stimulation, demonstrating that the negative results obtained with MCF-7 was not due to problems with the western blotting procedure.

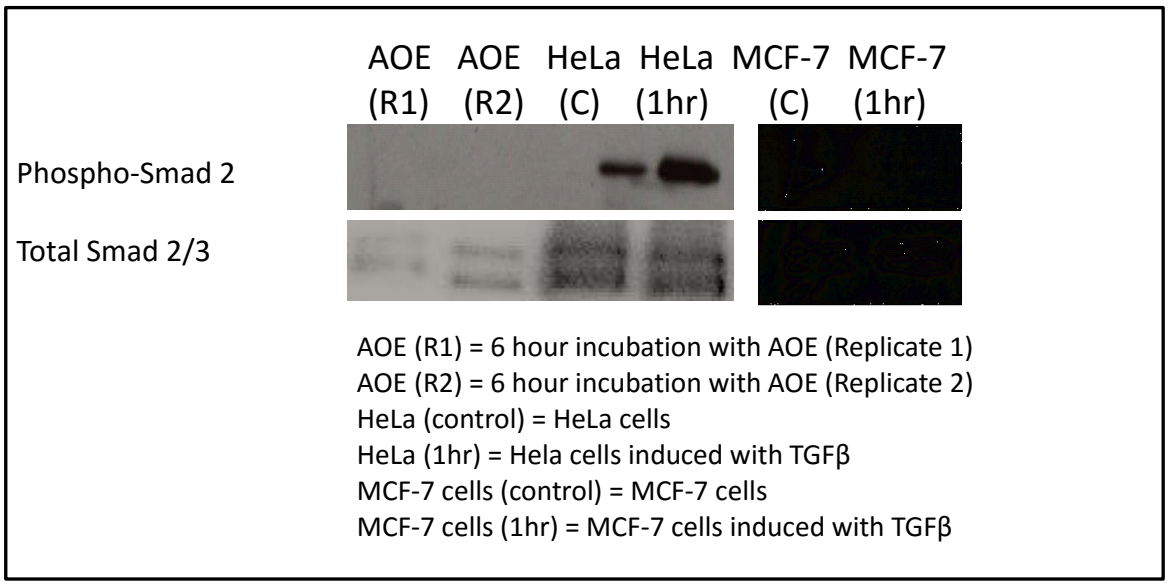


Figure 4.14. **Analysis of phospho-Smad2 and total Smad 2/3 in permeabilised MCF-7 cell incubated with AOE for 6 hours**. Hela and MCF-7 cells were stimulated with 10ng/mL TGF-β1 for 1 hour as positive control for antibody validation.

4.3 Discussion

The first step in the present study involved the optimisation of incubation time for cell permeabilisation before oocyte extract treatment. This is due to the strategy of reprogramming which relies on the transitory permeabilisation with digitonin of the plasma and nuclear membranes of the cells to be reprogrammed with digitonin. Efficient permeabilisation and uptake of regulatory extract components are clearly crucial to obtain proficient reprogramming. Therefore optimisation of several factors is necessary to establish the optimum conditions for cell permeabilisation. Digitonin treatment is a potential detrimental process for the cells as it causes formation of pores in the plasma and nuclear membranes. Different factors that need to be consider are cell concentration, type of cells culture (adherent or suspension), concentration of digitonin, length of exposure to digitonin, quality of extract and time given for resealing of membrane pores and recovery (Agarwal, 2006).

The ultimate results require striking a balance between maximum exposure to digitonin and the health and recovery of the cells after treatment. The conditions optimised for MCF-7 cells in this study allowed about 57.01%±19.14 recovery, with a good level of permeabilisation (84.17±7.06%). Unfortunately it was not possible to achieve 100% permeabilisation without loss of cell viability. Although this represents a limitation of this study, previous studies have shown that these conditions are conducive to reprogramming of somatic and cancer cells *in vitro* and *in vivo* (Allegrucci et al., 2011, Bian et al., 2009, Alberio et al., 2005). Treated cells with AOE extract were plated overnight (12 hour after reprogramming) to allow resealing of the cellular membranes after digitonin treatment. The total number of AOE treated cell recovered was significantly lower as compared to the untreated group. This condition might be due to the growth inhibitory effect of AOE treatment resulting in cell death as well to cells not surviving after the extract incubation in suspension at low temperature. However, the latter explanation seems unlikely since the untreated cells were also exposed to the same incubation conditions. A small reduction in the total number of cells recovered from 2D after four days in culture, albeit not statistically

significant.

An alternative method based on 5-ethynyl-2'-deoxyuridine (EdU) instead of Bromodeoxyuridine (BrdU) incorporation was chosen to assess proliferation of cells treated with AOE. The use of EdU requires a simple, fast protocol for reproducible results as it eliminates the denaturation step that is instead required for BrdU incorporation. Other advantage of using EdU is that the staining is easily multiplexed for relevant broad antibody based targets. EdU is incorporated into cellular DNA throughout DNA replication and accumulates in the nucleus (Yamakoshi et al., 2011). Successful incorporation of EdU in actively replicating cells was detected by fluorescence activated cell sorter (FACS). Several studies reported the used of EdU incorporation to evaluate proliferation of ovarian cancer and osteosarcoma cell lines (Sun et al., 2013, Ryu et al., 2010). EdU was also found to efficiently bind into DNA of replicating cells including NIH 3T3 cells, HeLa cells, and the human T-cell leukemia Jurkat cell line (Yu et al., 2009). Together with the EdU incorporation, double staining with propidium iodide (PI) allowed the identification of cells in other phases of the cell cycle.

An early response of growth inhibition mediated by AOE can explain the result of the cell cycle analysis at 12 hour after reprogramming. There was a significant reduction in the cell number recovery with a reduced but not significant percentage of cells in Go/G1 and S phase in AOE treated group. However, a significant increase in the percentage of cells in G2/M phase was observed at this time point. This effect could be due to the characteristic of vertebrate oocytes that are naturally arrested as maturation begins in G2/M during prophase I of meiosis (Eyers et al., 2005). The activities contained in the AOE may therefore trigger the cell cycle block during the incubation and this could be sustained after 12 hour reprogramming. This G2/M block is physiologically overcome by stimulation with progesterone and resulting in progression of oocytes through meiosis (Tunquist and Maller, 2003).

Viable cells for AOE-treated and control groups were then grown for four days. The morphology of cells in 2D culture did not show any difference between the two

experimental groups, but a significant reduction in the percentage of cells in S phase was measured suggesting a cell cycle arrest before S phase in prolonged cultures. This result is consistent with the cell cycle block observed in the AOE treated xenografts tumours.

In order to understand the molecular mechanisms at the basis of the growth arrest response, the next step in the study was to analyse the expression of genes and the activity of signalling pathways involved in cell cycle regulation. Regulated expression of genes that encode cell cycle proteins is necessary for cell cycle progression (Stein et al., 2001). After receiving signals from growth factor-mediated or intracellular feedback mechanisms, the complex of cyclins and cyclin-dependent kinases (CDKs) catalyses the phosphorylation of transcription factors to direct the expression of cell cycle-regulated genes and structural proteins that are involved in cell proliferation (Stein et al., 2001).

Results obtained in this chapter suggest that some key regulatory genes affected after AOE could mediate cell cycle block and inhibit cell growth. They include CDK4, CDKN1B, CDKN2B and E2F5. A small reduction of CDK4 and upregulation of E2F5 were measured after 6 hours reprogramming, although not significant. A significant difference of expression was instead observed at 12 hours in CDKN1B, gene encoding for the cyclin-dependent kinase inhibitor p27 (Malanga et al., 2012). This protein is particularly important to control cell cycle progression from G1 phase by preventing the activation of cyclin E-CDK2 (Canbay et al., 2010). Upregulation of expression upon the AOE incubation demonstrates the ability to reactivate protein expression of p27 that is frequently reduced in breast cancer thus functioning as tumour suppressor (Belletti and Baldassarre, 2012). The expression of p27 was also upregulated in AOE treated xenografts (see Chapter 3) and therefore this result suggests that reactivation of this tumour suppressor gene is an early event during the reprogramming process. For the cell cycle modulating genes, a significant decreased of expression was demonstrated for JUN after 6 hour of incubation with AOE. JUN encodes c-JUN protein and, in combination with c-FOS, establishes the activator protein 1 (AP-1),

which is an early response transcription factor which is activated by phosphorylation by growth factors signalling (Zhang et al., 2007). During cell cycle progression, c-JUN functions as a regulator of transcriptional levels of cyclin D1 (major Rb kinase). Expression of c-Jun/AP-1 is associated with invasive characteristics of aggressive breast cancer (Vleugel et al., 2006). A study describing the overexpression of c-JUN in the breast cancer cell line MCF-7 demonstrated enhanced AP-1 activity, motility and invasiveness of the cells *in vitro* and tumour incidence in nude mice (Zhang et al., 2007). The decreased expression observed after 6 hours reprogramming is consistent with its function as an early response gene. c-JUN is required to maintain sufficient cyclin D1 for cell cycle progression (Wisdom et al., 1999) and reduced expression of JUN expression could be responsible for the observed cell cycle block in G1/S phase. TOB1, BTG2, MAPK9, MLL5 showed a small change in expression between AOE treated and untreated group, but not significant. The changes of expression in these genes may also contribute to the block of cell cycle.

An important limitation of the reprogramming experiments for the determination of gene expression that are presented in this chapter was that a large variation in responses was observed across biological replicates. Although a clear trend was generally observed in all the biological replicates (either by upregulation or downregulation of gene expression), data were not found always significant when statistically analysed as the fold difference in gene expression were variable. Different strategies were adopted, including logarithmic transformation of the fold differences of analysis by two-way ANOVA with “replication” considered as a covariant, but no major difference in statistical significance was achieved.

Signalling pathways transduce various extracellular inputs during G1 phase that affect DNA replication and cell division. In breast epithelial cells, the signals are generally received from growth factors, steroid hormones (i.e estrogen and progesterone), cytokines, and the extracellular matrix. These signals converge on pRb inactivation by influencing the levels and activity of cyclins and CDK inhibitors to regulate the G1/S phase (Caldon et al., 2006). In late G1 phase, p44/42 MAPK activation increases the activity of CDKs and also inhibit CDKIs activity to allow the transition to S phase

(Chambard et al., 2007).

Therefore, the significant reduction of phosphorylation after AOE treatment could be responsible for the observed cell cycle arrest. p44/42 MAPK is activated mainly due to mitogens and growth factors and contributes to important functions in various cellular responses, including cell proliferation, cell differentiation and cell survival (Torii et al., 2006). In invasive breast cancer and in most of the experimental models of breast cancer progression, signalling through the p44/42 MAPK pathway has been shown to be crucial (Whyte et al., 2009). Mutation in p44/42 MAPK pathway and constitutive activation was observed in 30% of human breast cancers; though, the nature of the deregulation varies depending on cancer subtypes and individual tumours (Whyte et al., 2009).

Another pathway tested in MAPK family was p38 MAPK. This pathway it stimulated by cellular stress such as UV irradiation, heat shock, high osmotic stress, lipopolysaccharides, protein synthesis inhibitors, proinflammatory cytokines and specific mitogens (Zarubin and Han, 2005). p38 MAPK has dual function as a regulator of cell death. It can either mediate cell survival or cell death depending on the type of stimulus and specific cell types (Koul et al., 2013). In additions, it has an important role in modulating cell migration and invasion (Koul et al., 2013).

Treatment with AOE significantly reduced p38 MAPK phosphorylation levels. This results seems to contradict the classical role of p38 MAPK which is to induce cell cycle arrest and apoptosis when activated and not inhibited. Through this mechanism, activation of p38MAPK is able to regulate G1/S checkpoint via activation of p53. This activation results in the accumulation of p21 and further inactivation CDK2 (Thornton and Rincon, 2009). Besides, p38 MAPK is able to directly phosphorylate and stabilize p21 *in vivo* (Kim et al., 2002). Moreover, p38 MAPK could adversely control cyclin D1 at the level of transcription by phosphorylation and stabilization of HMG-box protein 1 (HBP1). It can also phosphorylate cyclin D1 thus resulting in cyclin D1 ubiquitination and proteosomal degradation (Yee et al., 2004). Additionally, p38 MAPK is able to

phosphorylate and activate the degradation of Cdc25A, thus establishing G1/S checkpoint (Goloudina et al., 2003). Other possible mechanisms of G1/S arrest are via regulation of p16INK4a and p19ARF. These proteins encoded in the *INK4a/ARF* locus regulate the G1/S checkpoint in specific ways. p16 activates G1/S checkpoint by inhibiting Cdk4/6 activation, whereas p19 promotes G1/S checkpoint by regulating p53 function (Thornton and Rincon, 2009). However, a non-classical role of p38MARK has been recognised. P38MAPK is also involved in cell cycle checkpoint and cell survival under certain circumstances, for instance in response to DNA damage Some studies have also showed p38 MAPK activation in survival of tumour cells independently of DNA damage, and suggested that p38 MAPK can contributes to metastasis (Thornton and Rincon, 2009).

Another interesting finding from the bioinformatics analysis of microarray data suggested an inhibition of apoptosis in the AOE xenografts. Apoptosis inhibition was also found in MCF-7 lacking p38α mitogen-activated protein kinase (Mendoza et al., 2011) and it is a reported characteristic of growth arrest induced by quiescence (Coller et al., 2006). Therefore further study of p38MAPK signalling in the AOE treated xenografts would be needed to define the role of this pathway in the reprogrammed tumour phenotype.

An additional pathway of the MAPK family tested was JNK. Activation of this pathway is stimulated by mitogens, cytokines, DNA-damaging agents, and cellular stress. JNK signalling pathways is involved in the regulation of gene expression, T-cell differentiation, cell mortality and cell-cycle progression through phosphorylation of diverse substrates including transcription factors, cytoskeletal components, and bcl-2 family members (MacCorkle and Tan, 2005). JNK pathway exerts these diverse functions subject to cell type, stimulus, and context (Wagner and Nebreda, 2009). The signalling is also involved in cell growth and proliferation of unstressed cells. Under some conditions, JNK plays a protective role in promoting cell survival thus stimulating cell proliferation, cell transformation and tumour progression, and embryonic morphogenesis (Davis, 2000). After AOE treatment, no significant difference in the levels of JNK phosphorylation was observed, suggesting that this

pathway might not be responsible for the growth inhibition caused by the treatment.

Two other assessed signalling pathways regulated via tyrosine kinase activation were Akt and mTOR signalling pathways. As a central signal transduction pathway, PI3-kinase-Akt-mTOR pathway controls various critical aspects of cell physiology such as cell survival, proliferation, apoptosis, morphology and migration, protein synthesis, and integration of metabolism (Lauring et al., 2013). To activate the transition of G1/S phase, phosphorylation by Akt causes deactivation of glycogen synthase kinase 3 (GSK-3) that inhibits degradation of cyclin D1 and β-catenin. Thus, TCF/LEF transcription factor target genes are increased rapidly including cyclin D1 (Alao, 2007). Besides, Akt potentially activates cyclin D1 translation by direct phosphorylation of mTOR (Hay and Sonenberg, 2004). Also, Akt is capable either indirectly or directly to regulate CDK inhibitors (p21 and p27) to allow cell cycle transition. p27 is phosphorylated at Thr157 and this prevents its nuclear import (Denicourt and Dowdy, 2004).

The level of Akt phosphorylation after AOE treatment was significantly increased and no significant change was observed for mTOR. Increased level of Akt phosphorylation after AOE treatment could be related to overcoming a G2/M cell cycle checkpoint induced by DNA damage. The mechanism involved may occur via overexpression of a constitutively active form or via the loss of PTEN. Activated Akt also induces the reduction in CDC2 activity and mitotic index after exposure to DNA damage (Kandel et al., 2002).

Finally, the last studied pathway possibly involved in the cell cycle block was TGFβ. Three isoforms of TGF-β (TGF-β1, TGF-β2 and TGF-β3) are expressed in mammalian tissues and these act via the same receptor signalling pathways (Hall et al., 2013). TGFβ controls cell cycle progression by promoting arrest in G1 through expression of cyclin-dependent kinase inhibitors (CDKIs) which in turn inactivate specific CDK activity (Lebrun, 2012). TGFβ-induced gene transcription of p15 and p21 is mediated by Smad association with specific transcription factors, such as FoxO forkhead and Sp1, that then interact with either CDK4 or CDK6 or with CDK4-cyclin D or CDK6-cyclin

D , CDK2-cyclin A or CDK2-cyclin E complexes (Erol, 2011, Lebrun, 2012). Activation of TGFβ signalling pathway was not observed in MCF-7 cells treated with AOE as there was no Smad 2 (TGFβ receptor-associated signalling molecules) phosphorylation was detected. Activation of TGFβ was also not observed after stimulation of MCF-7 with TGFβ for 1 hour. However, at the gene expression level, TGFβ3 expression was increased in response to the treatment after 12 hour reprogramming with AOE, although not significant when averaged across biological replicates, and in AOE reprogrammed xenografts. So it is possible that the failed activation of this pathway was due to insufficient time for reprogramming or of exposure to exogenous TGFβ.

4.4 Conclusions

In this chapter, the expression of several cell cycle regulator and modulating genes was assessed at two different time points (6 hour incubation with AOE and further culture extract-free overnight-12 hour after reprogramming, respectively). A decreased expression of JUN was observed as an early response to the oocyte extract, whereas activation of p27 was found after 12 hours. This represented a significant finding that could be directly implicated in the growth inhibition of xenografts as this time point represents the time of injection if cells into immunocompromised mice.

The involvement of signalling pathways involved during G1/S transition were next investigated. This study was only performed after 6 hours of reprogramming to avoid the confounding effect on cell growth induced by the presence of serum in prolonged culture after reprogramming. Two signalling pathways of the MAPK family demonstrated a significant reduction in phosphorylation level, the p44/42 MAPK and p38 MAPK. Whilst reduction of p44/42 MAPK activity is constituent with cell cycle arrest, reduced activity of p38 MAPK may suggested in inhibiting breast cancer cell survival and an induction of quiescence. No activation of mTOR or TGFβ was observed after AOE treatment, whereas an increased phosphorylation of Akt was measured after 6 hours of reprogramming. This activation might be associated to overwhelm a G_2/M cell cycle checkpoint that is induced by DNA damage. However, the study of interconnected signalling pathways in the short time window of reprogramming

resulted very complex and not all the responses could be easily explained. Therefore, detailed signalling pathway analyses of the xenografts will be needed to clarify their involvement in the growth arrested phenotype of the treated tumours as most of these pathways have context dependent effects and could act in cooperation to mediate an arrest of cell growth which is not associated with cell death.

In summary, the work presented in this chapter proves the ability of AOE to modulate gene expression of important cell cycle regulators. Furthermore, the determination of phosphorylation levels of selected cell signalling pathways revealed that complex mechanisms might be involved during reprogramming with AOE. Therefore, identification of specific mechanisms underlying AOE reprogramming requires the analysis of signalling pathways in the tumour xenografts and use of specific inhibitors of cell signalling involved in the regulation of cell cycle in long-term *in vitro* studies to establish the relative contribution of specific oocyte extract activities to the reprogramming process associated with tumour growth arrest.

Chapter 5: Epigenetic remodelling of histone marks by AOE

5.1 Introduction

The previous experimental chapters presented an insight into the genes and signaling pathways that are affected by the treatment of cancer cells with AOE extracts. The AOE treatment responsible for the growth arrest of breast cancer cells *in vivo* is

accomplished by only 6 hours' exposure. Therefore, another important aspect that is essential for the elucidation of the mechanism of the tumour reversion is the epigenetic effect of the extract in remodelling the chromatin of cancer cells and its impact on gene expression. Indeed, epigenetic modifications of the chromatin act a fundamental role in regulating gene expression without inducing any modifications to the DNA sequence (Bannister and Kouzarides, 2011). Epigenetic modifications are plastic, can respond to environmental clues and are reversible (Shah and Allegrucci, 2013).

The reversibility of epigenetic chromatin modifications makes them a valuable target to counteract malignancy. During modification of the chromatin structure, histones can be methylated, phosphorylated, acetylated, sumolyated, ubiquitinated and ADP-ribosylated and this regulates key cellular processes such as transcription and DNA replication and repair (Sharma et al., 2010). Histone modifications, together with methylation of DNA determine two different states of chromatin configuration, euchromatin or heterochromatin. While the first identifies an open chromatin state that allows binding of transcription factors and gene expression, the latter defines a closed configuration which is not permissive to transcription (Apostolou and Hochedlinger, 2013). Histone tail biochemical modifications are very dynamic and reversible. They constitute a proper "histone code" that can be recognised by other effectors and regulate gene expression (Cohen et al., 2011).

Lysine acetylation is generally associated with chromatin accessibility and activation of transcriptional activity. Methylation of histones, which occurs either at arginine or lysine tails, does not influence the net charge of the chromatin and therefore does not directly impact on DNA-histone interactions. Histone methylation affects transcriptional activity instead by becoming an establishing template for effector proteins modifying the chromatin structure and directing either repression or activation. Hence, histone methylation is able to associate with activation or repression of transcription subjected on which effector protein is associated to lead to specific chromatin states (Cloos et al., 2008). Methylation of several histone marks such as H3-K4, H3-K36 and H3-K79 are mostly associated with transcriptionally active

genes (active marks), whilst methylation of H3-K9, H3-K27 and H4-K20 mark silent genes or heterochromatin (repressive marks) (Vakoc et al., 2006).

The switch from heterochromatin (transcriptionally silent) to euchromatin (transcriptionally active) can be facilitated by chromatin remodelling enzymes involving erasers, writers or readers (Helin and Dhanak, 2013) (Figure 6.1). The erasers comprise histone deacetylases (HDACs) and histone demethylases (HDMs), functioning in removing post-translational modifications (PTMs) from the histone tails. The "writers" enzymes include histone acetyltransferase (HATs), kinases, DNA as well as histone methyltransferases (HMTs) and ubiquitin ligases (Helin and Dhanak, 2013, Chervona and Costa, 2012). The "readers" instead recognise to act on particular modified histone residues, at an epigenetic code level (Rotili and Mai, 2011) (Figure 5.1).

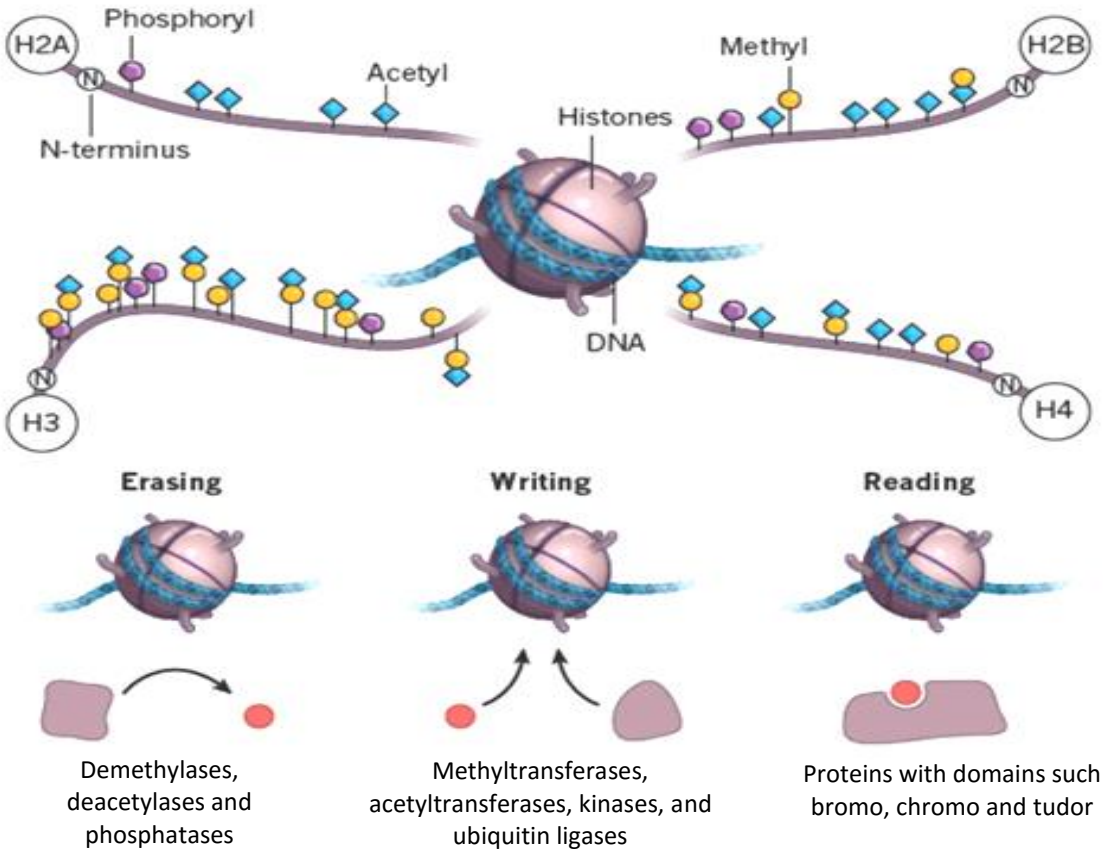


Figure 5.1. **Epigenetic regulators involved in post-translational modification of histones.** The erasers include histone deacetylases and histone demethylases; writers comprise histone acetylases, kinases, DNA and histone methyltransferases and ubiquitin ligases and the readers include proteins with specific domains, such as bromo-, chromo-, tudor, MBT-, PWWP-, WD40- and PHD-domains, which bind to specific modifications (Helin and Dhanak, 2013).

Histone modifications play a crucial function in chromatin remodelling, gene transcriptional regulation, stem cell maintenance and differentiation (Zhang et al. 2009). Misregulation of histone modifying enzymes such as histone deacetylase (HDACs), histone acetyltransferase (HATs), histone methyl transferases (HMTs) and histone demethylases (HDMs*),* can lead to aberrant histone modification. Therefore, occurrence of miswriting, misreading, and miserasing of histone acetylation and methylation marks is able to induce carcinogenesis and tumour proliferation. In most cases, methylation of Arg and Lys tails is stably maintained, in an irreversible manner as a consequence of slow turnover of methyl groups in the chromatin (Rotili and Mai, 2011). Deficiency of histone acetylation and methylation, including loss occurring primarily at the acetylated Lys16 and trimethylated Lys20 residues of histone H4 has been reported in cancer cells, including in the majority of breast cancer cases. Increased levels of total H4 histone acetylation and methylation were also identified mainly connected to a favourable prognosis, with detection mainly found in luminal-like breast tumours (Chervona and Costa, 2012).

Analysis done using Chromatin-immunoprecipitation (ChIP) on breast cancer tissues demonstrated the centromeric satellites (SAT2) are enriched with repressive histone marks H3K9me3 and H4K20me3 which induce gene silencing and define pericentric heterochromatin. In addition, abnormal expression of several histone modifying enzymes was identified being associated to prognosis of breast cancer. Up-regulated expression of EZH2, which is recognised as part of Polycomb Repressive Complex 2 (PRC2) and catalysing methylation of H3-K27, is associated to breast cancer aggressiveness and poor patient prognosis. Lysine-specific demethylase 1 (LSD1) otherwise known as Lysine-specific histone demethylase 1A (KDM1A) are highly expressed in estrogen receptor (ER)-negative tumours. Also, breast cancer patients with elevated expression of histone deacetylase 6 (HDAC6) have a better prognosis compared to those with low levels of the enzyme, when referring to disease free-survival (Chervona and Costa, 2012).

Restoring normal chromatin modifications, either at the level of histone modifications or DNA methylation, is an important goal of cancer therapy. Epigenetic

therapies are currently being developed for the treatment of both liquid and solid tumours, with several clinical trials in progress (Valdespino and Valdespino, 2015). Therefore, understanding how epigenetic alterations contribute to tumorigenesis and how they can be targeted is a very important aspect for novel cancer drugs discovery. The process of nuclear reprogramming can be instrumental for the understanding of the process of tumorigenesis as nuclear reprogramming can be defined as an epigenetic process involving a change from a differentiated state to another cellular one (Halley-Stott et al., 2013).

At the pathological level cancer is generally defined by a loss of differentiation and therefore can mimic the process of reprogramming (Campos-Sanchez et al., 2011). During development, oocytes are naturally able to reprogram differentiated sperm cells to give rise to a totipotent embryo. This process is characterised by a profound reprogramming process in which the sperm chromatin is remodelled into a zygote nucleus. Nuclear transfer experiments with somatic cells have shown that oocytes are also able to reprogram somatic chromatin with remodelling of histone variants, histone tail modifications and DNA methylation (Teperek and Miyamoto, 2013). Importantly, oocytes-specific factors actively participate in the reprogramming process and are readily incorporated into the reprogrammed cells (Jullien et al., 2014).

Extracts prepared from oocytes have shown to be a versatile and useful experimental system to study the molecular and transcriptional events occurring during reprogramming (Leno, 1998, Miyamoto et al., 2009, Halley-Stott et al., 2013). Seminal work with amphibian oocyte has elucidated the molecular events of nuclear reprogramming (Halley-Stott et al., 2013, Jullien et al., 2014, Gurdon, 2013) and extracts made from these oocytes have been used to dissect the epigenetic events participating in reprogramming of somatic cells (Allegrucci et al., 2011, Bian et al., 2009, Bui et al., 2012, Wang et al., 2014). Urodele amphibians (salamander), mostly studies for their regeneration abilities, have also been used as a model for studying nuclear reprogramming. Mammalian and urodele developmental gene circuitries have been demonstrated to be conserved (Dixon et al., 2010, Swiers et al., 2010) and

axolotl oocyte extracts have been used to reprogram breast cancer cells and induce tumour reversion (Allegrucci et al., 2011). The reprogramming process involved reactivation of silenced tumour suppressor genes via promoter DNA demethylation and remodelling of histone modifications. Importantly, these changes were promoted without reprogramming of cancer cells to pluripotency, as re-activation of pluripotency gene expression would be an undesirable outcome in cancer (Allegrucci et al., 2011).

Mammalian oocytes can be also used to study epigenetic reprogramming (add reference Bui et al 2012), but less efficiently than amphibian oocytes due to constraints related to availability, costs and ethical considerations. Bovine oocyte extracts have been shown capable to reactivate silenced tumour suppressor genes with no increased of pluripotency-associated genes (Wang et al., 2013a, Wang et al., 2013b, Wang et al., 2014). Although both amphibian and mammalian oocyte extracts have proved efficient systems to induce cancer cell reprogramming mechanisms, the oocyte molecules responsible for the epigenetic remodelling and tumour reversion effect still remain elusive.

This chapter aims at answering some of these outstanding questions by looking at the epigenetic reprogramming of histone modifications induced by axolotl oocyte extracts at the genome-wide level in the context of breast cancer tumour reversion and induction of growth arrest and quiescence *in vivo*.

The objectives of this chapter were as follow:

i. To determine the remodelling effect of AOE on selected histone marks; trimethylation of histone H3 at lysine 27 (H3K27me3), trimethylation of histone H3 at lysine 9 (H3K9me3), dimethylation of histone H3 at lysine 9 (H3K9me2), trimethylation lysine 4 at histone 3 (H3K4me3), acetylation of histone H3 lysine 9 (H3K9Ac), monomethylation of histone H4 at lysine 20 (H4K20me1), dimethylation of histone H4 at lysine 20 (H4K20me2) and trimethylation of histone H4 at lysine 20 (H4K20me3).

ii. To assess the stability of histone marks at two different time points (6 hour incubation with AOE and further culture extract-free overnight - 12 hour after reprogramming) to elucidate their role in the initiation of tumour reversion.

iii. To assess the impact of epigenetic reprogramming inhibition by the use epigenetic modifiers inhibitors added to the AOE.

5.2 Results

5.2.1 Optimisation of western blotting for the study of histone marks

To identify the most suitable amount of histone proteins to be loaded for western blotting analysis, three different protein loading (2.5, 5 and 10 µg/lane) were assessed for two different histone marks and the canonical histone H3 (used as reference) (Figure 5.2). Based on the results obtained, 2.5 µg of histone loaded gave a good loading and not saturated signal, therefore optimal to allow semi-quantitative measurements.

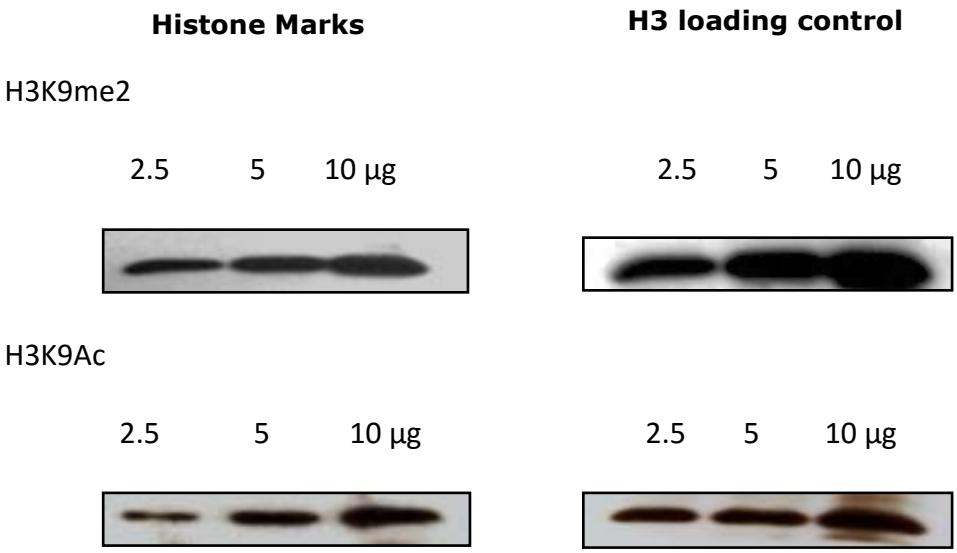


Figure 5.2. Levels of two different histone marks in MCF-7 cells with different amount of histone proteins loaded.

5.2.2 Effect of AOE on repressive histone marks

To assess the ability of AOE to remodel repressive histone marks (H3K9me3, H3K9me2 and H3K27me3) during reprogramming, 2.5µg/lane of histone proteins were loaded and the level of total histone marks were assessed at two different time points by western blotting (after treatment with AOE for 6 hours and further culture extract-free overnight-12 hour after reprogramming). The 6 hour time point was

conducted in order to assess the histone marks changes immediately after AOE treatment, whereas the 12 hour time point gave an overview the condition of the cells before injection into the mice. Results demonstrated that after 6 hour of incubation there was a significant reduction of H3K9me3 and H3K9me2 levels in AOE-treated cells in comparison to the untreated group (Figure 5.3 (a)(b)). However, level of H3K27me3 was significantly increased after 6-hour incubation with AOE (Figure 5.3 (c)). For 12-hour reprogramming, the same pattern of all histone marks was observed as seen at 6 hours of AOE incubation. These results demonstrate the potential of AOE to modulate repressive histone marks in MCF-7 cells and the stability of the epigenetic reprogramming over 12 hours.

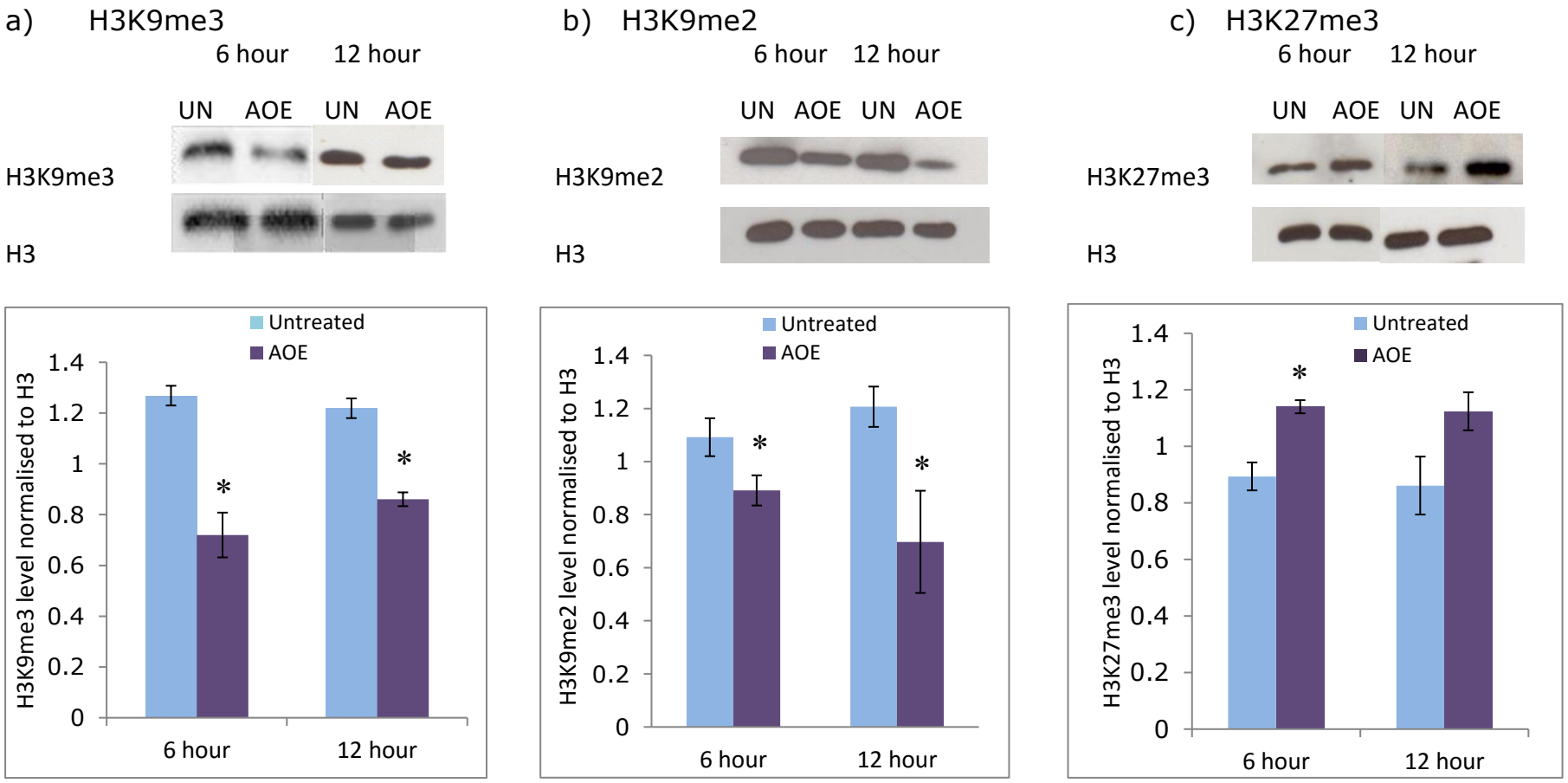


Figure 5.3: **Relative repressive histone modification changes in MCF-7 cells after 6 and 12 hours of reprogramming with AOE (a) (b) (c)**. The values for modified histone levels were normalised to values for H3 histone levels (n=3, mean ± SD, independent biological replicates) $p<0.05$ by Unpaired t-test. UN: Untreated, AOE: Axolotl Oocyte Extract.

5.2.3 Effect of AOE incubation on active histone marks

Two active histone marks H3K9Ac and H3K4me3 were determined after 6-hour incubation in AOE and 12-hour reprogramming by western blotting. As shown in Figure 5.4 (a) (b), the level of H3K9Ac and H3K4me3 histone marks in AOE-treated group was significantly increased after 6 hour incubation with AOE. H3K9Ac higher levels were maintained at 12 hours. However, after 12- hour reprogramming the levels of H3K4me3 were significantly decreased. These results show that factors contained in AOE are able to induce active histone marks that are commonly repressed in cancer cells.

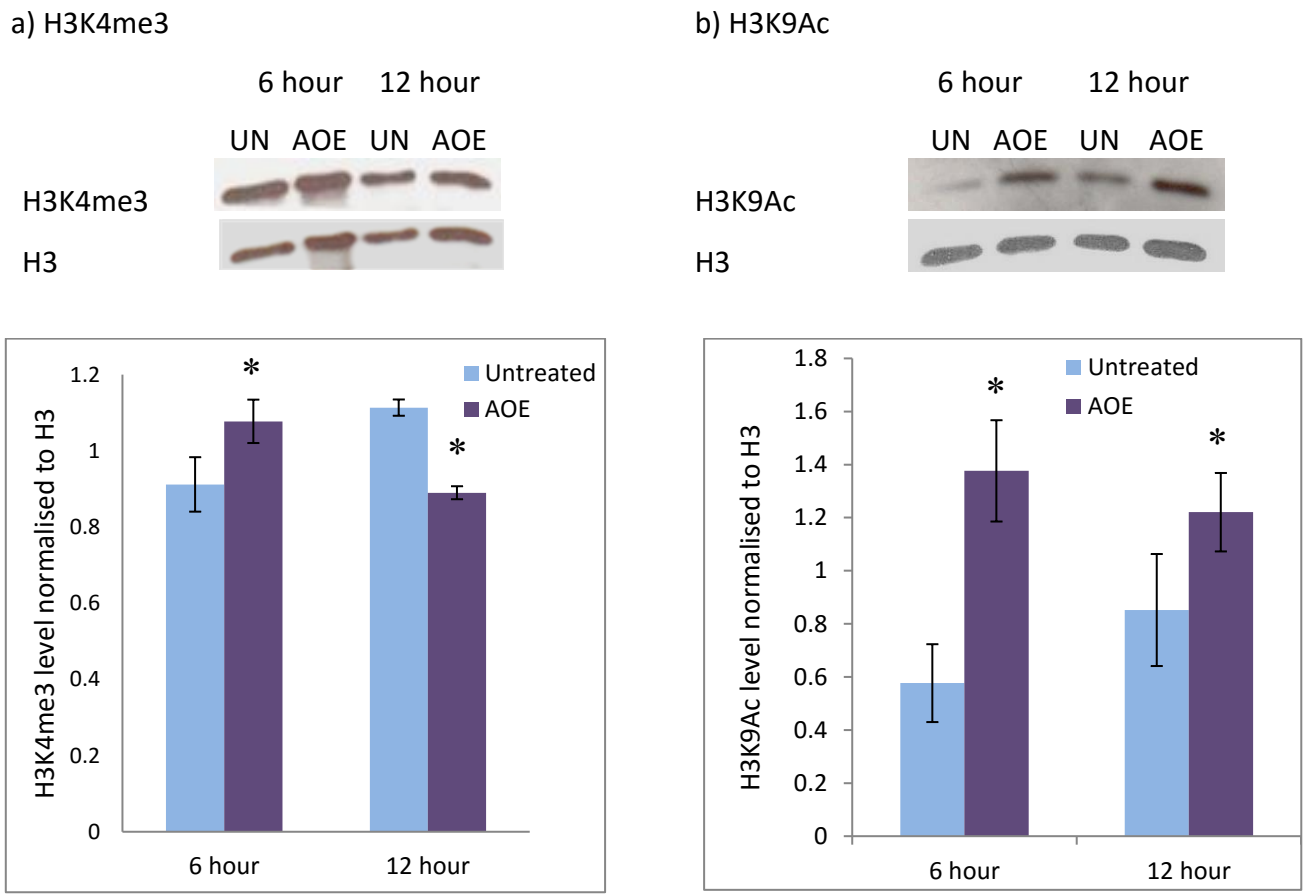


Figure 5.4. **Relative active histone modification changes in MCF-7 cells after 6 and 12 hours of reprogramming with AOE (a) (b)**. The values for modified histone levels were normalised to values for H3 histone levels (n=3, mean ± SD, independent biological replicates) $p<0.05$ by Unpaired T test. UN: Untreated, AOE: Axolotl Oocyte Extract.

5.2.4 Effect of AOE on cell proliferation- and quiescence-associated active and repressive histone marks

Next, other histone marks such as H4K20me1, H4K20me2 and H3K20me3 were assessed by western blotting as directly related to cell proliferation and quiescence. Results in Figure 5.5 (a) (b) (c) show that levels of the active proliferation mark H4K20me1 was reduced after 6 hour of treatment, whereas the levels of quiescence-related H4K20me2 and H4K20me3 were significantly increased. No difference was found at 12 hours for H4K20me1, H4K20me2 and H4K20me3. This might occur due to serum interference when the cells were plated overnight as the quiescence was overcome by the addition of serum in the culture medium.

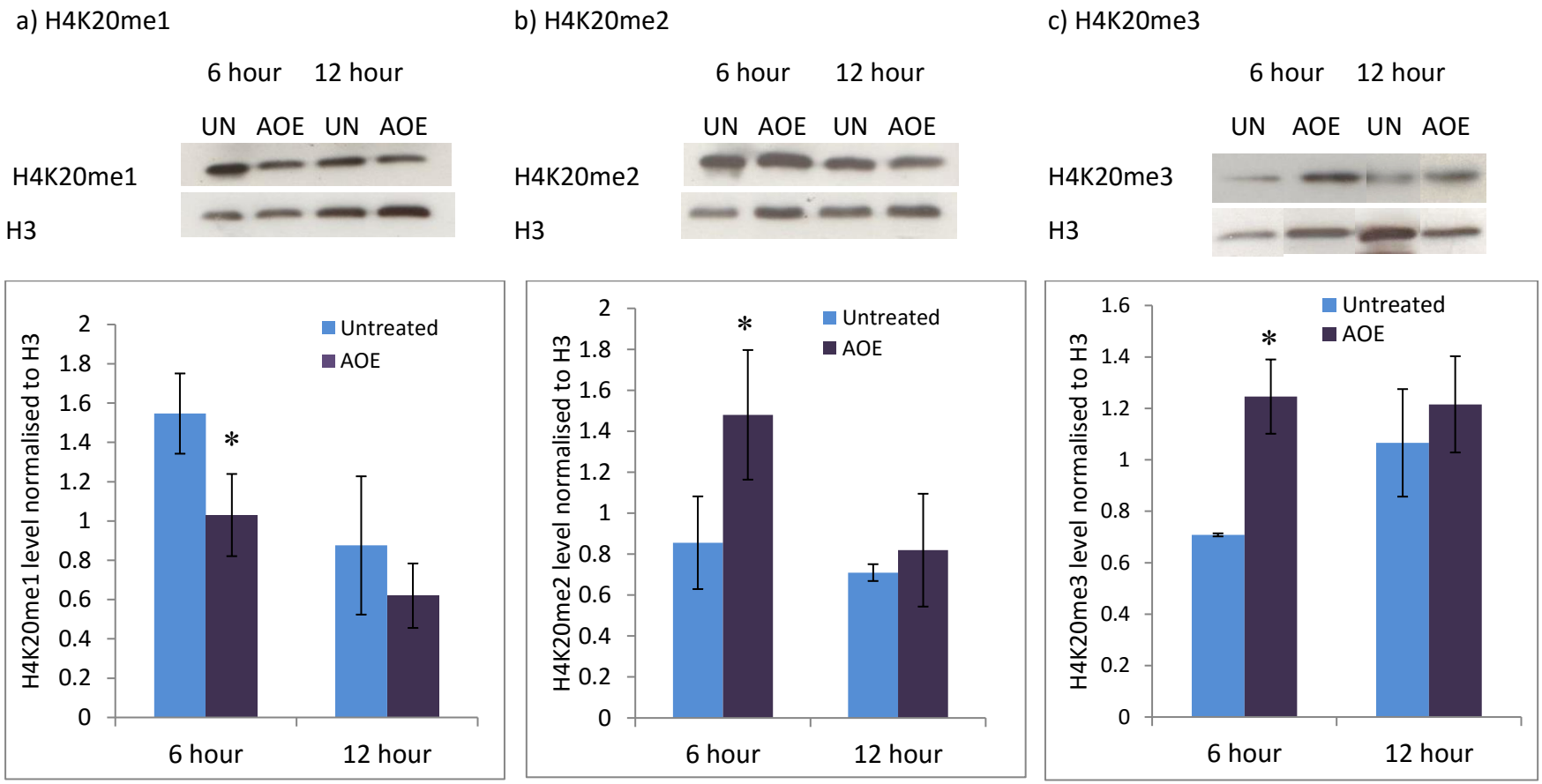


Figure 5.5. Relative histone modification changes associated to cell proliferation and quiescence in MCF-7 cells after 6 and 12 hours of reprogramming with AOE (a) (b) (c). The values for modified histone levels were normalised to values for H3 histone values (n=2 independent biological replicates, mean ± SD $p<0.05$ by Unpaired T test). UN: Untreated, AOE: Axolotl Oocyte Extract.

5.2.5 Effect of epigenetic inhibitors on associated histone marks

To assess the functional role of quiescence-related histone mark changes induced by AOE, after 6 hours reprogramming specific inhibitors of epigenetic modifiers were applied during reprogramming process and the associated histone marks were then identified by western blotting analysis.

5.2.5.1 H4K20me1 after treatment with by Daminozide (PHF8 histone demethylase inhibitor)

The PHF8 histone demethylase is involved in the demetylation of H4K20me1, so the specific inhibitor Daminozide (Tocris Bioscience) was used this enzyme to interfere with the change of this histone mark during reprogramming process in the AOE. Daminozide was reported to selectively inhibit the KDM2/7 enzyme family, including KDM2A, PHF8, KIAA1718 and KDM3A, at the concentration tested (Rose et al., 2012). The concentration of 10 µM was selected as no toxic effect was observed on cells when the concentrations of 1, 5, 10 µM were tested on MCF-7 cells for 24 hour in normal culture conditions (Figure 5.6). Treatment with 10µM Daminozide in AOE for 6 hours decreased the level of H4K20me1 demethylation caused by incubation with the oocyte extract. The level of H4K20me1 was significantly decreased in AOE treated cells compared to the buffer untreated as expected, but the levels of H4K20me1 remained reduced in the presence if the inhibitor instead of being increased (Figure 5.7).

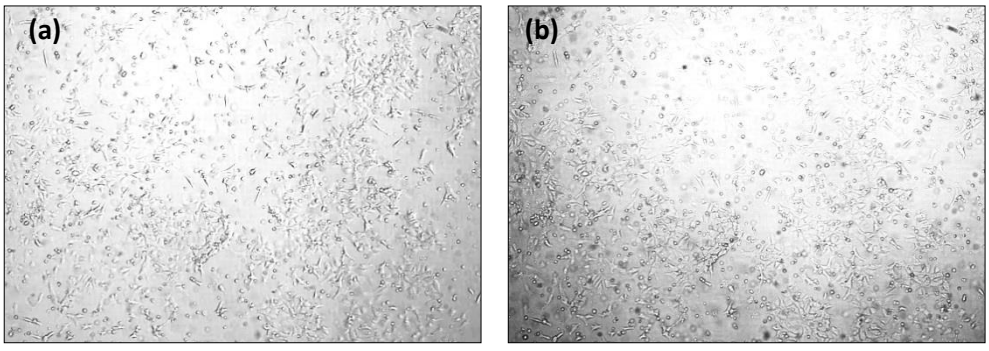


Figure 5.6 Morphology of MCF-7 cells in absence (a) and presence (b) of 10 µM Daminozide. The cells were cultured for 24 hours (10X magnification).

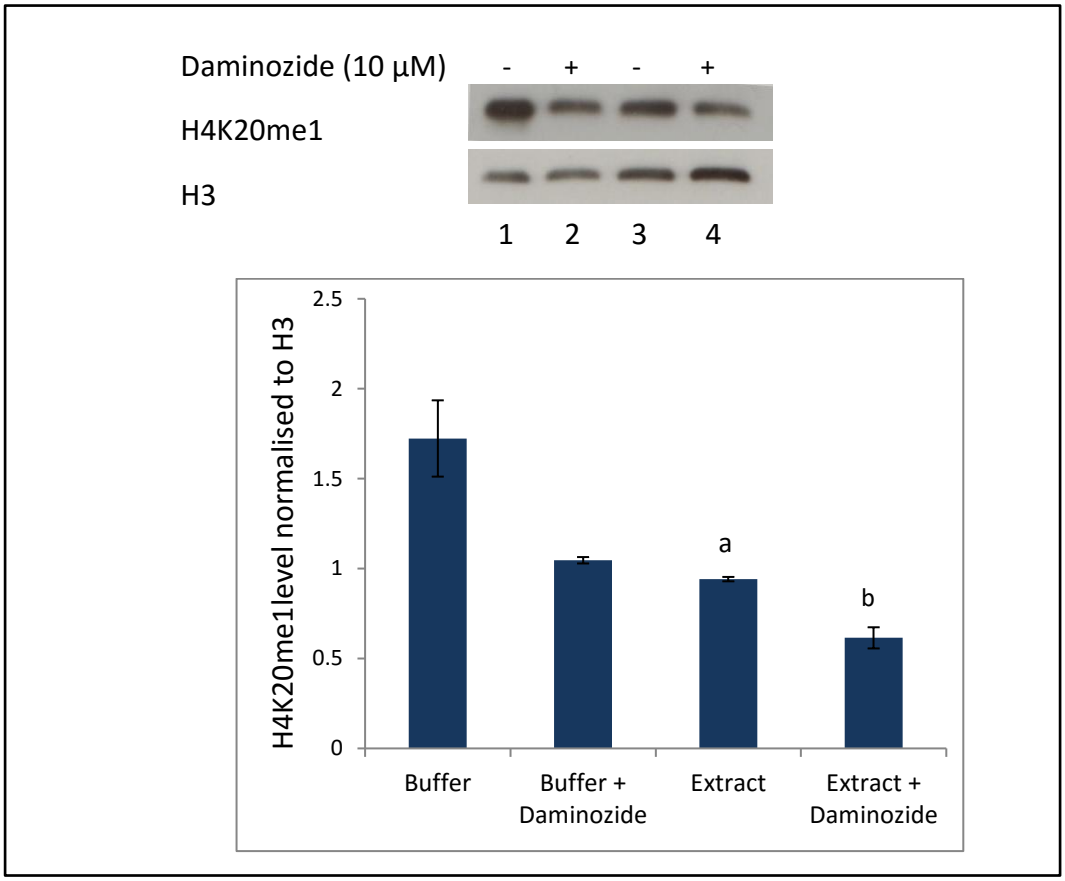


Figure 5.7. Relative histone modification changes of H4K20me1 in MCF-7 cells after 6 hours incubation in AOE in the presence of 10 µM Daminozide (PHF8 inhibitor). The values for modified histone levels were normalised to values for H3 histone values (n=2; independent biological replicates). Lane 1: Buffer (Control), lane 2: Buffer + 10 µM Daminozide, lane 3: Extract and lane 4: Extract + 10 µM Daminozide. Different lower case letters indicate significant differences between different groups in *post hoc* Tukey tests (p<0.05): a= Buffer vs Extract, b= Buffer+Daminozide vs Extract+Daminozide.

5.2.5.2 H4K20me2 and H4K20me3 after treatment with A196 (SUV420H1/H2 histone methyltransferases inhibitor)

SUV420H1 and SUV420H2 are two histone methyltransferases that mediate H4K20me2 and H4K20me3, respectively (Jørgensen et al., 2013). A-196 is the only inhibitor available for those two enzymes (provided by SSG, http://www.thesgc.org). 10 µM of A-196 was used as no toxic effect was observed when this concentration was tested on MCF-7 cells in normal culture conditions (Figure 5.8).

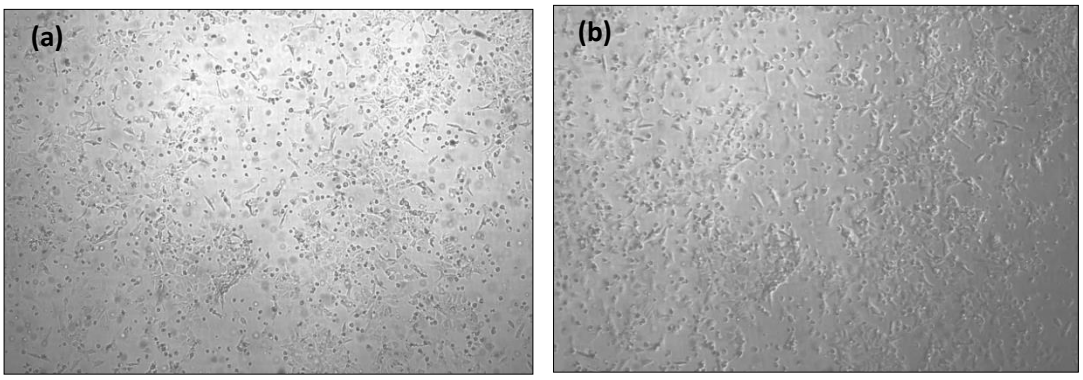


Figure 5.8 Morphology of MCF-7 cells in absence (a) and presence (b) of 10 µM A-196. The cells were cultured for 24 hours (10X magnification).

Results in Figure 5.9 demonstrate the effect on H4K20me2 changes after incorporating the A-196 inhibitor during reprogramming. After 6-hour reprogramming, the levels of H4K20me2 in both groups treated with AOE (AOE and AOE + A196) were increased as compared to other the buffer control groups.

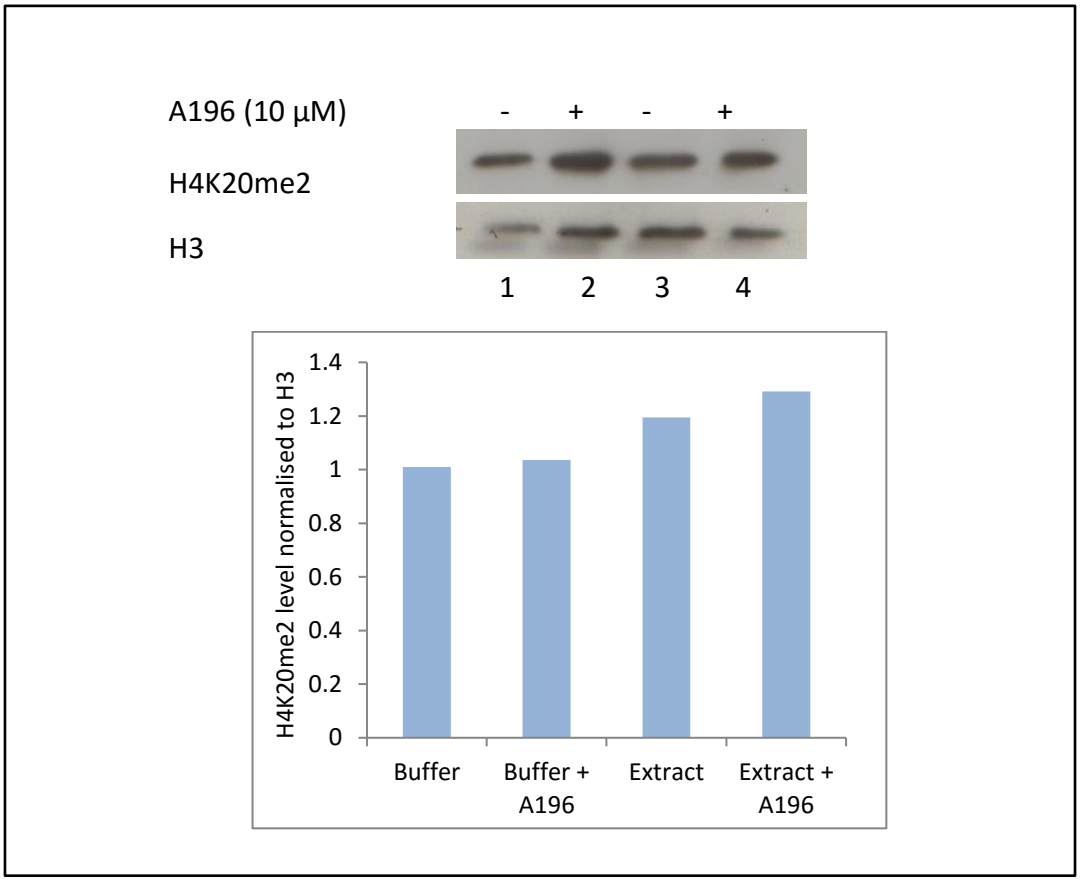


Figure 5.9. Relative histone modification changes of H4K20me2 in MCF-7 cells after 6-hours incubation in AOE in the presence of 10 µM A-196 (SUV420H1/H2 inhibitor). The values for modified histone levels were normalised to values for H3 histone values, n=1. Lane 1: Buffer (Control), lane 2: Buffer + 10µM A-196, lane 3: Extract and lane 4: Extract + 10µM A-196. Different lower case letters indicate significant differences between different groups in post hoc Tukey tests ($p<0.05$): a= Buffer vs Extract, b= Buffer+A196 vs Extract+A196.

Results in Figure 5.10 reveal the changes of H4K20me3 in the presence of the inhibitor. The level of H4K20me3 was increased following treatment of AOE but the inhibitor did not reduce this response.

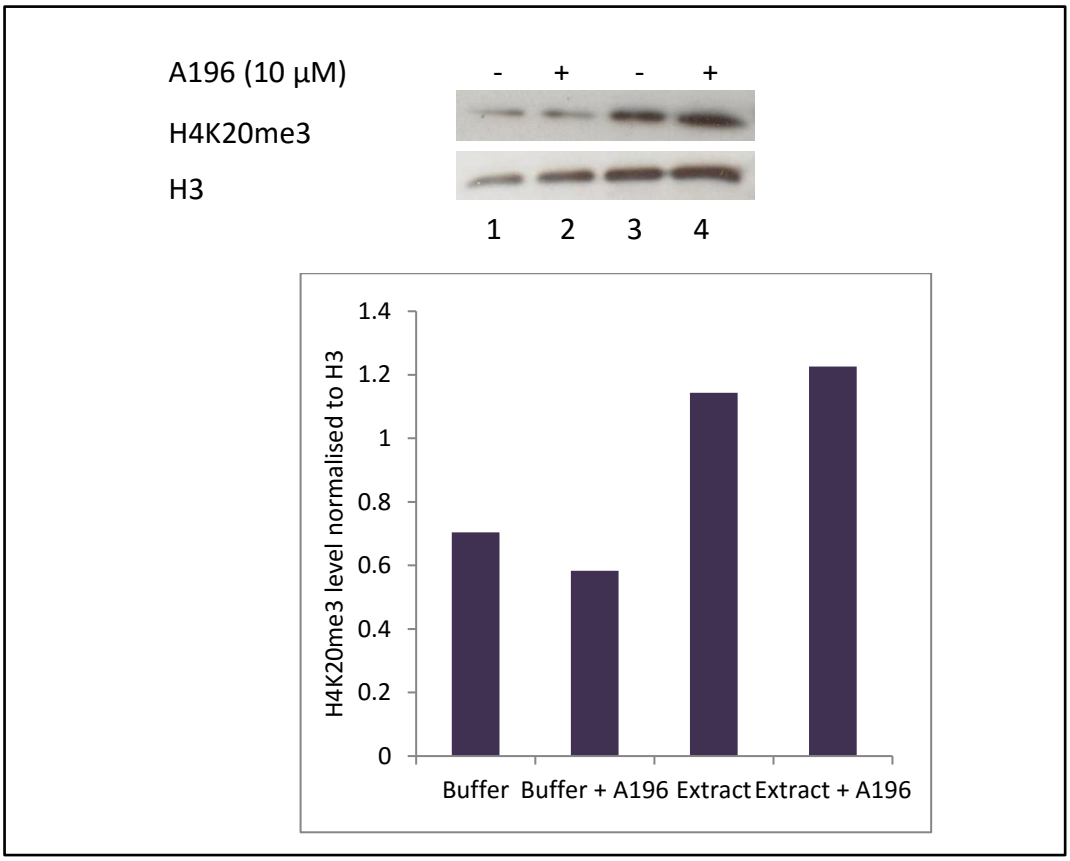


Figure 5.10. **Relative histone modification changes of H4K20me3 in MCF-7 cells after 6-hour incubation in AOE in the presence of 10µM A196 (SUV420H1/H2 inhibitor)**. The values for modified histone levels were normalised to values for H3 histone values, n=1. Lane 1: Buffer (Control), lane 2: Buffer + 10 µM A-196, lane 3: Extract and lane 4: Extract + 10 µM A-196. Different lower case letters indicate significant differences between different groups in post hoc Tukey tests ($p<0.05$): a= Buffer vs Extract, b= Buffer+A196 vs Extract+A196.

5.3 Discussion

Histone modification is one of the key events in the epigenetic reprogramming approach for cancer treatment. Due its reversible nature, histone modification could provide a great target for promising applications towards novel treatments for breast cancer (Falahi et al., 2014). In this study, axolotl oocytes extracts (AOE) were used for their reprogramming capacity to modify several histone marks in breast cancer cells (Allegrucci et al., 2011). Histone modifications regulate gene expression and their alteration are observed in cancer (Chervona and Costa, 2012). Several aberrant histone modifications in breast cancer have been reported. They include H4K16ac

(low or absent in most of breast cancer cases), H3K9ac, H3K18ac, H4K12ac, H3K4me2, H4K20me3 and H4R3me2 (moderate to low levels) and were detected in carcinomas of poorer prognostic subtypes, involving basal carcinomas and HER-2-positive tumours (Elsheikh et al., 2009).

In this study, histone marks were remodelled after 6 hours treatment in the extracts and generally those changes were maintained at 12 hours, indicating the stability of the epigenetic reprogramming. Two possibilities could be at the basis of the histone modification reprogramming. Either this changes could be mediated by specific activities in the extract or by an activation of cell-endogenous enzymes following the treatment. Both possibilities are valid and results obtained in this study cannot discriminate them. AOE demonstrated the ability to reduce level of the repressive histone marks H3K9me3 and H3K9me2 at genome-wide level. These results are consistent with the previous work did by Chromatin immunoprecipitation (ChIP) analysis on gene promoters regions (Allegrucci et al., 2011). Another study reported by Bian et al. (2009) also demonstrated that the level of these marks was reduced when somatic cells were treated with axolotl oocytes to remodel chromatin. Two demethylases enzymes are potentially able to eliminate hypermethylation, the lysine-specific demetylases (LSD1) and the Jumonji (JmJC) domain protein family. Demethylase activity have been recognised for histone H3 lysine 4 (H3K4), H3K9, H3K27 as well as H3K36 (Nottke et al., 2009).

A study by (Metzger et al., 2005) described the ability of LSD1 to demethylate H3K9me2 when co-localised with the androgen receptor to stimulate transcription in normal human prostate and prostate tumours. Moreover, LSD1/KDM1 also has been demonstrated to function as H3K9me2/1 demethylase in two homologs of S. pompe resulting in slow growth phenotype (Lan et al., 2007). In breast cancer, LSD1 was found highly expressed in estrogen receptor (ER)-negative tumours. LSD1 is important for mammalian development as well as during tumorigenesis (Zhao et al., 2012). Inhibition and knockdown of LSD1 affected growth of breast cancer cells and revealed the ability of LSD1 to be recruited at several genes related to proliferation such as p21, ERBB2 and CCNA2. LSD1 also offers a prognostic marker for aggressive

tumour biology and represents A convincing therapeutic target for the treatment of ER-negative breast cancers (Lim et al., 2010). Moreover, the proliferation of estrogen receptor alpha positive and negative breast cancer cells were changed when LSD1 inhibitor were incorporated, proving the importance of LSD1 in breast cancer (Pollock et al., 2012).

The other class of enzymes reported with demethylase activity, JmjC-domain-proteins includes 30 different JmjC domains-containing proteins with 17 being able to demethylate specific lysines or arginines in the H3 tail (Cloos et al., 2008, Helin and Dhanak, 2013). JmjC-domain-proteins are able of demethylating mono-, di- and tri-methylated substrates (Grant et al., 2004). The importance of JHDM2A/KDM3A to mediate the demethylation of H3K9me2 has been demonstrated in spermatogenesis further affecting expression of transition nuclear protein 1 (*Tnp1*) and protamine 1 (*Prm1*) genes. These two genes are required for compaction and condensation of sperm chromatin (Okada et al., 2007). In another study, the JMJD2A/KDM4A family were demonstrated to be able to demethylate trimethylated H3-K9 to dimethylated but not mono- or unmethylated products in HeLa cells (Whetstine et al., 2006).

Results of another repressive histone mark, H3K27me3 were different as reported previously by ChIP analysis on gene promoters (Allegrucci et al., 2011). In this study, the level of H3K27me3 was significantly increased in response to AOE. This finding could be linked to the increased activity of enhancer of zeste (EZH) 2, an enzyme responsible for methylation of H3K27me3, in the extracts. EZH2 is a component of polycomb repressive complex 2 (PRC2) that is associated to chromatin condensation. Besides, H3K27me3 epigenetic mark is elevated in quiescent neural cells and reduction of this mark is associated with cell differentiation (Bertonnier-Brouty, 2014). Elevated level of EZH2 with silencing of EZH2 target genes correlated to cancer aggressiveness and poor prognosis in breast, kidney and lung (Yoo and Hennighausen, 2012, Lim et al., 2010). In follicular lymphoma, evidence revealed mutations occurring in the SET domain of EZH2 affecting the diffusion of large B-cell lymphoma (DLBCL) due to upregulation of H3K27me3. Therefore, these results propose that deregulated level of H3K27me3 can occur via overexpression or point

mutation of EZH2, further suggesting an interesting approach for enzyme therapeutics.

In contrast, inactivating mutations of EZH2 were demonstrated in myelodysplastic syndrome, which might propose a possible function as tumour suppressor. Hence the role of EZH2 and H3K27 methylation in progressing or inhibiting tumour development is context dependent. Therefore, the increased level of H3K27me3 after AOE treatment could potentially act as a tumour suppressor further suggesting a contribution to reduced cell proliferation. Two active histone marks were also tested for the reprogramming capacity of AOE. The increased level of H3K9Ac and H3K4me3 were consistent with previous findings on reactivation of gene promoters after AOE and *Xenopus* oocyte extracts (Allegrucci et al., 2011). Results in this chapter showed a significant increase of H3K9Ac in AOE-treated. This finding is also consistent with results demonstrated in somatic cells as reported by Bian et al. (2009) that showed massive increased of H3K9Ac after oocyte extract incubation.

Similar findings were also reported using porcine GV stage oocyte extracts, whereby cells were greatly acetylated in response to GV-extract treatment (Miyamoto et al., 2009). In cancer cells, the loss of acetylation mediated by HDACs causes gene repression. HDACs are normally found overexpressed in many cancer types. To work in corporation with HDACs in sustaining low histone acetylation levels, HATs also need also to be changed in cancer (Sharma et al., 2010). Therefore, the result obtained with AOE treatment could be potentially due to the increase of expression of HATs to modify chromatin from heterochromatin to euchromatin. Histone acetylation results in a negative charge of the modified lysine residue, influencing a decreased contact between the histone and DNA that is normally connected with active transcription (Cloos et al., 2008).

Another active histone mark, H3K4me3, was increased after AOE treatment. Thus far, several specific H3K4 methyltransferases have been identified including SET domain-containing methyltransferases, varied lineage leukemia 1–5 (MLL1−5), SET1A/B, SET7/9, SET and MYND domain-containing protein 1–3 (SMYD1−3), Absent,

Small, or Homeotic 1-like (ASH1L), SET domain and Mariner transposase fusion gene (SETMAR), and PR domain zinc finger protein 9 (PRDM9). In addition, with modifications related to transcription initiation, H3K4me3 takes place in particular promoter regions of dynamically transcribed genes (Guenther et al., 2007).

Next, three histone marks that are linked to proliferating cells and quiescence were assessed: H4K20me1, H4K20me2 and H4K20me3. Histone H4 lysine 20 (H4K20) characterises one of the nucleosomal core histone modifications which contributes to several cellular processes. H4K20me1 plays an important role in transcriptional activation and cell cycle regulation. H4K20me2 is involved in DNA replication and repair. H4K20me3, the mark most significantly enriched in quiescent chromatin, is connected with repression of transcription when found at promoters (Wang et al., 2008). Besides, H4K20me3 is crucial for silencing of repetitive DNA and transposons (Schotta et al., 2008). Hence, the loss of H4K20me3 together with reduction of H4K16ac has been recognised as a hallmark of human cancers (Fraga et al., 2005).

The reduction of H4K20me1 and increased levels of H4K20me2 and H4K20me3 after AOE treatment pointed out a chromatin state associated with quiescence. A study conducted by Evertts et al. (2013) demonstrated that levels of H4K20me2 and H4K20me3 were increased in quiescence cells and other histone modifications were seen at no different levels in proliferating cell and quiescence cells. Besides, the level of H4K20me1 was increased after S phase as it was converted to H4K20me2 and H4K20me3 moving into quiescence. H4K20me1 is very dynamic all through the cell cycle transition and progresses to highly enriched in S phase to peak at G2/M (Huen et al., 2008, Schotta et al., 2008, Luo et al., 2013) and on mitotic chromosomes (Jørgensen et al., 2013).

There are at least three methyltransferases (PR-Set7, Suv4-20h1, and Suv4-20h2) and one demethylase (PHF8) directly regulating H4K20 methylation (Beck et al., 2012). Demethylation is crucial for cell cycle control, mainly for indicating the starting point for DNA replication (Kuo et al., 2012) as well as required for DNA damage response. The two major enzymes inducing H4K20me2 and H4K20me3 are Suv4-20h1 and

SUV4-20H2, respectively (Schotta et al., 2008). The loss of H4K20me3 genome-wide was found in numerous cancer types including breast cancer. When established human breast cancer cell lines (MCF-7, MDA-MB-231 and MDA-MB-231(S30) were classified based on prominent epigenetic alterations, MDA-MB- 231 had a major loss of DNA methylation together with modified expression of DNMT1, methyl-binding proteins MeCP2 and MBD2, reduced H4K20 me3 and hyperacetylation of histone H4 when compared to MCF-7 cells.

The reduced in H4K20me3 in MDA-MB-231 cells was simultaneous to decreased expression of Suv4-20h2 histone methyltransferase (Tryndyak et al., 2006). Moreover, the low level of H4K20me3 was highly connected with lower disease-free survival and might be used as an independent marker of poor prognosis in breast cancer patients (Yokoyama et al., 2014).

To further elucidate the involvement of oocyte molecules to induce histone modification changes, specific inhibitors targeting enzymes that catalyse modification of histone marks were used. To block the demethylase activity that modified H4K20me1 in AOE, Daminozide (N-dimethylamino) succinamic acid, 160 Da, a plant growth regulator that selectively inhibits the KDM2/7 JmjC subfamily, including PHF8 activity, was used during reprogramming. Results suggest Daminozide did not block the demetylation during AOE incubation. The inhibition of enzymatic activity might not have occurred possibly due the need of longer treatment time for the inhibitor to exert its effect. Another factor that could influence this result was the incompatibility of this inhibitor with human cells as no other study reported on its inhibiting activity. Daminozide inhibitory activity has been reported in plants as a plant growth retardant. However, due to toxicity issue this inhibitor was no longer used for that purpose (Rose et al., 2012).

Another inhibitor used, A-196 act specifically to block histone methyltransferase for both homologous enzymes, SUV420H1 and SUV420H2. The effect of inhibition

SUV420H1 enzyme activity in AOE could be seen at 12-hour reprogramming as the level of H4K20me2 was slightly lower as compared to AOE group alone. However, the inhibitor used seemed not responsive to block SUV420H2 activity. The level of H4K20me3 was slightly increased compared to AOE alone for both time points. This condition is believed possibly due to longer time of exposure required for the inhibitor to response. Thus far, very limited information revealed on this inhibitor act on animal and human cell lines.

6.4 Conclusions

The results obtained show that treatment with AOE induced epigenetic reprogramming by decreasing levels of H3K9me3, H3K9me2 and H4K20me1, and increasing H3K27me3, H3K9Ac, H3K4me3, H4K20me2 and H4K20me3 after 6-hour incubation. 12 hours after reprogramming, level of H3K9me3, H3K9me2, H3K27me3, H3K9Ac, H4K20me2 and H4K20me3 maintained at the same pattern obtained after 6 hour of incubation. These results may suggest the epigenetic reprogramming induced by the extract is stable up to this time point and that cells do not re-acquire their altered epigenetic memory after being re-plated in culture. The elevated levels of H3K27me3, H3K9Ac, H4K20me2 and H4K20me3 as well as decreased level of H4K20me1 strongly suggest that cells undergo quiescence after AOE treatment (Everttts et al., 2013, Hansen et al., 2008, Kouzarides, 2007).

Different enzymatic activities in the extracts might cause the reprogramming process directing the modification of histone marks. Application of specific inhibitors such as Daminozide (to block histone demethylase) and A-194 (to block histone methyltransferases) that act on particular enzymes during reprogramming process had the potential to revealed the function of specific enzymes in the extract that could potentially direct the epigenetic reprogramming. However, the results obtained with these inhibitors remain inconclusive and more biological replicates will be needed to draw any conclusion. Nevertheless, in view of the fundamental

contribution in the different stages of carcinogenesis, approaches aimed at remodelling of histone modifications provide good biomarkers for disease progression and targets for treatment.

Chapter 6: General Discussion

6.1 Summary of work

The reprogramming capacity of oocytes has been reported by several studies (Gurdon and Wilmut, 2011, Bui et al., 2012, Allegrucci et al., 2011). Specific transcription factors and chromatin remodellers contribute to the oocyte's natural epigenetic reprogramming capacity (Awe and Byrne, 2013). Hence, this project aimed to utilise the reprogramming capacity of oocytes to scrutinise the mechanisms behind the reprogramming of breast cancer cells. Published results on epigenetic reprogramming of breast cancer cells by axolotl oocyte extracts (Allegrucci et al. 2011) were used as a starting point for the present study. As presented in Chapter 3, analysis of extract treated tumour xenografts microarrays highlighted that oocyte-mediated reprogramming of breast cancer cells involves a block of cell cycle in G_1/S transition. The absence of staining of the cell proliferation marker BrdU in treated xenografts further confirmed the involvement of a cell cycle block. Hence, the relationship between cell signalling and chromatin remodelling was studied and the hypothesis for this work was that epigenetic remodelling of cancer cell chromatin by axolotl extract would make cells susceptible to the effects of signalling pathways that arrest the cell cycle to inhibit malignant growth.

In Chapter 4, cell cycle analysis was conducted to further understand cell cycle progression after 6-hour incubation and after 12-hour reprogramming with AOE (same time points used for injection of MCF-7 cells into the mice). The results obtained revealed a significant reduction of cells in G2/M phase in the AOE group compared to the untreated after 12-hour reprogramming. In long-term culture (four days), AOE treatment reduced the percentage of cells in S-phase. By gene expression analysis, JUN expression was found reduced as an immediate response to the AOE treatment, whereas activation of p27 was found after 12 hours. Furthermore, a significant reduction of phosphorylation levels of p44/42 MAPK was observed in the AOE-treated group which correlated to cell cycle arrest. Given the critical function in

various cellular responses, including cell proliferation, cell differentiation and cell survival, mutation in p44/42 MAPK pathway and constitutive activation is observed in 30% of human breast cancers (Torii et al., 2006, Whyte et al., 2009). Therefore, the observed inhibition of this pathway after treatment represents a significant finding. In Chapter 5, the reprogramming ability of breast cancer cells were correlated to reduced levels of pathway after treatment represents a significant finding.

In Chapter 5, the repressive histone marks such as H3K9me2 and H3K9me3. In breast cancer, aberrant DNA hypermethylation of some important genes as well as mutated histone methyltransferases are frequently found (Falahi et al., 2014, Berdasco and Esteller, 2010). Treatment with AOE also revealed the ability to increase levels of two active histone marks, H3K9Ac and H3K4me3. In addition, decreased level of H4K20me1 together increased level of H4K20me2, H4K20me3 and H3K27me3 suggested AOE-treated cells underwent a status of cell cycle arrest leading to quiescence. Finally, application of histone modifiers inhibitors during reprogramming were implemented to identify possible activities that could be responsible for the observed growth arrest after treatment with axolotl oocyte extracts.

6.2 Cell cycle arrest mediated by oocyte extracts

Gene ontology and IPA analysis of tumour samples from an *in vivo* study that was previously conducted in our laboratory (Allegrucci et al., 2011) suggested the involvement of cyclins and cell cycle regulation in cancer growth inhibition. Specifically, it was predicted that oocyte-mediated reprogramming of breast cancer cells might involve a block of cell cycle in G_1/S transition. In Figure 6.1 are presented several important regulators which participate during this transition such as cyclin-kinases complexes CDK4/6-cyclin D and CDK2-cyclin E as well as extracellular or intrinsic stimuli such as TGFβ, DNA damage, UV stress, replicative senescence, growth factor removal and contact inhibition.

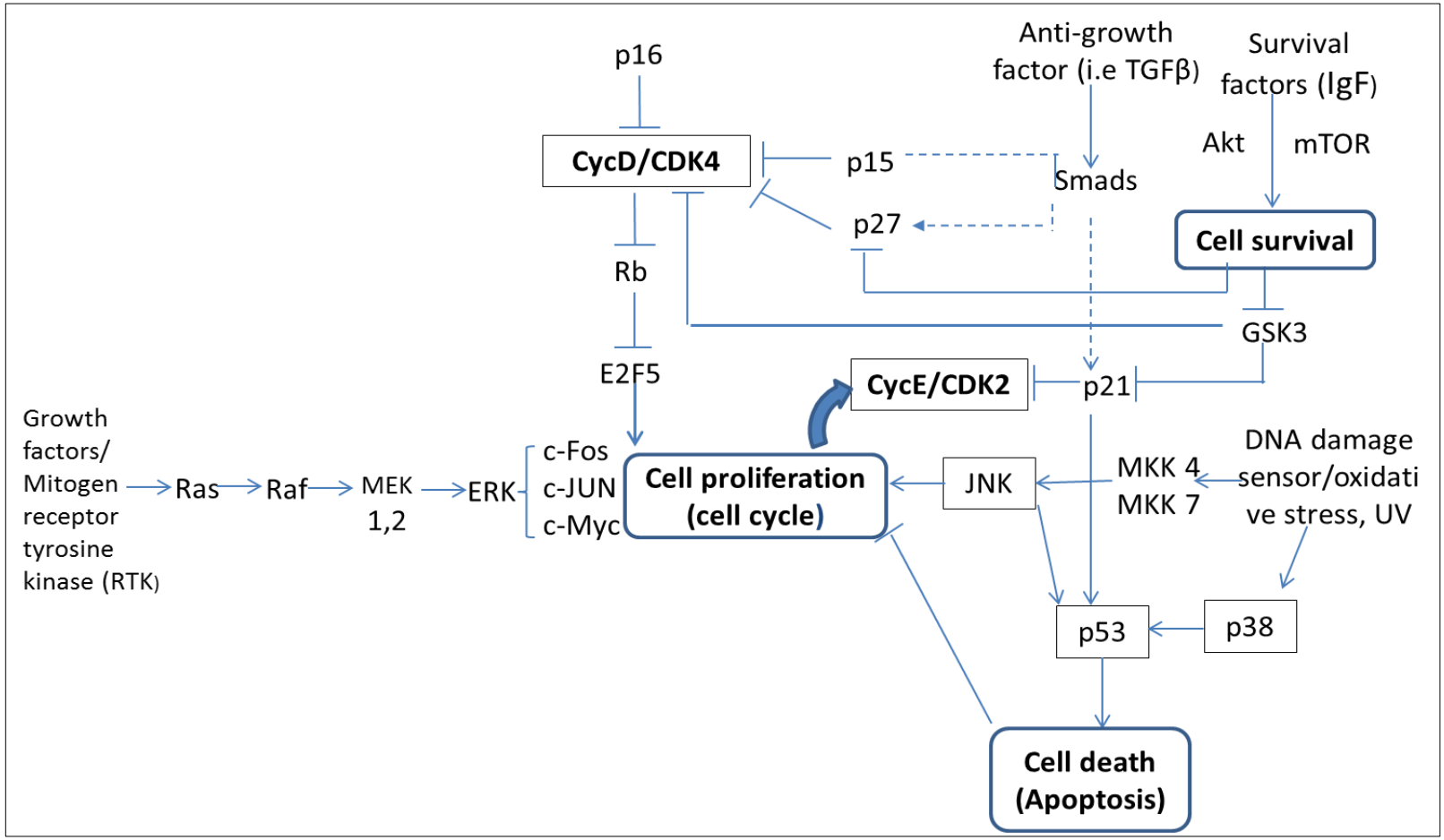


Figure 6.1. Key molecules participating during G1/S phase transition in the cell cycle.

Moreover, the main transcriptions factors involved are indicated. They comprise retinoblastoma protein (Rb), the transcription factor E2Fs as well as CDK inhibitors that function as negative regulators on the activity of CDK complexes. This diagram was therefore used to guide the experimental design of this project. The main finding of the present study highlighted JUN, p27 and MAPK signalling pathways being significantly involved in reprogramming of AOE-treated cells. The reduction of JUN (early response gene), upregulation of p27 (tumour suppressor gene) together with reduced phosphorylation of p44/42 MAPK after AOE treatment strongly suggests that the inhibition of cell proliferation of breast cancer cells occurs via modulation of cell cycle by p44/42 MAPK signalling. The important involvement of JUN/AP-1 in tumorigenesis and metastasis in breast cancer has been proved both *in vivo* and *in vitro* studies (Zhang et al., 2007, Smith et al., 1999).

For p27 regulation, its decreased expression has been correlated to the development of several cancers, including breast cancer (Chu et al., 2007, Guan et al., 2010). Furthermore, low level or loss of p27 expression is correlated to reduced survival and unfavourable prognosis for breast cancer (Guan et al., 2010). The involvement of MAPK signalling cascade has been implicated in breast cancer with activation p44/42 MAPK pathway associated in breast cancer growth (Whyte et al., 2009). The relationship between p27 and MAPK has been studied and reports showed that p44/42 MAPK activation regulates phosphorylation of p27 which is associated with its degradation (Osaki and Gama, 2013). In turn, p27 can inhibit MAPK activation by sequestering H-Ras (Fabris et al., 2015). In quiescent normal cells, p27 is localised in the nucleus and oncogenic activation of MAPK can promote its cytoplasmic accumulation, as observed in many cancer types (Chu et al., 2008), where it promotes cell motility and metastasis. The AOE treatment not only increased the expression of p27 but also changed the localisation from cytoplasmic to nuclear. This is certainly an important finding as reduced p27 expression is associated with increased proliferation, high tumour grade and ER and PR negative status and poor prognosis.

Molecules contained in the oocyte extracts could be involved in this response. A study conducted by (Jullien et al., 2014) revealed that oocytes-specific factors

dynamically contribute to the reprogramming process and are assimilated into reprogrammed cells (Jullien et al., 2014). Non-ovulated oocytes are arrested in prophase of meiosis I and this arrest is dependent on cAMP and cGMP which are downstream of the G-coupled receptor GPR3. Activation of this orphan receptor induces increase of these second messengers and inhibition of cyclin B accumulation (Yang et al., 2012). Elevate cAMP drives activation of PKA type I (PKAI) which in turn inhibits p44/42 MAP phosphorylation which is essential for meiosis progression and oocyte maturation (Li et al., 2012). Therefore, it is possible that oocyte molecules could be directly responsible for the block of p44/42 MAPK activity.

Although breast cancer is a complex disease, promising findings are reported on Ras-Raf-MEK-ERK (MAPK pathway) contribution in breast tumorigenesis. As the aberrant activation of MAPK pathway occurs due to up-regulated or mutated receptor tyrosine kinases, Ras or B-Raf mutations, Raf kinase and MEK inhibitors have been developed such as BAY 43-9006, V600E B-Raf and CI-1040. The inhibitors work conclusively with tumours with fundamentally active or aberrant Ras or Raf signalling, nevertheless they can also be used in case of unregulated MAPK signal transduction as a consequence of overexpression of upstream growth factors or their receptors (Sridhar et al., 2005). Combining MAPK inhibitors with other drugs targeting important pathways in breast cancer such as phosphoinositide-3-kinase or EGFR could be an efficient and promising therapeutic approach. Also, combination of chemotherapy and/or radiation therapy may also be predominantly successful, and could prevent recurrence of cells in between cycles of treatment (Roberts and Der, 2007).

6.3 Inter-relationship of cell signalling and epigenetic regulation of cell cycle

Together with the identified pathways and genes that possibly contributed to growth inhibition, histone marks changes also were also studied. Signalling pathways mediate integration and interpretation of extracellular signals into regulated gene transcription. These transcriptional states are regulated by modifications of the chromatin structure that can allow or repress gene expression. Even though much is

known about signal transduction and associated pathways, the relationship between signalling and epigenetics is still unclear (Arzate-Mejía et al., 2011). In this project several signalling pathways together with histone changes involved in the regulation of G1 to S phase transition were investigated. Figure 6.2 represent a working model where the interplay between cell cycle arrest and histone modification is presented.

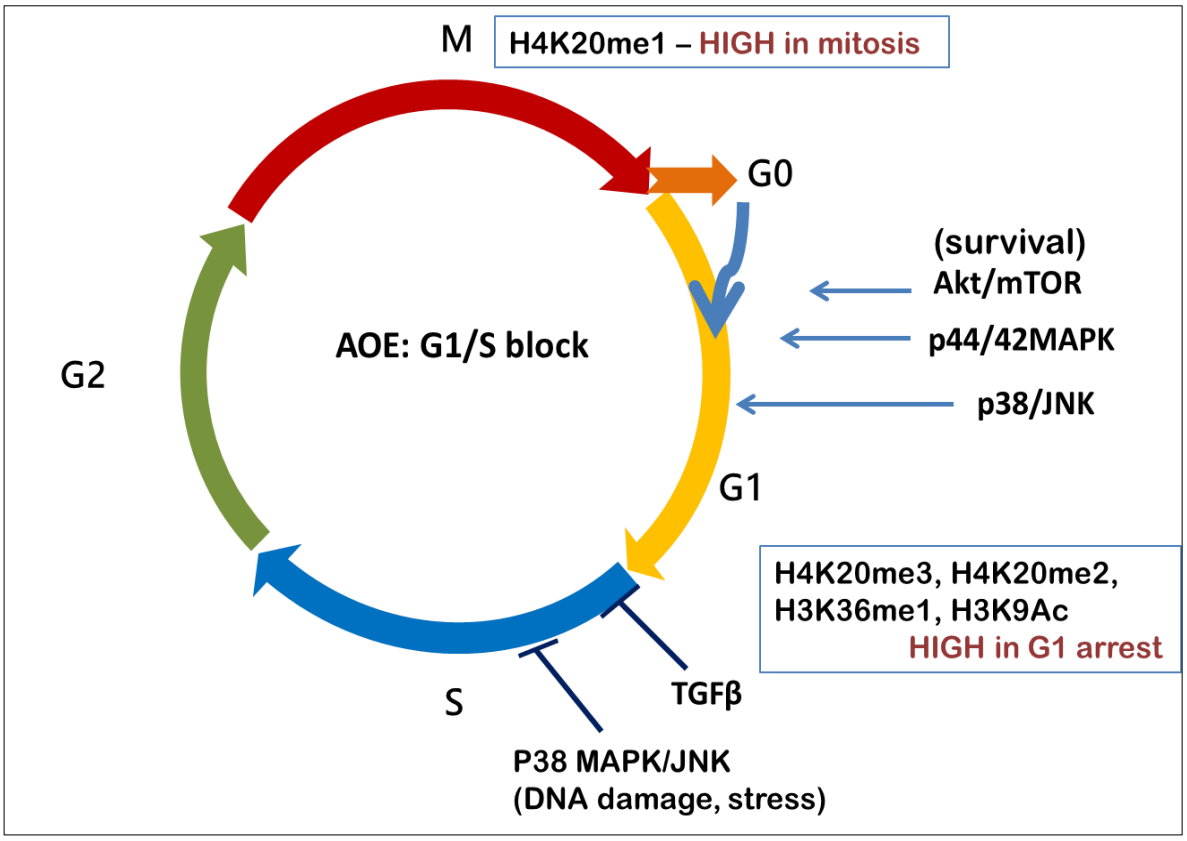


Figure 6.2. Working hypothesis on relationship of signalling pathways and histone modifications involvement during G1/S transition.

The histone code is dynamically regulated during cell cycle progression. During G1/S transition, the DNA is less compacted as cells are just beginning DNA synthesis. H4K20me can be di- and tri-methylate with the involvement of Suv4-20h enzymes (Jørgensen et al., 2013). Besides, at this stage PRC2 complex binds to the H3K27me3 mark and co-localises in G1 phase with sites of continuing DNA replication. The level of enzymes involved (i.e EZH2, SUZ12 and EED) during cell proliferation are stable to ensure PRC2 complex binding to regions enriched in H3K27me3 (Hansen et al., 2008).

During S-phase, replication of DNA occurs without alteration in chromatin structure, with the exception of replication forks. DNA replication begins at precise positions of the genome known as replication origins where the DNA is replicated from multiple sites specifically by binding of the origin recognition complex (ORC) (Sclafani and Holzen, 2007). Early-firing origins with open chromatin structure have been reported with high levels of H3K9Ac, H3K14Ac, HeK16Ac, H3K18Ac, H3K27Ac and H4K4me3 and low levels of the silencing H3K9me3 mark (Rampakakis et al., 2009, Kheir and Lund, 2010). Late-firing origins on the other hand, are associated with a close chromatin conformation until their activation, and thus marked with low levels of H3K9Ac, H3K14Ac and H3K4me3 and increased H3K9me3 marks (Kheir and Lund, 2010).

By the end of S phase, chromatin transcriptional activation and DNA replication should be suppressed to assure that replication is initiated once and not repeated (He et al., 2011). Because of the crucial function of histone acetylation in S-phase transition, deacetylation might occur at the end of S-phase. In contrast, H4K20 and H3K79 methylations were not detected on newly deposited histones until later time points in the cell cycle. On the other hand, H3K27 is rapidly monomethylated on newly synthesized histones, which is probably due to the localization of EZH2 (the H3K27 methyltransferase) to sites of replication. H4K20me3 is detected at its lowest levels in S-phase, in agreement with the predicted negative correlation with H4K16Ac (Kheir and Lund, 2010, Strahl and Allis, 2000).

The chromatin begins condensing during the G2-phase and condensation is completed at metaphase. Since the G2-phase of the cell cycle is a short, documentation of post-translational histone modifications often merges the G2-phase with the following M-phase. Histone H3 phosphorylation is low until late interphase and spreads throughout the genome until prophase, where it constitutes a mitotic hallmark (Hendzel et al., 1997, Kheir and Lund, 2010). Transcription is believed to be turned off during mitosis, which is in accordance with an observed global deacetylation of histones. A dramatic decrease in acetylation has been reported on histones H3K9, H3K18, H3K23, H4K5, H4K8, H4K12 and H4K16 during this

stage. Lysine deacetylation has been also observed on histone H2A and H2B, in particular H2AK5, H2BK12, H2BK15 and H2BK20 (Kheir and Lund, 2010).

After AOE treatment, levels of H3K9me repressive chromatin marks were reduced but other repressive marks such as H3K27me3 and H4K20me2/3 were increased. There was also an increase of the active marks H3K9ac and H3K4me3. These observations do not point to a specific cell cycle stage, but rather to a specific chromatin configuration.

Accumulation of H4K20me2/3 and a decrease ion H4K20me1 are observed in quiescent cells. These modifications mediate chromatin compaction in quiescent cells and they are found concomitant with less pronounced increased H2K27me3, H3K36me1 and H3K9ac (Evertts et al., 2013). This is consistent with the results presented in this study and therefore it can be hypothesised that the outcome of the reprogramming by AOE is to induce a quiescence-associated chromatin state. In general, histone lysine methylation is considered a stable modification with low changeover as compared to acetylation or phosphorylation. Currently, it is uncertain whether H4K20me2 and H4K20me3 are dynamically replaced. However, the control mechanism for H4K20me1 is more established. To control H4K20me1 state in S phase, SET8 (H4K20me1 demethylase) is maintained at low levels to provide newly integrated histones with low level H4K20 methylation (Wu et al., 2010). In late S/G2 phase, levels of SET8 are increased to allow unmodified H4K20 conversion to mono-methylated by the PR-Set7 enzyme (Jørgensen et al., 2013). Indeed, the new formed modification is sheltered from subsequent conversion by SUV4-20H enzymes until end of mitosis. Accurate regulation of these dynamic and diverse fluctuations in H4K20 methylation throughout the cell cycle is of great importance to preserve cellular homeostasis (Jørgensen et al., 2013).

H4K20me3 is enriched at condensed pericentric chromatin and it requires the presence of H3K9me3. However, in quiescent cells H3K20me3 is also present at ribosomal DNA sequences and this is independent of H3K9 methylation (Bierhoff et al., 2014). Because a reduction of H3K9me3 was observed after AOE treatment, it is

possible that these genomic regions could be the target of H4K20me silencing after reprogramming.

The question is what could be the mechanism leading to this quiescent state. A long non-coding RNA (lncRNa) based mechanism has been found to trigger H4K20me3 by guiding SUV4-20H. LncRNAs transcribed antisense from ribosomal RNA regions have been termed PAPAS and they are involved in this type of transcriptional silencing during quiescence (Bierhoff et al., 2014). Interestingly, PAPAS and H4K20me3 levels are increased in MCF-7 cells after cell-cycle arrest mediated by treatment with an estrogen receptor antagonist (Bierhoff et al., 2014).

Oocytes express promoter associated lncRNA (Hamazaki et al., 2015) and therefore this mechanism could be at the basis of the reprogramming to a quiescent state. They also express H4K20me modifying enzymes (Oliveri et al., 2007) and therefore the incorporation of this histone mark after reprogramming could be directly due to the effect of oocyte enzymatic activities on breast cancer cells which have been shown to express reduced levels of H4K20me2/3 (Yokoyama et al., 2014). Another mechanism involved in H4K20me3 stabilisation is mediated by the Rb protein. Rb is active (not phosphorylated) in quiescent cells and it can directly recruit SUV-20H histone methyltransferases (Gonzalo et al., 2005). Because the activation of Rb is dependent on phosphorylation by CDKs, the reduced expression of these together with the observed reduced activity of p44/42 MAPK after AOE treatment could suggest a direct relationship between cell signalling and epigenetic modification of the chromatin.

MAPKs regulate their effects via phosphorylation, triggering or deactivating variety of protein substrates including MAPK-activated protein kinases and other groups of kinases. In response to the extracellular activation, this signalling transduction cascade could activate transcription factors as well as integral parts of chromatin structure. For instance, phosphorylation of Ser10 and Ser28 of the histone H3 by ERK and p38MAPK functions as the main modification in sustaining condensed mitotic chromosomes to allow chromosome segregation. Therefore, the reduced

phosphorylation seen on p42/42 MAPK pathway could lead to unphosphorylated Ser10 and Ser28 on H3 and further preventing chromatin condensation for mitotic division (Arzate-Mejia et al., 2011).

6.4 Cell cycle arrest and quiescence: therapeutic opportunities

Results of BrdU and p27 staining performed on tumour xenografts and gene expression analysis of MCF-7 cells strongly suggest that cells undergo quiescence after AOE treatment. Furthermore, modification of histone marks H4K20me1, H4K20me and H4K20me3 further confirms that cells are arrested in G0-G1 phase of the cell cycle.

Other than methylation at lysine 20 of histone H4, modification of H3K4me3 and H3K27me3 have been recognised to modulate the quiescent state (Cheung and Rando, 2013). Results obtained in this project have shown increased H3K27me3 and H3K4me3 after reprogramming. Thus, the dual functions of these histone marks that positively and negatively regulate of transcription might explain this finding. The chromatin regions that are marked by both H3K4me3 and H3K27me3 are known as bivalent domains that normally positioned close to transcription start sites. Most of genes that own bivalent chromatin properties are able to regulated cell lineages as well as maintain ES cells in a poised state to allow flexibility for lineage choices (Cheung and Rando, 2013).

Entry into G0 is characterized by a shut-down of cyclin genes and an inactivation of specific gene programmes depending on cellular function. The quiescent state is maintained and conserved via inhibition of CDKs by CDKIs such as p21, p27 and p57 (Cheung and Rando, 2013). The increased expression of p27 after AOE treatment possibly exerts the effect as explained in stem cells study conducted by Zou et al. (2011), whereby elevated p27 and p57 halt the process of nuclear transport for the HSC70-cyclin D1 complex which is crucial to progress in cell cycle. Moreover, p21 is also required to keep the quiescent state of stem cells as it regulates the entry into the cell cycle. In the neural stem cell, p15, p21, p16 and p27 suppress cell proliferation

by reducing phosphorylation of Rb, which can thus inhibit E2F-regulated promoters, CDKs as well as cyclins (Bertonnier-Brouty, 2014).

Induction of quiescence by AOE treatment offers exciting therapeutic possibilities. First, it would be desirable to maintain the quiescent state so that cancer could be treated as a chronic condition whereby growth of malignant cells can be kept at bay. Second, quiescent cells could be specifically targeted to be destroyed so that no minimal residual disease would be present (Aguirre-Ghiso, 2007). Induction of quiescence using specific drugs (chemoquiescence) is a novel approach to cancer therapy and prevention. Differentiation therapy and targeting of niche signals such as Wnt, BMP, Notch represent good strategies. Several drugs have been proved to induce chemoquiescence in cancer cells, including chloroquine, metformin, Sonic Hedgehog inhibitors, cyclopamine, cerulin, and proton pump inhibitors (Kangwan et al., 2014). In addition, cell cycle arrest mediated by targeting of Rb-EF2 pathway trough CDK4/6 inhibition has proved effective in breast cancer treatment (Johnson et al., 2016).

Finally, epigenetic drugs such as the demethylating agent 5-azacytidin and HDAC inhibitors can induce cell cycle arrest associated with quiescence either alone or in combination with the differentiation inducer all-trans retinoic acid (Sosa et al., 2014). Therefore, a full understanding of the mechanisms behind the tumour reversion and induction of quiescence in breast cancer cells mediated by AOE would greatly contribute to the development of novel therapies. This project has provided some insight into the AOE activities and it represents a fist stepping stone for future discoveries.

6.5 Conclusions

This project represents the first effort to understand the molecular mechanisms involved in the breast tumour reversion mediated by axolotl oocyte extract

treatment. Microarray analyses of treated tumours and subsequent experiments have highlighted that cell cycle arrest and induction of quiescence could be the main features of the tumour arrested phenotype. The main findings of this project were the upregulation of CDK inhibitors, such as p15 and p27 together with a reduction of the MAPK signalling. These signalling changes were concomitant with a profound epigenetic reprogramming of active and repressive histone modifications mediated by the extract treatment. However, the interrelationship between the epigenetic machinery and cell signalling pathways is still unclear. Complex mechanisms and the ability to work in context-dependent fashion for many of the signalling pathways may affect the relationship between signalling and epigenetics and have a great influence in the way they regulate gene expression ultimately leading to growth inhibition.

The results obtained are more consistent with an effect of AOE on cell signalling that could influence epigenetic reprogramming and not viceversa, as originally hypothesised. However, this will still need further clarification. This project also demonstrated that axolotl oocytes contain activities that function synergistically to remodel the epigenetic landscape and signalling pathways in breast cancer cells. Further comprehensive work needs to be performed to understand the nature of breast cancer that is multi-complex. The elucidation of tumour reversion mechanisms and reversible targets will provide insights for effective therapies to tackle the disease and to treat and prevent its incidence (Suter and Marcum, 2007).

6.6 Future work

Although the project provided very important insights into tumour reversion of cancer cells, more work will be needed in the future to fully understand how the oocyte extract can produce such a dramatic effect on growth inhibition. A limitation of the project was the variability of the response of the extract across different biological replicates. Across experiments it was clear that the extract in able to produce a consistent effect, but the magnitude of the response proved variable. Therefore, more biological replicates will be needed in the future to further validate

some of the results. Experiments will need to be reproduced to support any conclusion.

In addition, some components of the pathways analysed were not evaluated. These include genes like MYC, p53, Rb and histone modifications such as H4K16ac. These will be studied in the future.

This project focused on cell cycle arrest as main mechanism responsible for growth inhibition but many more pathways and genes were found to be involved by microarray analysis. These could be the focus of future projects as they may also play an important role. The project also focused on understanding the events that might have initiated the tumour reversion, after treatment with the oocyte extract and before injection of cells into immunocompromised mice. Therefore, the pathways studied will need to be also evaluated in the xenograft tumours. Therefore, additional xenograft experiments need to be performed to obtain more biological replicates.

One important aspect of the project is to identify specific activities in the oocytes that are responsible for tumour suppression. During this project, a collaborator's laboratory (Dr Johnson) obtained the full transcriptomics of axolotl oocytes and therefore future work will look into identify possible oocyte specific molecules involved in reprogramming by constructing gene expression libraries that could be tested in cancer cells. Another possibility would be to undertake an immune-depletion approach so that specific proteins could be depleted from the extract so that their function in the reprogramming process can be evaluated. This approach will be next undertaken to dissect the relationship between MAPK inhibition and H4K20me2/3 accumulation in reprogrammed cells by immune-depletion of SUV4-H20 enzymes.

And finally, in order to identify oocyte molecules involved in tumour reversion, high throughput *in vivo* screening methods will be needed. One method that will be developed in the future is the zebrafish cancer transplantation model whereby only a small amount of treated cells can be transplanted in fish embryos and followed for

a short time (up to 10 day) of development (Veinotte et al., 2014). This will allow a faster, cheaper and more ethically acceptable alternative to mouse xenografts. Together with this, the development in of *in vitro* models that closely resemble *in vivo* conditions will be developed. To this end, in this project preliminary experiments were undertaken to develop a 3D model with the aim to recapitulate the *in vivo* tumour reversion effect. Over the past two decades, various 3D cell culture models have been established to allow cell-cell and cell-ECM interactions that function to maintain tissue homeostasis (Kim et al., 2004).

The most used matrix used in 3D cell cultures is a laminin-rich basement membrane extract (BME) (also called Matrigel) which is composed by the normal ECM molecules present in the basement membrane (i.e., laminin, collagen IV, heparin sulfate proteoglycan, and nidogen/entactin) (Hickman et al., 2014). Only when cultured in 3D in the presence of ECM proteins (floating collagen membrane) mouse mammary epithelial cells are able to differentiate by forming acini and secreting milk casein (Campbell and Watson, 2009). In addition, 3D cultures of breast cancer cells can form alveoli-like structures and milk proteins secretions into the hollow lumen (Mroue and Bissell, 2013). Importantly, culture of breast cancer spheres in 3D Matrigel enables the maintenance of the original tumour molecular subtype classification. In this system, triple negative cell lines generally develop invasive network structures, while luminal A and basal subtypes form round or grape-like colonies (Mroue and Bissell, 2013, Hickman et al., 2014)

In this project, MCF-7 cells proliferated and formed aggregates and rounded colonies after 4 days in Matrigel culture (Figure 6.3).

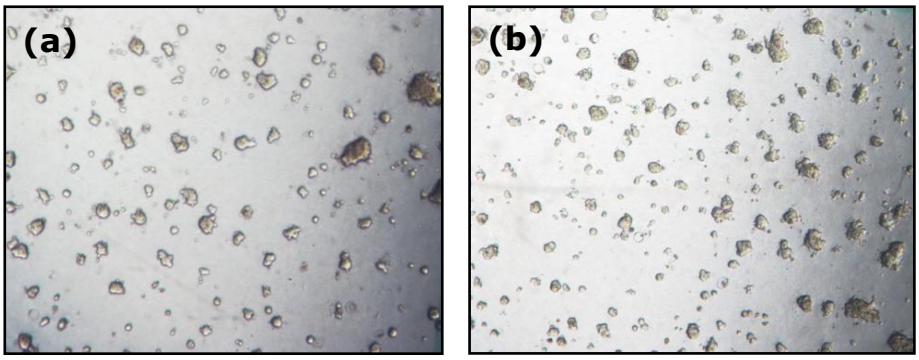


Figure 6.3 Growth morphology of permeabilised MCF-7 cells after four days culture in 3D on-top assay. (a) MCF-7 cells in control conditions (b) MCF-7 cells treated with AOE (10X magnification).

Cells labelled with EdU at the end of the culture showed smaller clusters of cells with incorporated EdU compared to the control group (Figure 6.4).

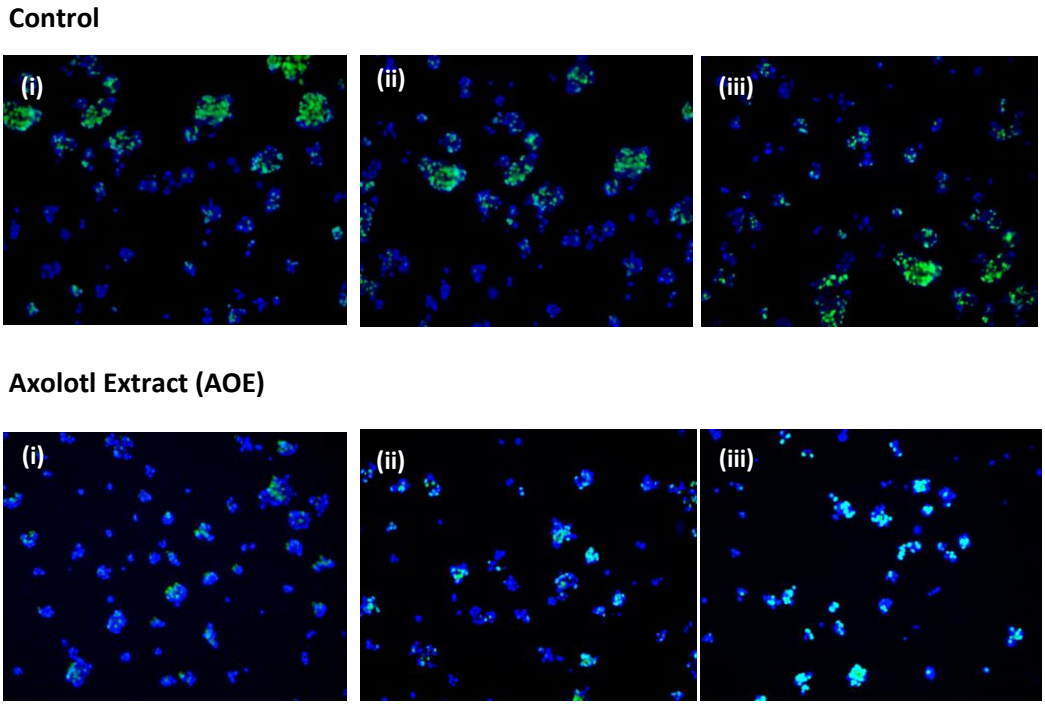


Figure 6.4. EdU incorporation was visualized by staining with Alexafluor 488 (green). The nucleus was stained with Hoechst 33342 (blue) and images were taken on a Leica fluorescence microscope (10X magnification). Pictures in each group show three representative fields of view per sample (i, ii, iii).

A significant decrease in the number of cells recovered from the AOE treated 3D cultures was also measured but no major differences in cells across phases of the cell cycle were observed (Figure 6.5).

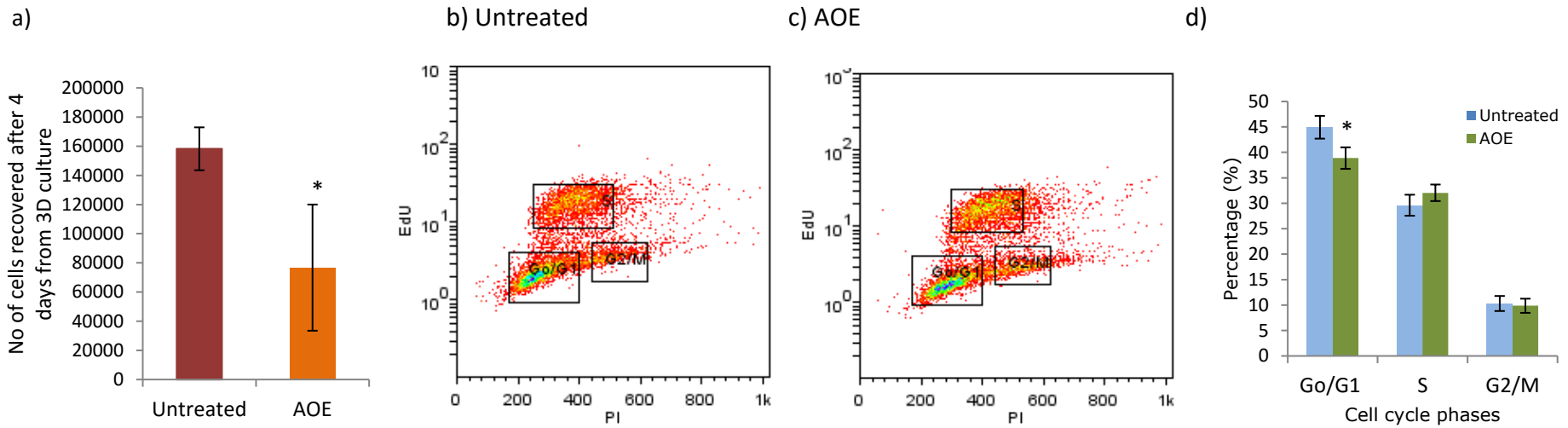


Figure 6.5. Number of cell recovered before flow cytometric analysis of EdU staining and cell cycle phases in untreated and AOE treated cells after four days cultured in 3D matrigel. The number of cells recovered after four days before EdU and PI staining (a) Go/G1, S and G2/M phases populations were gated (b,c) and the percentage of cells (±SD) in each cell cycle phase are indicated by the bar graph (d). Data represent three biological replicates (n=3). *= $p<0.05$.

These preliminary results show that the 3D matrigel system cannot fully recapitulate the phenotype of the previous *in vivo* study. It is possible that the lack of *in vivo* microenvironmental cues may influence the tumour reprogramming process. The mammary gland stroma has an important role in breast cancer development by playing a dual function: progression or prevention of cancer development in mammary epithelial cells. As a major component of the stroma, fibroblasts are crucial for the secretion of growth factors, extracellular matrix, angiogenic and immunological chemokines that stimulate development. Stromal fibroblasts have been identified to modify and persistently co-evolve with tumour epithelial cells' alteration as well as progression. Thus, tumour associated fibroblasts are an important element in the tumour malignant progression and good targets for cancer treatment (Li and Lu, 2011). Therefore, 3D co-culture with fibroblasts will be pursued in for future experiments.

Furthermore, additional experiments using other types of cancer cells lines should be performed to allow the determination of the broad range activities of AOE. This would also be important for making general conclusions on ability of AOE to act as anticancer agent.

Hopefully this proposed plan of work will significantly advance the use of oocyte molecules to induce tumour reversion and contribute to the development of new therapies for the treatment of breast cancer, especially of those types of cancers, like triple negative breast cancer, for which at present there is no effective cure.

References

ABBA, M. C., HU, Y., SUN, H., DRAKE, J. A., GADDIS, S., BAGGERLY, K., SAHIN, A. & ALDAZ, C. M. 2005. Gene expression signature of estrogen receptor α status in breast cancer. *BMC Genomics,* 6**,** 1-13.

ABD EL-REHIM, D. M., BALL, G., PINDER, S. E., RAKHA, E., PAISH, C., ROBERTSON, J. F., MACMILLAN, D., BLAMEY, R. W. & ELLIS, I. O. 2005. High-throughput protein expression analysis using tissue microarray technology of a large well-characterised series identifies biologically distinct classes of breast cancer confirming recent cDNA expression analyses. *Int J Cancer,* 116, 340-50.

ADAMS, J. M. & CORY, S. 2007. The Bcl-2 apoptotic switch in cancer development and therapy. *Oncogene,* 26, 1324-37.

ADVANI, S. H. 2010. Targeting mTOR pathway: A new concept in cancer therapy. *Indian J Med Paediatr Oncol,* 31**,** 132-6.

AGARWAL, S. 2006. Cellular reprogramming. *Methods Enzymol,* 420, 265-83.

AGUIRRE-GHISO, J. A. 2007. Models, mechanisms and clinical evidence for cancer dormancy. *Nat Rev Cancer,* 7, 834-46.

AHMAD, A., OZPOLAT, B., ZHOU, G., YANG, H. L., HAAKENSON, J. K., GREEN, L. J., JIA, L. T., CHEN, M. J., MAXIMOV, P. Y. & YANG, A. G. 2012. *Cell Cycle Regulatory Proteins in Breast Cancer: Molecular Determinants of Drug Resistance and Targets for Anticancer Therapies*, INTECH Open Access Publisher.

AHMED, M. & RAHMAN, N. 2006. ATM and breast cancer susceptibility. *Oncogene,* 25**,** 5906-11.

ALAO, J. P. 2007. The regulation of cyclin D1 degradation: roles in cancer development and the potential for therapeutic invention. *Molecular Cancer,* 6, 1-16.

ALBERIO, R., CAMPBELL, K. H. & JOHNSON, A. D. 2006. Reprogramming somatic cells into stem cells. *Reproduction,* 132, 709-20.

ALBERIO, R., JOHNSON, A. D., STICK, R. & CAMPBELL, K. H. 2005. Differential nuclear remodeling of mammalian somatic cells by Xenopus laevis oocyte and egg cytoplasm. *Exp Cell Res,* 307, 131-41.

ALLEGRUCCI, C., RUSHTON, M. D., DIXON, J. E., SOTTILE, V., SHAH, M., KUMARI, R., WATSON, S., ALBERIO, R. & JOHNSON, A. D. 2011. Epigenetic reprogramming of breast cancer cells with oocyte extracts. *Mol Cancer,* 10, 7.

ANDERSON, J. S., REISZ, R. R., SCOTT, D., FROBISCH, N. B. & SUMIDA, S. S. 2008. A stem batrachian from the Early Permian of Texas and the origin of frogs and salamanders. *Nature,* 453, 515-518.

ANWAR, T. & KHAN, A. U. 2008. Identification of a point mutation causing splitting of antigenic domain in M1 protein of H5N1 strain from 2006 outbreak in India. *Journal of Proteomics & Bioinformatics,* 2008.

APOSTOLOU, E. & HOCHEDLINGER, K. 2013. Chromatin dynamics during cellular reprogramming. *Nature,* 502, 462-471.

ARCARO, A. & GUERREIRO, A. S. 2007. The Phosphoinositide 3-Kinase Pathway in Human Cancer: Genetic Alterations and Therapeutic Implications. *Current Genomics,* 8, 271-306.

ARMSTRONG, L., LAKO, M., DEAN, W. & STOJKOVIC, M. 2006. Epigenetic modification is central to genome reprogramming in somatic cell nuclear transfer. *Stem Cells,* 24, 805-14.

ARZATE-MEJIA, R. G., VALLE-GARCIA, D. & RECILLAS-TARGA, F. 2011. Signaling epigenetics: novel insights on cell signaling and epigenetic regulation. *IUBMB Life,* 63, 881-95.

ARZATE-MEJÍA, R. G., VALLE-GARCÍA, D. & RECILLAS-TARGA, F. 2011. Signaling epigenetics: Novel insights on cell signaling and epigenetic regulation. *IUBMB Life,* 63, 881-895.

AWE, J. P. & BYRNE, J. A. 2013. Identifying candidate oocyte reprogramming factors using cross-species global transcriptional analysis. *Cell Reprogram,* 15, 126-33.

AZAB, S. S. 2013. Targeting the mTOR Signaling Pathways in Breast Cancer: More Than the Rapalogs. *Journal of Biochemical and Pharmacological Research,* 1**,** 75-83.

AZIZ, S. W. & AZIZ, M. H. 2013. Major Signaling Pathways Involved in Breast Cancer. *In:* AHMAD, A. (ed.) *Breast Cancer Metastasis and Drug Resistance: Progress and Prospects.* New York, NY: Springer New York.

BAE, K.-M., SU, Z., FRYE, C., MCCLELLAN, S., ALLAN, R. W., ANDREJEWSKI, J. T., KELLEY, V., JORGENSEN, M., STEINDLER, D. A., VIEWEG, J. & SIEMANN, D. W. 2010. Expression of Pluripotent Stem Cell Reprogramming Factors by Prostate Tumor Initiating Cells. *The Journal of urology,* 183, 2045-2053.

BAKER, S. J. & REDDY, E. P. 2012. CDK4: A Key Player in the Cell Cycle, Development, and Cancer. *Genes Cancer,* 3, 658-69.

BALUK, P., HASHIZUME, H. & MCDONALD, D. M. 2005. Cellular abnormalities of blood vessels as targets in cancer. *Curr Opin Genet Dev,* 15, 102-11.

BANNISTER, A. J. & KOUZARIDES, T. 2011. Regulation of chromatin by histone modifications. *Cell Research,* 21, 381-395.

BARANZINI, S. E. 2014. The role of antiproliferative gene Tob1 in the immune system. *Clinical & experimental neuroimmunology,* 5, 132-136.

BARLOW, W. E., WHITE, E., BALLARD-BARBASH, R., VACEK, P. M., TITUS-ERNSTOFF, L., CARNEY, P. A., TICE, J. A., BUIST, D. S., GELLER, B. M., ROSENBERG, R., YANKASKAS, B. C. & KERLIKOWSKE, K. 2006. Prospective breast cancer risk prediction model for women undergoing screening mammography. *J Natl Cancer Inst,* 98, 1204-14.

BASELGA, J. 2011. Targeting the phosphoinositide-3 (PI3) kinase pathway in breast cancer. *Oncologist,* 16 Suppl 1, 12-9.

BASERGA, R., PERUZZI, F. & REISS, K. 2003. The IGF-1 receptor in cancer biology. *Int J Cancer,* 107, 873-7.

BAYLIN, S. B. & JONES, P. A. 2011. A decade of exploring the cancer epigenome — biological and translational implications. *Nat Rev Cancer,* 11, 726-734.

BECK, D. B., ODA, H., SHEN, S. S. & REINBERG, D. 2012. PR-Set7 and H4K20me1: at the crossroads of genome integrity, cell cycle, chromosome condensation, and transcription. *Genes Dev,* 26, 325-37.

BEILSTEN-EDMANDS, V., GORDIYENKO, Y., KUNG, J. C. K., MOHAMMED, S., SCHMIDT, C. & ROBINSON, C. V. 2015. eIF2 interactions with initiator tRNA and eIF2B are regulated by post-translational modifications and conformational dynamics. *Cell Discovery,* 1, 15020.

BELLETTI, B. & BALDASSARRE, G. 2012. New light on p27(kip1) in breast cancer. *Cell Cycle,* 11, 3701-3702.

BERDASCO, M. & ESTELLER, M. 2010. Aberrant epigenetic landscape in cancer: how cellular identity goes awry. *Dev Cell,* 19, 698-711.

BERGAMASCHI, A., HJORTLAND, G. O., TRIULZI, T., SØRLIE, T., JOHNSEN, H., REE, A. H., RUSSNES, H. G., TRONNES, S., MÆLANDSMO, G. M., FODSTAD, O., BORRESEN-DALE, A.-L. & ENGEBRAATEN, O. 2009. Molecular profiling and characterization of luminal-like and basal-like in vivo breast cancer xenograft models. *Molecular Oncology,* 3, 469-482.

BERNS, K., HORLINGS, H. M., HENNESSY, B. T., MADIREDJO, M., HIJMANS, E. M., BEELEN, K., LINN, S. C., GONZALEZ-ANGULO, A. M., STEMKE-HALE, K., HAUPTMANN, M., BEIJERSBERGEN, R. L., MILLS, G. B., VAN DE VIJVER, M. J. & BERNARDS, R. 2007. A functional genetic approach identifies the PI3K pathway as a major determinant of trastuzumab resistance in breast cancer. *Cancer Cell,* 12, 395-402.

BERTOLI, C., SKOTHEIM, J. M. & DE BRUIN, R. A. 2013a. Control of cell cycle transcription during G1 and S phases. *Nat Rev Mol Cell Biol,* 14, 518-28.

BERTOLI, C., SKOTHEIM, J. M. & DE BRUIN, R. A. M. 2013b. Control of cell cycle transcription during G1 and S phases. *Nat Rev Mol Cell Biol,* 14, 518-528.

BERX, G. & VAN ROY, F. 2009. Involvement of members of the cadherin superfamily in cancer. *Cold Spring Harb Perspect Biol,* 1, a003129.

BHAT, M., ROBICHAUD, N., HULEA, L., SONENBERG, N., PELLETIER, J. & TOPISIROVIC, I. 2015. Targeting the translation machinery in cancer. *Nat Rev Drug Discov,* 14, 261-278.

BHOWMICK, N. A., NEILSON, E. G. & MOSES, H. L. 2004. Stromal fibroblasts in cancer initiation and progression. *Nature,* 432, 332-7.

BIAN, Y., ALBERIO, R., ALLEGRUCCI, C., CAMPBELL, K. H. & JOHNSON, A. D. 2009. Epigenetic marks in somatic chromatin are remodelled to resemble pluripotent nuclei by amphibian oocyte extracts. *Epigenetics,* 4, 194-202.

BIERHOFF, H., DAMMERT, M. A., BROCKS, D., DAMBACHER, S., SCHOTTA, G. & GRUMMT, I. 2014. Quiescence-induced LncRNAs trigger H4K20 trimethylation and transcriptional silencing. *Mol Cell,* 54, 675-82.

BLANCO, M. A. & KANG, Y. 2011. Signaling pathways in breast cancer metastasis - novel insights from functional genomics. *Breast Cancer Research : BCR,* 13**,** 206-206.

BLAGOSKLONNY, M. V. & PARDEE, A. B. 2002. The restriction point of the cell cycle. *Cell Cycle,* 1**,** 103-10.

BLELLOCH, R. H., HOCHEDLINGER, K., YAMADA, Y., BRENNAN, C., KIM, M., MINTZ, B., CHIN, L. & JAENISCH, R. 2004. Nuclear cloning of embryonal carcinoma cells. *Proc Natl Acad Sci U S A,* 101, 13985-90.

BOMMER, U. A., HENG, C., PERRIN, A., DASH, P., LOBOV, S., ELIA, A. & CLEMENS, M. J. 2009. Roles of the translationally controlled tumour protein (TCTP) and the double-stranded RNA-dependent protein kinase, PKR, in cellular stress responses. *Oncogene,* 29, 763-773.

BONNAL, S., VIGEVANI, L. & VALCARCEL, J. 2012. The spliceosome as a target of novel antitumour drugs. *Nat Rev Drug Discov,* 11, 847-59.

BRAMBRINK, T., HOCHEDLINGER, K., BELL, G. & JAENISCH, R. 2006. ES cells derived from cloned and fertilized blastocysts are transcriptionally and functionally indistinguishable. *Proc Natl Acad Sci U S A,* 103, 933-8.

BRIGGS, R. & KING, T. J. 1952. Transplantation of living nuclei from blastula cells into enucleated frogs' eggs. *Proceedings of the National Academy of Sciences,* 38, 455-463.

BRODY, L. C. & BIESECKER, B. B. 1998. Breast cancer susceptibility genes. BRCA1 and BRCA2. *Medicine (Baltimore),* 77**,** 208-26.

BROWAEYS-POLY, E., PERDEREAU, D., LESCUYER, A., BURNOL, A. F. & CAILLIAU, K. 2009. Akt interaction with PLC(gamma) regulates the G(2)/M transition triggered by FGF receptors from MDA-MB-231 breast cancer cells. *Anticancer Res,* 29, 4965-9.

BUI, H. T., KWON, D. N., KANG, M. H., OH, M. H., PARK, M. R., PARK, W. J., PAIK, S. S., VAN THUAN, N. & KIM, J. H. 2012. Epigenetic reprogramming in somatic cells induced by extract from germinal vesicle stage pig oocytes. *Development,* 139, 4330-40.

BUI, H. T., WAKAYAMA, S., KISHIGAMI, S., KIM, J. H., VAN THUAN, N. & WAKAYAMA, T. 2008. The cytoplasm of mouse germinal vesicle stage oocytes can enhance somatic cell nuclear reprogramming. *Development,* 135, 3935-45.

BUMGARNER, R. 2013. DNA microarrays: Types, Applications and their future. *Current protocols in molecular biology / edited by Frederick M. Ausubel ... [et al.],* 0 22, Unit-22.1.

BYLER, S., GOLDGAR, S., HEERBOTH, S., LEARY, M., HOUSMAN, G., MOULTON, K. & SARKAR, S. 2014. Genetic and epigenetic aspects of breast cancer progression and therapy. *Anticancer Res,* 34, 1071-7.

CAILLIAU, K., PERDEREAU, D., LESCUYER, A., CHEN, H., GARBAY, C., VILAIN, J. P., BURNOL, A. F. & BROWAEYS-POLY, E. 2005. FGF receptor phosphotyrosine 766 is a target for Grb14 to inhibit MDA-MB-231 human breast cancer cell signaling. *Anticancer Res,* 25, 3877-82.

CALDON, C. E., DALY, R. J., SUTHERLAND, R. L. & MUSGROVE, E. A. 2006. Cell cycle control in breast cancer cells. *J Cell Biochem,* 97, 261-74.

CAMPBELL, J. J. & WATSON, C. J. 2009. Three-dimensional culture models of mammary gland. *Organogenesis,* 5, 43-49.

CAMPOS-SANCHEZ, E., SÁNCHEZ GARCÍA, I. & COBALEDA, C. 2011. Plasticity and Tumorigenicity.

CANBAY, E., ERALTAN, I. Y., CERCEL, A., ISBIR, T., GAZIOGLU, E., AYDOGAN, F., CACINA, C., CENGIZ, A., FERAHMAN, M., ZENGIN, E. & UNAL, H. 2010. CCND1 and CDKN1B polymorphisms and risk of breast cancer. *Anticancer Res,* 30, 3093-8.

CARDIFF, R. D. & WELLINGS, S. R. 1999. The comparative pathology of human and mouse mammary glands. *J Mammary Gland Biol Neoplasia,* 4, 105-22.

CAREY, L. A., PEROU, C. M., LIVASY, C. A., DRESSLER, L. G., COWAN, D., CONWAY, K., KARACA, G., TROESTER, M. A., TSE, C. K., EDMISTON, S., DEMING, S. L., GERADTS, J., CHEANG, M. C., NIELSEN, T. O., MOORMAN, P. G., EARP, H. S. & MILLIKAN, R. C. 2006. Race, breast cancer subtypes, and survival in the Carolina Breast Cancer Study. *Jama,* 295, 2492-502.

CARIOU, S., DONOVAN, J. C., FLANAGAN, W. M., MILIC, A., BHATTACHARYA, N. & SLINGERLAND, J. M. 2000. Down-regulation of p21WAF1/CIP1 or p27Kip1 abrogates antiestrogen-mediated cell cycle arrest in human breast cancer cells. *Proc Natl Acad Sci U S A,* 97**,** 9042-6.

CAVA, C., BERTOLI, G. & CASTIGLIONI, I. 2015. Integrating genetics and epigenetics in breast cancer: biological insights, experimental, computational methods and therapeutic potential. *BMC Systems Biology,* 9, 62.

CAVALLARO, U. & CHRISTOFORI, G. 2004. Cell adhesion and signalling by cadherins and Ig-CAMs in cancer. *Nat Rev Cancer,* 4, 118-132.

CECH, T. R. 2004. Beginning to understand the end of the chromosome. *Cell,* 116, 273-9.

CHAMBARD, J.-C., LEFLOCH, R., POUYSSÉGUR, J. & LENORMAND, P. 2007. ERK implication in cell cycle regulation. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research,* 1773, 1299-1310.

CHEANG, M. C., CHIA, S. K., VODUC, D., GAO, D., LEUNG, S., SNIDER, J., WATSON, M., DAVIES, S., BERNARD, P. S., PARKER, J. S., PEROU, C. M., ELLIS, M. J. & NIELSEN, T. O. 2009. Ki67 index, HER2 status, and prognosis of patients with luminal B breast cancer. *J Natl Cancer Inst,* 101, 736-50.

CHEN, Z., ZANG, J., KAPPLER, J., HONG, X., CRAWFORD, F., WANG, Q., LAN, F., JIANG, C., WHETSTINE, J., DAI, S., HANSEN, K., SHI, Y. & ZHANG, G. 2007. Structural basis of the recognition of a methylated histone tail by JMJD2A. *Proc Natl Acad Sci USA,* 104.

CHERVONA, Y. & COSTA, M. 2012. Histone modifications and cancer: biomarkers of prognosis? *American Journal of Cancer Research,* 2, 589-597.

CHEUNG, T. H. & RANDO, T. A. 2013. Molecular regulation of stem cell quiescence. *Nature reviews. Molecular cell biology,* 14, 10.1038/nrm3591.

CHRISTOU, C. M. & KYRIACOU, K. 2013. BRCA1 and Its Network of Interacting Partners. *Biology,* 2**,** 40-63.

CHO, S. & HWANG, E. S. 2012. Status of mTOR activity may phenotypically differentiate senescence and quiescence. *Mol Cells,* 33, 597-604.

CIDADO, J. & PARK, B. H. 2012. Targeting the PI3K/Akt/mTOR pathway for breast cancer therapy. *J Mammary Gland Biol Neoplasia,* 17**,** 205-16.

CHOI, J. D. & LEE, J.-S. 2013. Interplay between Epigenetics and Genetics in Cancer. *Genomics & Informatics,* 11, 164-173.

CHOONG, M. K. & TSAFNAT, G. 2012. Genetic and epigenetic biomarkers of colorectal cancer. *Clinical Gastroenterology and Hepatology,* 10, 9-15.

CHOONG, P. F., TEH, H. X., TEOH, H. K., ONG, H. K., CHOO, K. B., SUGII, S., CHEONG, S. K. & KAMARUL, T. 2014. Heterogeneity of osteosarcoma cell lines led to variable responses in reprogramming. *Int J Med Sci,* 11, 1154-60.

CHU, I., SUN, J., ARNAOUT, A., KAHN, H., HANNA, W., NAROD, S., SUN, P., TAN, C. K., HENGST, L. & SLINGERLAND, J. 2007. p27 phosphorylation by Src regulates inhibition of cyclin E-Cdk2. *Cell,* 128.

CHU, I. M., HENGST, L. & SLINGERLAND, J. M. 2008. The Cdk inhibitor p27 in human cancer: prognostic potential and relevance to anticancer therapy. *Nat Rev Cancer,* 8, 253-267.

CIDADO, J. & PARK, B. H. 2012. Targeting the PI3K/Akt/mTOR pathway for breast cancer therapy. *J Mammary Gland Biol Neoplasia,* 17, 205-16.

CLOOS, P. A., CHRISTENSEN, J., AGGER, K. & HELIN, K. 2008. Erasing the methyl mark: histone demethylases at the center of cellular differentiation and disease. *Genes Dev,* 22, 1115-40.

COHEN, I., PORĘBA, E., KAMIENIARZ, K. & SCHNEIDER, R. 2011. Histone Modifiers in Cancer: Friends or Foes? *Genes & Cancer,* 2, 631-647.

COLLAS, P. & HAKELIEN, A. M. 2003. Teaching cells new tricks. *Trends Biotechnol,* 21, 354-61.

COLLER, H. A., SANG, L. & ROBERTS, J. M. 2006. A new description of cellular quiescence. *PLoS Biol,* 4, e83.

COLLINS, N., WOOSTER, R. & STRATTON, M. R. 1997. Absence of methylation of CpG dinucleotides within the promoter of the breast cancer susceptibility gene BRCA2 in normal tissues and in breast and ovarian cancers. *Br J Cancer,* 76**,** 1150-6.

CONNOLLY, R. & STEARNS, V. 2012. Epigenetics as a Therapeutic Target in Breast Cancer. *Journal of mammary gland biology and neoplasia,* 17, 191-204.

CRANE, R. F. & RUDERMAN, J. V. 2006. Using Xenopus oocyte extracts to study signal transduction. *Methods Mol Biol,* 322, 435-43.

CURTIS, C., SHAH, S. P., CHIN, S.-F., TURASHVILI, G., RUEDA, O. M., DUNNING, M. J., SPEED, D., LYNCH, A. G., SAMARAJIWA, S., YUAN, Y., GRAF, S., HA, G., HAFFARI, G., BASHASHATI, A., RUSSELL, R., MCKINNEY, S., LANGEROD, A., GREEN, A., PROVENZANO, E., WISHART, G., PINDER, S., WATSON, P., MARKOWETZ, F., MURPHY, L., ELLIS, I., PURUSHOTHAM, A., BORRESEN-DALE, A.-L., BRENTON, J. D., TAVARE, S., CALDAS, C. & APARICIO, S. 2012. The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. *Nature,* 486, 346-352.

DAI, X., CHEN, A. & BAI, Z. 2014. Integrative investigation on breast cancer in ER, PR and HER2-defined subgroups using mRNA and miRNA expression profiling. *Scientific Reports,* 4, 6566.

DAI, X., LI, T., BAI, Z., YANG, Y., LIU, X., ZHAN, J. & SHI, B. 2015. Breast cancer intrinsic subtype classification, clinical use and future trends. *American Journal of Cancer Research,* 5, 2929-2943.

DAVIDOFF, A. M., KERNS, B.-J. M., IGLEHART, J. D. & MARKS, J. R. 1991. Maintenance of p53 alterations throughout breast cancer progression. *Cancer research,* 51**,** 2605-2610.

DAVIS, R. J. 2000. Signal Transduction by the JNK Group of MAP Kinases. *Cell,* 103, 239-252.

DENICOURT, C. & DOWDY, S. F. 2004. Cip/Kip proteins: more than just CDKs inhibitors. *Genes Dev,* 18, 851-5.

DENNLER, S., GOUMANS, M. J. & TEN DIJKE, P. 2002. Transforming growth factor beta signal transduction. *J Leukoc Biol,* 71, 731-40.

DESHPANDE, A., SICINSKI, P. & HINDS, P. W. 2005. Cyclins and cdks in development and cancer: a perspective. 24**,** 2909-2915.

DEWI, D., ISHII, H., HARAGUCHI, N., NISHIKAWA, S., KANO, Y., FUKUSUMI, T., OHTA, K., MIYAZAKI, S., OZAKI, M., SAKAI, D., SATOH, T., NAGANO, H., DOKI, Y. & MORI, M. 2012. Reprogramming of gastrointestinal cancer cells. *Cancer Sci,* 103, 393-9.

DHANASEKARAN, S. M., BARRETTE, T. R., GHOSH, D., SHAH, R., VARAMBALLY, S., KURACHI, K., PIENTA, K. J., RUBIN, M. A. & CHINNAIYAN, A. M. 2001. Delineation of prognostic biomarkers in prostate cancer. *Nature,* 412, 822-826.

DHILLON, V. S., SHAHID, M. & HUSAIN, S. A. 2004. CpG methylation of the FHIT, FANCF, cyclin-D2, BRCA2 and RUNX3 genes in Granulosa cell tumors (GCTs) of ovarian origin. *Mol Cancer,* 3**,** 33.

DIAZ-MORALLI, S., TARRADO-CASTELLARNAU, M., MIRANDA, A. & CASCANTE, M. 2013. Targeting cell cycle regulation in cancer therapy. *Pharmacol Ther,* 138, 255-71.

DICKSON, M. A. & SCHWARTZ, G. K. 2009. Development of cell-cycle inhibitors for cancer therapy. *Current Oncology,* 16**,** 36-43.

DIMRI, G., BAND, H. & BAND, V. 2005. Mammary epithelial cell transformation: insights from cell culture and mouse models. *Breast Cancer Res,* 7, 171-9.

DIXON, J. E., ALLEGRUCCI, C., REDWOOD, C., KUMP, K., BIAN, Y., CHATFIELD, J., CHEN, Y.-H., SOTTILE, V., VOSS, S. R., ALBERIO, R. & JOHNSON, A. D. 2010. Axolotl Nanog activity in mouse embryonic stem cells demonstrates that ground state pluripotency is conserved from urodele amphibians to mammals. *Development (Cambridge, England),* 137, 2973-2980.

DIXON, K. & KOPRAS, E. 2004. Genetic alterations and DNA repair in human carcinogenesis. *Semin Cancer Biol,* 14, 441-8.

DOKMANOVIC, M. & MARKS, P. A. 2005. Prospects: histone deacetylase inhibitors. *J Cell Biochem,* 96.

DRABSCH, Y. & TEN DIJKE, P. 2011. TGF-β Signaling in Breast Cancer Cell Invasion and Bone Metastasis. *Journal of Mammary Gland Biology and Neoplasia,* 16**,** 97-108.

EGGER, G., LIANG, G., APARICIO, A. & JONES, P. A. 2004. Epigenetics in human disease and prospects for epigenetic therapy. *Nature,* 429, 457-63.

ELLIOTT, R. L. & BLOBE, G. C. 2005. Role of transforming growth factor Beta in human cancer. *J Clin Oncol,* 23, 2078-93.

ELSHEIKH, S. E., GREEN, A. R., RAKHA, E. A., POWE, D. G., AHMED, R. A., COLLINS, H. M., SORIA, D., GARIBALDI, J. M., PAISH, C. E., AMMAR, A. A., GRAINGE, M. J., BALL, G. R., ABDELGHANY, M. K., MARTINEZ-POMARES, L., HEERY, D. M. & ELLIS, I. O. 2009. Global histone modifications in breast cancer correlate with tumor phenotypes, prognostic factors, and patient outcome. *Cancer Res,* 69, 3802-9.

EROL, A. 2011. Genotoxic stress-mediated cell cycle activities for the decision of cellular fate. *Cell Cycle,* 10, 3239-3248.

EROLES, P., BOSCH, A., ALEJANDRO PÉREZ-FIDALGO, J. & LLUCH, A. 2012. Molecular biology in breast cancer: Intrinsic subtypes and signaling pathways. *Cancer Treatment Reviews,* 38, 698-707.

ESTELLER, M., SILVA, J. M., DOMINGUEZ, G., BONILLA, F., MATIAS-GUIU, X., LERMA, E., BUSSAGLIA, E., PRAT, J., HARKES, I. C., REPASKY, E. A., GABRIELSON, E., SCHUTTE, M., BAYLIN, S. B. & HERMAN, J. G. 2000. Promoter hypermethylation and BRCA1 inactivation in sporadic breast and ovarian tumors. *J Natl Cancer Inst,* 92.

EVERTTS, A. G., MANNING, A. L., WANG, X., DYSON, N. J., GARCIA, B. A. & COLLER, H. A. 2013. H4K20 methylation regulates quiescence and chromatin compaction. *Mol Biol Cell,* 24, 3025-37.

FABRIS, L., BERTON, S., PELLIZZARI, I., SEGATTO, I., D'ANDREA, S., ARMENIA, J., BOMBEN, R., SCHIAPPACASSI, M., GATTEI, V., PHILIPS, M. R., VECCHIONE, A., BELLETTI, B. & BALDASSARRE, G. 2015. p27(kip1) controls H-Ras/MAPK activation and cell cycle entry via modulation of MT stability. *Proceedings of the National Academy of Sciences of the United States of America,* 112, 13916-13921.

FALAHI, F., VAN KRUCHTEN, M., MARTINET, N., HOSPERS, G. A. & ROTS, M. G. 2014. Current and upcoming approaches to exploit the reversibility of epigenetic mutations in breast cancer.

FEINBERG, A. P., OHLSSON, R. & HENIKOFF, S. 2006. The epigenetic progenitor origin of human cancer. *Nat Rev Genet,* 7, 21-33.

FENGXIA, Y. I. N., HUI, L. I. U., SHORGAN, B. O. U. & GUANGPENG, L. I. 2014. Oocyte-associated transcription factors in reprogramming after somatic cell nuclear transfer: a review. *Frontiers of Agricultural Science and Engineering,* 1, 104-113.

FERLAY, J., SOERJOMATARAM, I., DIKSHIT, R., ESER, S., MATHERS, C., REBELO, M., PARKIN, D. M., FORMAN, D. & BRAY, F. 2015. Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012. *International Journal of Cancer,* 136, E359-E386.

FIDLER, I. J. 2003. Understanding bone metastases: the key to the effective treatment of prostate cancer. *Clin Adv Hematol Oncol,* 1, 278-9.

FINGAR, D. C., RICHARDSON, C. J., TEE, A. R., CHEATHAM, L., TSOU, C. & BLENIS, J. 2004. mTOR Controls Cell Cycle Progression through Its Cell Growth Effectors S6K1 and 4E-BP1/Eukaryotic Translation Initiation Factor 4E. *Molecular and Cellular Biology,* 24, 200-216.

FORD, D., EASTON, D. F., STRATTON, M., NAROD, S., GOLDGAR, D., DEVILEE, P., BISHOP, D. T., WEBER, B., LENOIR, G., CHANG-CLAUDE, J., SOBOL, H., TEARE, M. D., STRUEWING, J., ARASON, A., SCHERNECK, S., PETO, J., REBBECK, T. R., TONIN, P., NEUHAUSEN, S., BARKARDOTTIR, R., EYFJORD, J., LYNCH, H., PONDER, B. A., GAYTHER, S. A., ZELADA-HEDMAN, M. & ET AL. 1998. Genetic heterogeneity and penetrance analysis of the BRCA1 and BRCA2 genes in breast cancer families. The Breast Cancer Linkage Consortium. *Am J Hum Genet,* 62**,** 676-89.

FOSTER, D. A., YELLEN, P., XU, L. & SAQCENA, M. 2010. Regulation of G1 Cell Cycle Progression: Distinguishing the Restriction Point from a Nutrient-Sensing Cell Growth Checkpoint(s). *Genes & Cancer,* 1**,** 1124-1131.

FRAGA, M. F., BALLESTAR, E., VILLAR-GAREA, A., BOIX-CHORNET, M., ESPADA, J., SCHOTTA, G., BONALDI, T., HAYDON, C., ROPERO, S., PETRIE, K., IYER, N. G., PEREZ-ROSADO, A., CALVO, E., LOPEZ, J. A., CANO, A., CALASANZ, M. J., COLOMER, D., PIRIS, M. A., AHN, N., IMHOF, A., CALDAS, C., JENUWEIN, T. & ESTELLER, M. 2005. Loss of acetylation at Lys16 and trimethylation at Lys20 of histone H4 is a common hallmark of human cancer. *Nat Genet,* 37.

FRANK, B., HEMMINKI, K., MEINDL, A., WAPPENSCHMIDT, B., SUTTER, C., KIECHLE, M., BUGERT, P., SCHMUTZLER, R. K., BARTRAM, C. R. & BURWINKEL, B. 2007.

BRIP1 (BACH1) variants and familial breast cancer risk: a case-control study. *BMC Cancer,* 7**,** 1-4.

FUSTER, J. J., FERNÁNDEZ, P., GONZÁLEZ-NAVARRO, H., SILVESTRE, C., NABAH, Y. N. A. & ANDRÉS, V. 2010. Control of cell proliferation in atherosclerosis: insights from animal models and human studies. *Cardiovascular Research,* 86, 254-264.

GARRETT, M. D. 2001. Cell cycle control and cancer.

GE, F., ZHANG, L., TAO, S. C., KITAZATO, K., ZHANG, Z. P., ZHANG, X. E. & BI, L. J. 2011. Quantitative proteomic analysis of tumor reversion in multiple myeloma cells. *J Proteome Res,* 10, 845-55.

GHIASI, N., HABIBAGAHI, M., ROSLI, R., GHADERI, A., YUSOFF, K., HOSSEINI, A., ABDULLAH, S. & JABERIPOUR, M. 2013. Tumour suppressive effects of WEE1 gene silencing in breast cancer cells. *Asian Pac J Cancer Prev,* 14, 6605-11.

GLYNNE, R., GHANDOUR, G., RAYNER, J., MACK, D. H. & GOODNOW, C. C. 2000. B-lymphocyte quiescence, tolerance and activation as viewed by global gene expression profiling on microarrays. *Immunol Rev,* 176, 216-46.

GOKMEN-POLAR, Y., GOSWAMI, C. P., TORONI, R. A., SANDERS, K. L., MEHTA, R., SIRIMALLE, U., TANASA, B., SHEN, C., LI, L., IVAN, M., BADVE, S. & SLEDGE, G. W., JR. 2014. Gene Expression Analysis Reveals Distinct Pathways of Resistance to Bevacizumab in Xenograft Models of Human ER-Positive Breast Cancer. *J Cancer,* 5, 633-45.

GOLOUDINA, A., YAMAGUCHI, H., CHERVYAKOVA, D. B., APPELLA, E., FORNACE, A. J., JR. & BULAVIN, D. V. 2003. Regulation of human Cdc25A stability by Serine 75 phosphorylation is not sufficient to activate a S phase checkpoint. *Cell Cycle,* 2, 473-8.

GONDA, K., FOWLER, J., KATOKU-KIKYO, N., HAROLDSON, J., WUDEL, J. & KIKYO, N. 2003. Reversible disassembly of somatic nucleoli by the germ cell proteins FRGY2a and FRGY2b. *Nat Cell Biol,* 5, 205-10.

GONZALEZ-ANGULO, A. M. & MERIC-BERNSTAM, F. 2010. Metformin: a therapeutic opportunity in breast cancer. *Clin Cancer Res,* 16, 1695-700.

GONZALO, S., GARCIA-CAO, M., FRAGA, M. F., SCHOTTA, G., PETERS, A. H., COTTER, S. E., EGUIA, R., DEAN, D. C., ESTELLER, M., JENUWEIN, T. & BLASCO, M. A. 2005. Role of the RB1 family in stabilizing histone methylation at constitutive heterochromatin. *Nat Cell Biol,* 7, 420-8.

GORDON, K. J. & BLOBE, G. C. 2008. Role of transforming growth factor-β superfamily signaling pathways in human disease. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease,* 1782, 197-228.

GORSKI, B., NAROD, S. A. & LUBINSKI, J. 2005. A common missense variant in BRCA2 predisposes to early onset breast cancer. *Breast Cancer Res,* 7**,** R1023-7.

GRANT, G. M., FORTNEY, A., GORRETA, F., ESTEP, M., DEL GIACCO, L., VAN METER, A., CHRISTENSEN, A., APPALLA, L., NAOUAR, C., JAMISON, C., AL-TIMIMI, A., DONOVAN, J., COOPER, J., GARRETT, C. & CHANDHOKE, V. 2004. Microarrays in cancer research. *Anticancer Res,* 24, 441-8.

GREINER, D., BONALDI, T., ESKELAND, R., ROEMER, E. & IMHOF, A. 2005. Identification of a specific inhibitor of the histone methyltransferase SU(VAR)3-9. *Nat Chem Biol,* 1.

GUAN, X., WANG, Y., XIE, R., CHEN, L., BAI, J., LU, J. & KUO, M. T. 2010. p27(Kip1) as a prognostic factor in breast cancer: a systematic review and meta-analysis. *J Cell Mol Med,* 14, 944-53.

GUILLE, A., CHAFFANET, M. & BIRNBAUM, D. 2013. Signaling pathway switch in breast cancer. *Cancer Cell Int,* 13**,** 66.

GUENARD, F., PEDNEAULT, C. S., OUELLETTE, G., LABRIE, Y., SIMARD, J. & DUROCHER, F. 2010. Evaluation of the contribution of the three breast cancer susceptibility genes CHEK2, STK11, and PALB2 in non-BRCA1/2 French Canadian families with high risk of breast cancer. *Genet Test Mol Biomarkers,* 14**,** 515-26.

GUENTHER, M. G., LEVINE, S. S., BOYER, L. A., JAENISCH, R. & YOUNG, R. A. 2007. A Chromatin Landmark and Transcription Initiation at Most Promoters in Human Cells. *Cell,* 130, 77-88.

GUPTA, A., GODWIN, A. K., VANDERVEER, L., LU, A. & LIU, J. 2003. Hypomethylation of the synuclein gamma gene CpG island promotes its aberrant expression in breast carcinoma and ovarian carcinoma. *Cancer Res,* 63**,** 664-73.

GURDON, J. B. 2013. The egg and the nucleus: a battle for supremacy (Nobel Lecture). *Angew Chem Int Ed Engl,* 52, 13890-9.

GURDON, J. B. & MELTON, D. A. 2008. Nuclear Reprogramming in Cells. *Science,* 322, 1811-1815.

GURDON, J. B. & WILMUT, I. 2011. Nuclear transfer to eggs and oocytes. *Cold Spring Harb Perspect Biol,* 3.

HAKELIEN, A.-M., LANDSVERK, H. B., ROBL, J. M., SKALHEGG, B. S. & COLLAS, P. 2002. Reprogramming fibroblasts to express T-cell functions using cell 		extracts. *Nat Biotech,* 20, 460-466.

HALL, B. E., WANKHADE, U. D., KONKEL, J. E., CHERUKURI, K., NAGINENI, C. N., FLANDERS, K. C., ARANY, P. R., CHEN, W., RANE, S. G. & KULKARNI, A. B. 2013. Transforming Growth Factor-β3 (TGF-β3) Knock-in Ameliorates Inflammation Due to TGF-β1 Deficiency While Promoting Glucose Tolerance. *The Journal of Biological Chemistry,* 288, 32074-32092.

HALLEY-STOTT, R. P., PASQUE, V. & GURDON, J. B. 2013. Nuclear reprogramming. *Development,* 140, 2468-71.

HAMAZAKI, N., UESAKA, M., NAKASHIMA, K., AGATA, K. & IMAMURA, T. 2015. Gene activation-associated long noncoding RNAs function in mouse preimplantation development. *Development,* 142, 910-20.

HAN, J. & SIDHU, K. 2011. Embryonic stem cell extracts: use in differentiation and reprogramming. *Regen Med,* 6, 215-27.

HAN, J. W. & YOON, Y. S. 2011. Induced pluripotent stem cells: emerging techniques for nuclear reprogramming. *Antioxid Redox Signal,* 15, 1799-820.

HANAHAN, D. & WEINBERG, ROBERT A. 2011. Hallmarks of Cancer: The Next Generation. *Cell,* 144, 646-674.

HANDY, D. E., CASTRO, R. & LOSCALZO, J. 2011. Epigenetic Modifications: Basic Mechanisms and Role in Cardiovascular Disease. *Circulation,* 123, 2145-2156.

HANSEN, K. H., BRACKEN, A. P., PASINI, D., DIETRICH, N., GEHANI, S. S., MONRAD, A., RAPPSILBER, J., LERDRUP, M. & HELIN, K. 2008. A model for transmission of the H3K27me3 epigenetic mark. *Nat Cell Biol,* 10, 1291-300.

HANSIS, C., BARRETO, G., MALTRY, N. & NIEHRS, C. 2004. Nuclear reprogramming of human somatic cells by xenopus egg extract requires BRG1. *Curr Biol,* 14, 1475-80.

HARDWICK, L. J. A. & PHILPOTT, A. 2015. An oncologist's friend: How Xenopus contributes to cancer research. *Developmental Biology,* 408, 180-187.

HAY, N. & SONENBERG, N. 2004. Upstream and downstream of mTOR. *Genes Dev,* 18.

HE, H., YU, F. X., SUN, C. & LUO, Y. 2011. CBP/p300 and SIRT1 are involved in transcriptional regulation of S-phase specific histone genes. *PLoS One,* 6, e22088.

HELIN, K. & DHANAK, D. 2013. Chromatin proteins and modifications as drug targets. *Nature,* 502, 480-488.

HENDZEL, M. J., WEI, Y., MANCINI, M. A., VAN HOOSER, A., RANALLI, T., BRINKLEY, B. R., BAZETT-JONES, D. P. & ALLIS, C. D. 1997. Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. *Chromosoma,* 106, 348-60.

HERCEG, Z. & HAINAUT, P. 2007. Genetic and epigenetic alterations as biomarkers for cancer detection, diagnosis and prognosis. *Mol Oncol,* 1, 26-41.

HICKMAN, J. A., GRAESER, R., DE HOOGT, R., VIDIC, S., BRITO, C., GUTEKUNST, M., VAN DER KUIP, H. & CONSORTIUM, I. P. 2014. Three-dimensional models of cancer for pharmacology and cancer cell biology: capturing tumor complexity in vitro/ex vivo. *Biotechnol J,* 9, 1115-28.

HIRST, M. & MARRA, M. A. 2009. Epigenetics and human disease. *Int J Biochem Cell Biol,* 41, 136-46.

HOCHEDLINGER, K., BLELLOCH, R., BRENNAN, C., YAMADA, Y., KIM, M., CHIN, L. & JAENISCH, R. 2004. Reprogramming of a melanoma genome by nuclear transplantation. *Genes & Development,* 18, 1875-1885.

HOCHEDLINGER, K. & JAENISCH, R. 2006. Nuclear reprogramming and pluripotency. *Nature,* 441, 1061-1067.

HOLLIDAY, D. L. & SPEIRS, V. 2011. Choosing the right cell line for breast cancer research. *Breast Cancer Res,* 13, 215.

HOWE, L. R. & BROWN, A. M. C. 2004. Wnt Signaling and Breast Cancer. *Cancer Biology & Therapy,* 3, 36-41.

HOZYASZ, K. K., MOSTOWSKA, A., WÓJCICKI, P., LASOTA, A., OFFERT, B., BALCEREK, A., DUNIN-WILCZYŃSKA, I. & JAGODZIŃSKI, P. P. 2014. Nucleotide variants of the cancer predisposing gene CDH1 and the risk of non-syndromic cleft lip with or without cleft palate. *Familial Cancer,* 13**,** 415-421.

HUANG, C. & WU, J. C. 2012. Epigenetic Modulations of Induced Pluripotent Stem Cells: Novel Therapies and Disease Models. *Drug discovery today. Disease models,* 9, e153-e160.

HUANG DA, W., SHERMAN, B. T. & LEMPICKI, R. A. 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc,* 4, 44-57.

HUANG, D. W., SHERMAN, B. T. & LEMPICKI, R. A. 2008. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protocols,* 4, 44-57.

HUANG, Y., GREENE, E., MURRAY STEWART, T., GOODWIN, A. C., BAYLIN, S. B., WOSTER, P. M. & CASERO, R. A., JR. 2007. Inhibition of lysine-specific demethylase 1 by polyamine analogues results in reexpression of aberrantly silenced genes. *Proc Natl Acad Sci U S A,* 104, 8023-8.

HUANG, Y., NAYAK, S., JANKOWITZ, R., DAVIDSON, N. E. & OESTERREICH, S. 2011. Epigenetics in breast cancer: what's new? *Breast Cancer Research,* 13, 1-11.

HUANG, Y., STEWART, T. M., WU, Y., BAYLIN, S. B., MARTON, L. J., PERKINS, B., JONES, R. J., WOSTER, P. M. & CASERO, R. A., JR. 2009. Novel oligoamine analogues inhibit lysine-specific demethylase 1 and induce reexpression of epigenetically silenced genes. *Clin Cancer Res,* 15, 7217-28.

HUEN, M. S. Y., SY, S. M. H., VAN DEURSEN, J. M. & CHEN, J. 2008. Direct Interaction between SET8 and Proliferating Cell Nuclear Antigen Couples H4-K20 Methylation with DNA Replication. *The Journal of Biological Chemistry,* 283, 11073-11077.

IORIO, M. V., FERRACIN, M., LIU, C. G., VERONESE, A., SPIZZO, R., SABBIONI, S., MAGRI, E., PEDRIALI, M., FABBRI, M., CAMPIGLIO, M., MENARD, S., PALAZZO, J. P., ROSENBERG, A., MUSIANI, P., VOLINIA, S., NENCI, I., CALIN, G. A., QUERZOLI, P., NEGRINI, M. & CROCE, C. M. 2005. MicroRNA gene expression deregulation in human breast cancer. *Cancer Res,* 65, 7065-70.

JACKOWSKA, M., KEMPISTY, B., WOZNA, M., PIOTROWSKA, H., ANTOSIK, P., ZAWIERUCHA, P., BUKOWSKA, D., NOWICKI, M., JASKOWSKI, J. M. & BRUSSOW, K. P. 2013. Differential expression of GDF9, TGFB1, TGFB2 and TGFB3 in porcine oocytes isolated from follicles of different size before and after culture in vitro. *Acta Vet Hung,* 61, 99-115.

JAENISCH, R. & YOUNG, R. 2008. Stem cells, the molecular circuitry of pluripotency and nuclear reprogramming. *Cell,* 132, 567-582.

JANG, G. B., KIM, J. Y., CHO, S. D., PARK, K. S., JUNG, J. Y., LEE, H. Y., HONG, I. S. & NAM, J. S. 2015. Blockade of Wnt/beta-catenin signaling suppresses breast cancer metastasis by inhibiting CSC-like phenotype. *Sci Rep,* 5, 12465.

JASMINE, G. & YOGESHWER, S. 2008. Prognostic Factors of Male Breast Cancer: Proteomic Approaches for Early Detection and Treatment. *J Proteomics Bioinform,* 1**,** 112-127.

JENUWEIN, T. & ALLIS, C. D. 2001. Translating the histone code. *Science,* 293, 1074-80.

JIMENEZ-MARIN, A., COLLADO-ROMERO, M., RAMIREZ-BOO, M., ARCE, C. & GARRIDO, J. J. 2009. Biological pathway analysis by ArrayUnlock and Ingenuity Pathway Analysis. *BMC Proc,* 3 Suppl 4, S6.

JIN, B., LI, Y. & ROBERTSON, K. D. 2011. DNA Methylation: Superior or Subordinate in the Epigenetic Hierarchy? *Genes & Cancer,* 2, 607-617.

JIN, B. & ROBERTSON, K. D. 2013. DNA Methyltransferases (DNMTs), DNA Damage Repair, and Cancer. *Advances in experimental medicine and biology,* 754, 3-29.

JOHNSON, J., THIJSSEN, B., MCDERMOTT, U., GARNETT, M., WESSELS, L. F. A. & BERNARDS, R. 2016. Targeting the RB-E2F pathway in breast cancer. *Oncogene*.

JONES, P. A. & BAYLIN, S. B. 2007. The epigenomics of cancer. *Cell,* 128.

JOVANOVIC, J., RØNNEBERG, J. A., TOST, J. & KRISTENSEN, V. 2010. The epigenetics of breast cancer. *Molecular Oncology,* 4**,** 242-254.

JØRGENSEN, S., SCHOTTA, G. & SØRENSEN, C. S. 2013. Histone H4 Lysine 20 methylation: key player in epigenetic regulation of genomic integrity. *Nucleic Acids Research*.

JULLIEN, J., ASTRAND, C., HALLEY-STOTT, R. P., GARRETT, N. & GURDON, J. B. 2010. Characterization of somatic cell nuclear reprogramming by oocytes in which a linker histone is required for pluripotency gene reactivation. *Proc Natl Acad Sci U S A,* 107, 5483-8.

JULLIEN, J., MIYAMOTO, K., PASQUE, V., ALLEN, G. E., BRADSHAW, C. R., GARRETT, N. J., HALLEY-STOTT, R. P., KIMURA, H., OHSUMI, K. & GURDON, J. B. 2014. Hierarchical molecular events driven by oocyte-specific factors lead to rapid and extensive reprogramming. *Mol Cell,* 55, 524-36.

JUNTTILA, M. R., LI, S. P. & WESTERMARCK, J. 2008. Phosphatase-mediated crosstalk between MAPK signaling pathways in the regulation of cell survival. *FASEB J,* 22, 954-65.

KALARI, S. & PFEIFER, G. P. 2010. Identification of driver and passenger DNA methylation in cancer by epigenomic analysis. *Advances in genetics,* 70, 277-308.

KANDEL, E. S., SKEEN, J., MAJEWSKI, N., DI CRISTOFANO, A., PANDOLFI, P. P., FELICIANO, C. S., GARTEL, A. & HAY, N. 2002. Activation of Akt/protein kinase B overcomes a G(2)/m cell cycle checkpoint induced by DNA damage. *Mol Cell Biol,* 22**,** 7831-41.

KANGWAN, N., PARK, J. M., KIM, E. H. & HAHM, K. B. 2014. Chemoquiescence for ideal cancer treatment and prevention: where are we now? *J Cancer Prev,* 19, 89-6.

KATZ, L. H., LI, Y., CHEN, J.-S., MUÑOZ, N. M., MAJUMDAR, A., CHEN, J. & MISHRA, L. 2013. Targeting TGF-β signaling in cancer. *Expert opinion on therapeutic targets,* 17**,** 743-760

KEEN, J. C., YAN, L., MACK, K. M., PETTIT, C., SMITH, D., SHARMA, D. & DAVIDSON, N. E. 2003. A novel histone deacetylase inhibitor, scriptaid, enhances expression of functional estrogen receptor alpha (ER) in ER negative human breast cancer cells in combination with 5-aza 2'-deoxycytidine. *Breast Cancer Res Treat,* 81.

KHAN, O. & LA THANGUE, N. B. 2008. Drug Insight: histone deacetylase inhibitor-based therapies for cutaneous T-cell lymphomas. *Nat Clin Pract Oncol,* 5.

KHEIR, T. B. & LUND, A. H. 2010. Epigenetic dynamics across the cell cycle. *Essays in biochemistry,* 48, 107-120.

KIKYO, N., WADE, P. A., GUSCHIN, D., GE, H. & WOLFFE, A. P. 2000. Active remodeling of somatic nuclei in egg cytoplasm by the nucleosomal ATPase ISWI. *Science,* 289, 2360-2.

KIM, G. Y., MERCER, S. E., EWTON, D. Z., YAN, Z., JIN, K. & FRIEDMAN, E. 2002. The stress-activated protein kinases p38 alpha and JNK1 stabilize p21(Cip1) by phosphorylation. *J Biol Chem,* 277, 29792-802.

KIM, J. B., STEIN, R. & O'HARE, M. J. 2004. Three-dimensional in vitro tissue culture models of breast cancer-- a review. *Breast Cancer Res Treat,* 85, 281-91.

KIM, S. J., KANG, H. S., CHANG, H. L., JUNG, Y. C., SIM, H. B., LEE, K. S., RO, J. & LEE, E. S. 2008. Promoter hypomethylation of the N-acetyltransferase 1 gene in breast cancer. *Oncol Rep,* 19**,** 663-8.

KINGSLEY, K., ZUCKERMAN, J., DAVIS, M., MATTEUCCI, M. & KNAVEL, A. 2009. Induction of Differential Growth in vitro by High-risk Human Papillomavirus in Human Breast Cancer Cell Lines is Associated with Caspase Dysregulation. *J Cancer Sci Ther,* 1**,** 62-71.

KLEINSMITH, L. J. & PIERCE, G. B., JR. 1964. MULTIPOTENTIALITY OF SINGLE EMBRYONAL CARCINOMA CELLS. *Cancer Res,* 24, 1544-51.

KLUTTIG, A. & SCHMIDT-POKRZYWNIAK, A. 2009. Established and Suspected Risk Factors in Breast Cancer Aetiology. *Breast Care,* 4, 82-87.

KONTOROVICH, T., COHEN, Y., NIR, U. & FRIEDMAN, E. 2009. Promoter methylation patterns of ATM, ATR, BRCA1, BRCA2 and p53 as putative cancer risk modifiers in Jewish BRCA1/BRCA2 mutation carriers. *Breast Cancer Res Treat,* 116**,** 195-200.

KOUL, H. K., PAL, M. & KOUL, S. 2013. Role of p38 MAP Kinase Signal Transduction in Solid Tumors. *Genes & Cancer,* 4, 342-359.

KOUZARIDES, T. 2007. Chromatin modifications and their function. *Cell,* 128.

KUO, A. J., SONG, J., CHEUNG, P., ISHIBE-MURAKAMI, S., YAMAZOE, S., CHEN, J. K., PATEL, D. J. & GOZANI, O. 2012. The BAH domain of ORC1 links H4K20me2 to DNA replication licensing and Meier-Gorlin syndrome. *Nature,* 484, 115-9.

LAI, J., KONG, C. M., MAHALINGAM, D., XIE, X. & WANG, X. 2013. Elite Model for the Generation of Induced Pluripotent Cancer Cells (iPCs). *PLoS ONE,* 8, e56702.

LAN, F., ZARATIEGUI, M., VILLEN, J., VAUGHN, M. W., VERDEL, A., HUARTE, M., SHI, Y., GYGI, S. P., MOAZED, D. & MARTIENSSEN, R. A. 2007. S. pombe LSD1 homologs regulate heterochromatin propagation and euchromatic gene transcription. *Mol Cell,* 26, 89-101.

LAURING, J., PARK, B. H. & WOLFF, A. C. 2013. The phosphoinositide-3-kinase-Akt-mTOR pathway as a therapeutic target in breast cancer. *J Natl Compr Canc Netw,* 11, 670-8.

LAPIDUS, R. G., FERGUSON, A. T., OTTAVIANO, Y. L., PARL, F. F., SMITH, H. S., WEITZMAN, S. A., BAYLIN, S. B., ISSA, J. P. & DAVIDSON, N. E. 1996. Methylation of estrogen and progesterone receptor gene 5' CpG islands correlates with lack of estrogen and progesterone receptor gene expression in breast tumors. *Clin Cancer Res,* 2**,** 805-10.

LAPLANTE, M. & SABATINI, D. M. 2009. mTOR signaling at a glance. *Journal of Cell Science,* 122**,** 3589-3594.

LAWRENCE, M. S., STOJANOV, P., MERMEL, C. H., ROBINSON, J. T., GARRAWAY, L. A., GOLUB, T. R., MEYERSON, M., GABRIEL, S. B., LANDER, E. S. & GETZ, G. 2014. Discovery and saturation analysis of cancer genes across 21 tumour types. *Nature,* 505, 495-501.

LEBRUN, J.-J. 2012. The dual role of TGF in human cancer: from tumor suppression to cancer metastasis. *ISRN molecular biology,* 2012.

LEE, H., LIN, E. C., LIU, L. & SMITH, J. W. 2003. Gene expression profiling of tumor xenografts: In vivo analysis of organ-specific metastasis. *Int J Cancer,* 107, 528-34.

LEE, M. G., WYNDER, C., SCHMIDT, D. M., MCCAFFERTY, D. G. & SHIEKHATTAR, R. 2006. Histone H3 lysine 4 demethylation is a target of nonselective antidepressive medications. *Chem Biol,* 13, 563-7.

LEGESSE-MILLER, A., RAITMAN, I., HALEY, E. M., LIAO, A., SUN, L. L., WANG, D. J., KRISHNAN, N., LEMONS, J. M., SUH, E. J., JOHNSON, E. L., LUND, B. A. & COLLER, H. A. 2012. Quiescent fibroblasts are protected from proteasome inhibition-mediated toxicity. *Mol Biol Cell,* 23, 3566-81.

LEMONS, J. M., FENG, X. J., BENNETT, B. D., LEGESSE-MILLER, A., JOHNSON, E. L., RAITMAN, I., POLLINA, E. A., RABITZ, H. A., RABINOWITZ, J. D. & COLLER, H. A. 2010. Quiescent fibroblasts exhibit high metabolic activity. *PLoS Biol,* 8, e1000514.

LENO, G. H. 1998. Cell-free systems to study chromatin remodeling. *Methods Cell Biol,* 53, 497-515.

LESZINSKI, G., GEZER, U., SIEGELE, B., STOETZER, O. & HOLDENRIEDER, S. 2012. Relevance of histone marks H3K9me3 and H4K20me3 in cancer. *Anticancer Res,* 32**,** 2199-205.

LEU, Y. W., YAN, P. S., FAN, M., JIN, V. X., LIU, J. C., CURRAN, E. M., WELSHONS, W. V., WEI, S. H., DAVULURI, R. V., PLASS, C., NEPHEW, K. P. & HUANG, T. H. 2004. Loss of estrogen receptor signaling triggers epigenetic silencing of downstream targets in breast cancer. *Cancer Res,* 64**,** 8184-92.

LILLIE, E. O., BERNSTEIN, L. & URSIN, G. 2003. The role of androgens and polymorphisms in the androgen receptor in the epidemiology of breast cancer. *Breast Cancer Research,* 5**,** 164-173.

LIN, H. J., ZUO, T., LIN, C. H., KUO, C. T., LIYANARACHCHI, S., SUN, S., SHEN, R., DEATHERAGE, D. E., POTTER, D., ASAMOTO, L., LIN, S., YAN, P. S., CHENG, A. L., OSTROWSKI, M. C. & HUANG, T. H. 2008. Breast cancer-associated fibroblasts confer AKT1-mediated epigenetic silencing of Cystatin M in epithelial cells. *Cancer Res,* 68**,** 10257-66.

LIU, P., CHENG, H., ROBERTS, T. M. & ZHAO, J. J. 2009. Targeting the phosphoinositide 3-kinase (PI3K) pathway in cancer. *Nature reviews. Drug discovery,* 8**,** 627-644.

LIU, T., NIU, Y., FENG, Y., NIU, R., YU, Y., LV, A. & YANG, Y. 2008. Methylation of CpG islands of p16(INK4a) and cyclinD1 overexpression associated with progression of intraductal proliferative lesions of the breast. *Hum Pathol,* 39**,** 1637-46.

LI, J., MAO, G. & XIA, G. 2012. FSH modulates PKAI and GPR3 activities in mouse oocyte of COC in a gap junctional communication (GJC)-dependent manner to initiate meiotic resumption. *PLoS One,* 7, e37835.

LI, L., CONNELLY, M. C., WETMORE, C., CURRAN, T. & MORGAN, J. I. 2003. Mouse embryos cloned from brain tumors. *Cancer Res,* 63, 2733-6.

LI, L. & LU, Y. 2011. Optimizing a 3D Culture System to Study the Interaction between Epithelial Breast Cancer and Its Surrounding Fibroblasts. *Journal of Cancer,* 2, 458-466.

LIU, Y., SHEPHERD, E. G. & NELIN, L. D. 2007. MAPK phosphatases [mdash] regulating the immune response. *Nat Rev Immunol,* 7**,** 202-212.

LIEBERMANN, D. A., TRONT, J. S., SHA, X., MUKHERJEE, K., MOHAMED-HADLEY, A. & HOFFMAN, B. 2011. Gadd45 stress sensors in malignancy and leukemia. *Crit Rev Oncog,* 16, 129-40.

LIM, S., JANZER, A., BECKER, A., ZIMMER, A., SCHULE, R., BUETTNER, R. & KIRFEL, J. 2010. Lysine-specific demethylase 1 (LSD1) is highly expressed in ER-negative breast cancers and a biomarker predicting aggressive biology. *Carcinogenesis,* 31, 512-20.

LIU, Y.-C., PENNINGER, J. & KARIN, M. 2005. Immunity by ubiquitylation: a reversible process of modification. *Nat Rev Immunol,* 5, 941-952.

LU, Y., LIN, Y. Z., LAPUSHIN, R., CUEVAS, B., FANG, X., YU, S. X., DAVIES, M. A., KHAN, H., FURUI, T., MAO, M., ZINNER, R., HUNG, M. C., STECK, P., SIMINOVITCH, K. & MILLS, G. B. 1999. The PTEN/MMAC1/TEP tumor suppressor gene decreases cell growth and induces apoptosis and anoikis in breast cancer cells. *Oncogene,* 18**,** 7034-45.

LO, H.-W., WANG, S.-C. & HUNG, M.-C. 2006. Novel Signaling Pathways in Breast Cancer. *In:* PICCART, M. J., HUNG, M.-C., SOLIN, L. J., CARDOSO, F. & WOOD, W. C. (eds.) *Breast Cancer and Molecular Medicine.* Berlin, Heidelberg: Springer Berlin Heidelberg.

LUO, X. G., ZOU, J. N., WANG, S. Z., ZHANG, T. C. & XI, T. 2010. Novobiocin decreases SMYD3 expression and inhibits the migration of MDA-MB-231 human breast cancer cells. *IUBMB Life,* 62.

LUO, Y. B., MA, J. Y., ZHANG, Q. H., LIN, F., WANG, Z. W., HUANG, L., SCHATTEN, H. & SUN, Q. Y. 2013. MBTD1 is associated with Pr-Set7 to stabilize H4K20me1 in mouse oocyte meiotic maturation. *Cell Cycle,* 12, 1142-50.

LUSTBERG, M. B. & RAMASWAMY, B. 2009. EPIGENETIC TARGETING IN BREAST CANCER: THERAPEUTIC IMPACT AND FUTURE DIRECTION. *Drug news & perspectives,* 22**,** 369-381.

MACCORKLE, R. A. & TAN, T. H. 2005. Mitogen-activated protein kinases in cell-cycle control. *Cell Biochem Biophys,* 43, 451-61.

MACGREGOR, P. F. & SQUIRE, J. A. 2002. Application of microarrays to the analysis of gene expression in cancer. *Clin Chem,* 48, 1170-7.

MACPHERSON, I. 1965. Reversion in Hamster Cells Transformed by Rous Sarcoma Virus. *Science,* 148, 1731-1733.

MAHALINGAM, D., KONG, C. M., LAI, J., TAY, L. L., YANG, H. & WANG, X. 2012. Reversal of Aberrant Cancer Methylome and Transcriptome upon Direct Reprogramming of Lung Cancer Cells. *Scientific Reports,* 2, 592.

MALANGA, D., DE GISI, S., RICCARDI, M., SCRIMA, M., DE MARCO, C., ROBLEDO, M. & VIGLIETTO, G. 2012. Functional characterization of a rare germline mutation in the gene encoding the cyclin-dependent kinase inhibitor p27Kip1 (CDKN1B) in a Spanish patient with multiple endocrine neoplasia-like phenotype. *Eur J Endocrinol,* 166, 551-60.

MALESZEWSKA, M. & KAMINSKA, B. 2013. Is glioblastoma an epigenetic malignancy? *Cancers (Basel),* 5, 1120-39.

MALHOTRA, G. K., ZHAO, X., BAND, H. & BAND, V. 2010. Histological, molecular and functional subtypes of breast cancers. *Cancer Biol Ther,* 10, 955-60.

MAMMOTO, A., HUANG, S., MOORE, K., OH, P. & INGBER, D. E. 2004. Role of RhoA, mDia, and ROCK in cell shape-dependent control of the Skp2-p27kip1 pathway and the G1/S transition. *J Biol Chem,* 279, 26323-30.

MARCINIAK, S. J. & RON, D. 2006. Endoplasmic Reticulum Stress Signaling in Disease. *Physiological Reviews,* 86, 1133-1149.

MARKS, P. A., RICHON, V. M., MILLER, T. & KELLY, W. K. 2004. Histone deacetylase inhibitors. *Adv Cancer Res,* 91.

MARTIN, A. M. & WEBER, B. L. 2000. Genetic and hormonal risk factors in breast cancer. *J Natl Cancer Inst,* 92**,** 1126-35.

MASSAGUE, J. 2004. G1 cell-cycle control and cancer. *Nature,* 432, 298-306.

MASUDA, H., ZHANG, D., BARTHOLOMEUSZ, C., DOIHARA, H., HORTOBAGYI, G. N. & UENO, N. T. 2012. Role of Epidermal Growth Factor Receptor in Breast Cancer. *Breast cancer research and treatment,* 136**,** 10.1007/s10549-012-2289-9.

MATHIEU, J., ZHANG, Z., ZHOU, W., WANG, A. J., HEDDLESTON, J. M., PINNA, C. M. A., HUBAUD, A., STADLER, B., CHOI, M., BAR, M., TEWARI, M., LIU, A., VESSELLA, R., ROSTOMILY, R., BORN, D., HORWITZ, M., WARE, C., BLAU, C. A., CLEARY, M. A., RICH, J. N. & RUOHOLA-BAKER, H. 2011. HIF induces human embryonic stem cell markers in cancer cells. *Cancer research,* 71, 4640-4652.

MATSUMURA, H., TADA, M., OTSUJI, T., YASUCHIKA, K., NAKATSUJI, N., SURANI, A. & TADA, T. 2007. Targeted chromosome elimination from ES-somatic hybrid cells. *Nat Meth,* 4, 23-25.

MATTIE, M., CHRISTENSEN, A., CHANG, M. S., YEH, W., SAID, S., SHOSTAK, Y., CAPO, L., VERLINSKY, A., AN, Z., JOSEPH, I., ZHANG, Y., KUMAR-GANESAN, S., MORRISON, K., STOVER, D. & CHALLITA-EID, P. 2013. Molecular Characterization of Patient-Derived Human Pancreatic Tumor Xenograft Models for Preclinical and Translational Development of Cancer Therapeutics. *Neoplasia (New York, N.Y.),* 15, 1138-1150.

MAYA-MENDOZA, A., TANG, C. W., POMBO, A. & JACKSON, D. A. 2009. Mechanisms regulating S phase progression in mammalian cells. *Front Biosci (Landmark Ed),* 14, 4199-213.

MCDOWELL, G. S., HARDWICK, L. J. & PHILPOTT, A. 2014. Complex domain interactions regulate stability and activity of closely related proneural transcription factors. *Biochem Biophys Res Commun,* 450, 1283-90.

MCFARLAND, C. D., MIRNY, L. A. & KOROLEV, K. S. 2014. Tug-of-war between driver and passenger mutations in cancer and other adaptive processes. *Proceedings of the National Academy of Sciences,* 111, 15138-15143.

MEBRATU, Y. & TESFAIGZI, Y. 2009. How ERK1/2 Activation Controls Cell Proliferation and Cell Death Is Subcellular Localization the Answer? *Cell cycle (Georgetown, Tex.),* 8**,** 1168-1175.

MEERAN, S. M., AHMED, A. & TOLLEFSBOL, T. O. 2010. Epigenetic targets of bioactive dietary components for cancer prevention and therapy. *Clinical Epigenetics,* 1, 101-116.

MELOCHE, S. & POUYSSEGUR, J. 0000. The ERK1//2 mitogen-activated protein kinase pathway as a master regulator of the G1- to S-phase transition. *Oncogene,* 26**,** 3227-3239.

MENDOZA, R. A., MOODY, E. E., ENRIQUEZ, M. I., MEJIA, S. M. & THORDARSON, G. 2011. Tumorigenicity of MCF-7 human breast cancer cells lacking the p38alpha mitogen-activated protein kinase. *J Endocrinol,* 208, 11-9.

MENG, H., CAO, Y., QIN, J., SONG, X., ZHANG, Q., SHI, Y. & CAO, L. 2015. DNA methylation, its mediators and genome integrity. *Int J Biol Sci,* 11, 604-17.

METZGER, E., WISSMANN, M., YIN, N., MULLER, J. M., SCHNEIDER, R., PETERS, A. H., GUNTHER, T., BUETTNER, R. & SCHULE, R. 2005. LSD1 demethylates repressive histone marks to promote androgen-receptor-dependent transcription. *Nature,* 437, 436-9.

MI, Z., GUO, H., MARKOVIC, J. & KUO, P. C. 2011. Characterization of Osteopontin Binding Kinetics In MDA-MB231 Breast and SK-Hep-1 Liver Cancer Cells. *Journal of Cancer Science & Therapy,* 2009.

MIKI, Y., SWENSEN, J., SHATTUCK-EIDENS, D., FUTREAL, P. A., HARSHMAN, K., TAVTIGIAN, S., LIU, Q., COCHRAN, C., BENNETT, L. M., DING, W. & ET AL. 1994. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science,* 266**,** 66-71.

MILLER, R. A. & RUDDLE, F. H. 1976. Pluripotent teratocarcinoma-thymus somatic cell hybrids. *Cell,* 9, 45-55.

MIYAMOTO, K., NAGAI, K., KITAMURA, N., NISHIKAWA, T., IKEGAMI, H., BINH, N. T., TSUKAMOTO, S., MATSUMOTO, M., TSUKIYAMA, T., MINAMI, N., YAMADA, M., ARIGA, H., MIYAKE, M., KAWARASAKI, T., MATSUMOTO, K. & IMAI, H. 2011. Identification and characterization of an oocyte factor required for development of porcine nuclear transfer embryos. *Proceedings of the National Academy of Sciences,* 108, 7040-7045.

MIYAMOTO, K., TSUKIYAMA, T., YANG, Y., LI, N., MINAMI, N., YAMADA, M. & IMAI, H. 2009. Cell-free extracts from mammalian oocytes partially induce nuclear reprogramming in somatic cells. *Biol Reprod,* 80, 935-43.

MIYOSHI, N., ISHII, H., NAGAI, K.-I., HOSHINO, H., MIMORI, K., TANAKA, F., NAGANO, H., SEKIMOTO, M., DOKI, Y. & MORI, M. 2010. Defined factors induce reprogramming of gastrointestinal cancer cells. *Proceedings of the National Academy of Sciences of the United States of America,* 107, 40-45.

MONSLOW, J., GOVINDARAJU, P. & PURÉ, E. 2015. Hyaluronan–a functional and structural sweet spot in the tissue microenvironment. *Frontiers in immunology,* 6.

MOSES, H. & BARCELLOS-HOFF, M. H. 2011. TGF-β Biology in Mammary Development and Breast Cancer. *Cold Spring Harbor Perspectives in Biology,* 3, a003277.

MROUE, R. & BISSELL, M. J. 2013. Three-Dimensional Cultures of Mouse Mammary Epithelial Cells. *Methods in molecular biology (Clifton, N.J.),* 945, 221-250.

NAGA ANUSHA, P. 2011. Epidemiology of Genetic Alterations in Progression of Breast Cancer. *J Carcinogene Mutagene,* 2**,** 125.

NAGARAJ, N. S. & DATTA, P. K. 2010. Targeting the transforming growth factor-beta signaling pathway in human cancer. *Expert Opin Investig Drugs,* 19, 77-91.

NAGY, J. A., CHANG, S. H., SHIH, S. C., DVORAK, A. M. & DVORAK, H. F. 2010. Heterogeneity of the tumor vasculature. *Semin Thromb Hemost,* 36, 321-31.

NEGANOVA, I. & LAKO, M. 2008. G1 to S phase cell cycle transition in somatic and embryonic stem cells. *Journal of Anatomy,* 213**,** 30-44.

NEUZILLET, C., TIJERAS-RABALLAND, A., DE MESTIER, L., CROS, J., FAIVRE, S. & RAYMOND, E. 2014. MEK in cancer and cancer therapy. *Pharmacology & Therapeutics,* 141, 160-171.

NGUYEN, C. T., WEISENBERGER, D. J., VELICESCU, M., GONZALES, F. A., LIN, J. C., LIANG, G. & JONES, P. A. 2002. Histone H3-lysine 9 methylation is associated with aberrant gene silencing in cancer cells and is rapidly reversed by 5-aza-2'-deoxycytidine. *Cancer Res,* 62, 6456-61.

NISHIKAWA, S., ISHII, H., HARAGUCHI, N., KANO, Y., FUKUSUMI, T., OHTA, K., OZAKI, M., DEWI, D. L., SAKAI, D., SATOH, T., NAGANO, H., DOKI, Y. & MORI, M. 2012. microRNA-based cancer cell reprogramming technology. *Exp Ther Med,* 4, 8-14.

NOTTKE, A., COLAIÁCOVO, M. P. & SHI, Y. 2009. Developmental roles of the histone lysine demethylases. *Development (Cambridge, England),* 136, 879-889.

NOWAK, J., MOSOR, M., NOWICKA, K., REMBOWSKA, J. & JANUSZKIEWICZ, D. 2011. Is the NBN gene mutation I171V a potential risk factor for malignant solid tumors in children? *J Pediatr Hematol Oncol,* 33**,** e248-9.

O'BRIEN, K. M., COLE, S. R., TSE, C. K., PEROU, C. M., CAREY, L. A., FOULKES, W. D., DRESSLER, L. G., GERADTS, J. & MILLIKAN, R. C. 2010. Intrinsic breast tumor subtypes, race, and long-term survival in the Carolina Breast Cancer Study. *Clin Cancer Res,* 16, 6100-10.

O'MALLEY, S., SU, H., ZHANG, T., NG, C., GE, H. & TANG, C. K. 2009. TOB suppresses breast cancer tumorigenesis. *Int J Cancer,* 125, 1805-13.

OHSUMI, K., KATAGIRI, C. & KISHIMOTO, T. 1993. Chromosome condensation in Xenopus mitotic extracts without histone H1. *Science,* 262, 2033-5.

OKADA, Y., SCOTT, G., RAY, M. K., MISHINA, Y. & ZHANG, Y. 2007. Histone demethylase JHDM2A is critical for Tnp1 and Prm1 transcription and spermatogenesis. *Nature,* 450, 119-123.

OLIVERI, R. S., KALISZ, M., SCHJERLING, C. K., ANDERSEN, C. Y., BORUP, R. & BYSKOV, A. G. 2007. Evaluation in mammalian oocytes of gene transcripts linked to epigenetic reprogramming. *Reproduction,* 134, 549-58.

ONITILO, A. A., ENGEL, J. M., GREENLEE, R. T. & MUKESH, B. N. 2009. Breast Cancer Subtypes Based on ER/PR and Her2 Expression: Comparison of Clinicopathologic Features and Survival. *Clinical Medicine & Research,* 7, 4-13.

OSAKI, L. H. & GAMA, P. 2013. MAPK Signaling Pathway Regulates p27 Phosphorylation at Threonin 187 as Part of the Mechanism Triggered by Early-Weaning to Induce Cell Proliferation in Rat Gastric Mucosa. *PLoS ONE,* 8, e66651.

PAKNESHAN, P., SZYF, M., FARIAS-EISNER, R. & RABBANI, S. A. 2004. Reversal of the hypomethylation status of urokinase (uPA) promoter blocks breast cancer growth and metastasis. *J Biol Chem,* 279**,** 31735-44.

PAN, M. H., CHEN, W. J., LIN-SHIAU, S. Y., HO, C. T. & LIN, J. K. 2002. Tangeretin induces cell-cycle G1 arrest through inhibiting cyclin-dependent kinases 2 and 4 activities as well as elevating Cdk inhibitors p21 and p27 in human colorectal carcinoma cells. *Carcinogenesis,* 23, 1677-84.

PAPAGIANNOULI, F. & MECHLER, B. M. 2013. Modeling Tumorigenesis in Drosophila: Current Advances and Future Perspectives. *Future Aspects of Tumor Suppressor Genes": InTech publications*, 97-128.

PAREDES, J., ALBERGARIA, A., OLIVEIRA, J. T., JERONIMO, C., MILANEZI, F. & SCHMITT, F. C. 2005. P-cadherin overexpression is an indicator of clinical outcome in invasive breast carcinomas and is associated with CDH3 promoter hypomethylation. *Clin Cancer Res,* 11**,** 5869-77.

PARK, M. T. & LEE, S. J. 2003. Cell cycle and cancer. *J Biochem Mol Biol,* 36, 60-5.

PATHIRAJA, T. N., STEARNS, V. & OESTERREICH, S. 2010. Epigenetic regulation in estrogen receptor positive breast cancer - role in treatment response. *J Mammary Gland Biol Neoplasia,* 15.

PENG, H. B. 1991. Xenopus laevis: Practical uses in cell and molecular biology. Solutions and protocols. *Methods Cell Biol,* 36, 657-62.

PERROT, C. Y., JAVELAUD, D. & MAUVIEL, A. 2013. Insights into the Transforming Growth Factor-beta Signaling Pathway in Cutaneous Melanoma. *Ann Dermatol,* 25, 135-44.

PETRUCELLI, N., DALY, M. B. & FELDMAN, G. L. 2010. Hereditary breast and ovarian cancer due to mutations in BRCA1 and BRCA2. *Genet Med,* 12**,** 245-259.

PFAFFL, M. W., TICHOPAD, A., PRGOMET, C. & NEUVIANS, T. P. 2004. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper--Excel-based tool using pair-wise correlations. *Biotechnol Lett,* 26, 509-15.

PHIN, S., MOORE, M. & COTTER, P. D. 2013. Genomic rearrangements of PTEN in prostate cancer. *Frontiers in Oncology,* 3.

PIERCE, G. B. & DIXON, F. J., JR. 1959. Testicular teratomas. I. Demonstration of teratogenesis by metamorphosis of multipotential cells. *Cancer,* 12, 573-83.

PINHEIRO, H., CARVALHO, J., OLIVEIRA, P., FERREIRA, D., PINTO, M. T., OSÓRIO, H., LICASTRO, D., BORDEIRA-CARRIÇO, R., JORDAN, P. & LAZAREVIC, D. 2012. Transcription initiation arising from E-cadherin/CDH1 intron2: a novel protein isoform that increases gastric cancer cell invasion and angiogenesis. *Human molecular genetics***,** dds248.

POLLACK, R. E., GREEN, H. & TODARO, G. J. 1968. Growth control in cultured cells: selection of sublines with increased sensitivity to contact inhibition and decreased tumor-producing ability. *Proc Natl Acad Sci U S A,* 60, 126-33.

POLLOCK, J. A., LARREA, M. D., JASPER, J. S., MCDONNELL, D. P. & MCCAFFERTY, D. G. 2012. Lysine-specific histone demethylase 1 inhibitors control breast cancer proliferation in ERalpha-dependent and -independent manners. *ACS Chem Biol,* 7, 1221-31.

POSTOVIT, L.-M., MARGARYAN, N. V., SEFTOR, E. A., KIRSCHMANN, D. A., LIPAVSKY, A., WHEATON, W. W., ABBOTT, D. E., SEFTOR, R. E. B. & HENDRIX, M. J. C. 2008. Human embryonic stem cell microenvironment suppresses the tumorigenic phenotype of aggressive cancer cells. *Proceedings of the National Academy of Sciences,* 105, 4329-4334.

PUCCI, B., KASTEN, M. & GIORDANO, A. 2000. Cell Cycle and Apoptosis. *Neoplasia (New York, N.Y.),* 2, 291-299.

RAMANUJAN, V. K., REN, S., PARK, S. & FARKAS, D. L. 2010. Non-invasive, Contrast-enhanced Spectral Imaging of Breast Cancer Signatures in Preclinical Animal Models In vivo. *J Cell Sci Ther,* 1.

RAMANUJAN, V. K., ZHANG, J. H., BIENER, E. & HERMAN, B. 2005. Multiphoton fluorescence lifetime contrast in deep tissue imaging: prospects in redox imaging and disease diagnosis. *J Biomed Opt,* 10**,** 051407.

RAMPAKAKIS, E., DI PAOLA, D., CHAN, M. K. & ZANNIS-HADJOPOULOS, M. 2009. Dynamic changes in chromatin structure through post-translational modifications of histone H3 during replication origin activation. *J Cell Biochem,* 108, 400-7.

RASTOGI, N. & MISHRA, D. P. 2012. Therapeutic targeting of cancer cell cycle using proteasome inhibitors. *Cell Division,* 7, 26-26.

RATHBONE, A. J., LIDDELL, S. & CAMPBELL, K. H. 2013. Proteomic analysis of early reprogramming events in murine somatic cells incubated with Xenopus laevis oocyte extracts demonstrates network associations with induced pluripotency markers. *Cell Reprogram,* 15, 269-80.

REGAD, T. 2015. Targeting RTK Signaling Pathways in Cancer. *Cancers (Basel),* 7, 1758-84.

REIS-FILHO, J. S., WESTBURY, C. & PIERGA, J. Y. 2006. The impact of expression profiling on prognostic and predictive testing in breast cancer. *Journal of Clinical Pathology,* 59, 225-231.

RIVLIN, N., BROSH, R., OREN, M. & ROTTER, V. 2011. Mutations in the p53 Tumor Suppressor Gene: Important Milestones at the Various Steps of Tumorigenesis. *Genes & Cancer,* 2**,** 466-474.

ROBERTS, P. J. & DER, C. J. 2007. Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. *Oncogene,* 26, 3291-310.

RODENHISER, D. I., ANDREWS, J., KENNETTE, W., SADIKOVIC, B., MENDLOWITZ, A., TUCK, A. B. & CHAMBERS, A. F. 2008. Epigenetic mapping and functional analysis in a breast cancer metastasis model using whole-genome promoter tiling microarrays. *Breast Cancer Res,* 10, R62.

RODOLFA, K. T. 2008. Inducing pluripotency. In: StemBook ed 2008. The Stem Cell Research Community. Cambridge (MA): Harvard Stem Cell Institute.

RODRIGUEZ-PAREDES, M. & ESTELLER, M. 2011. Cancer epigenetics reaches mainstream oncology. *Nat Med*, 330-339.

ROSANIA, G. R., MERLIE, J., JR., GRAY, N., CHANG, Y. T., SCHULTZ, P. G. & HEALD, R. 1999. A cyclin-dependent kinase inhibitor inducing cancer cell differentiation: biochemical identification using Xenopus egg extracts. *Proc Natl Acad Sci U S A,* 96, 4797-802.

ROSE, N. R., WOON, E. C., TUMBER, A., WALPORT, L. J., CHOWDHURY, R., LI, X. S., KING, O. N., LEJEUNE, C., NG, S. S., KROJER, T., CHAN, M. C., RYDZIK, A. M., HOPKINSON, R. J., CHE, K. H., DANIEL, M., STRAIN-DAMERELL, C., GILEADI, C., KOCHAN, G., LEUNG, I. K., DUNFORD, J., YEOH, K. K., RATCLIFFE, P. J., BURGESS-BROWN, N., VON DELFT, F., MULLER, S., MARSDEN, B., BRENNAN, P. E., MCDONOUGH, M. A., OPPERMANN, U., KLOSE, R. J., SCHOFIELD, C. J. & KAWAMURA, A. 2012. Plant growth regulator daminozide is a selective inhibitor of human KDM2/7 histone demethylases. *J Med Chem,* 55, 6639-43.

ROTILI, D. & MAI, A. 2011. Targeting Histone Demethylases: A New Avenue for the Fight against Cancer. *Genes & Cancer,* 2, 663-679.

ROY, S., GARDINER, D. M. & BRYANT, S. V. 2000. Vaccinia as a tool for functional analysis in regenerating limbs: ectopic expression of Shh. *Dev Biol,* 218, 199-205.

RUSSO, G., ZEGAR, C. & GIORDANO, A. 2003. Advantages and limitations of microarray technology in human cancer. *Oncogene,* 22, 6497-507.

SADIKOVIC, B., AL-ROMAIH, K., SQUIRE, J. A. & ZIELENSKA, M. 2008. Cause and Consequences of Genetic and Epigenetic Alterations in Human Cancer. *Current Genomics,* 9, 394-408.

SALIC, A. & MITCHISON, T. J. 2008. A chemical method for fast and sensitive detection of DNA synthesis in vivo. *Proceedings of the National Academy of Sciences,* 105, 2415-2420.

SANCHEZ-ALVAREZ, R., MARTINEZ-OUTSCHOORN, U. E., LAMB, R., HULIT, J., HOWELL, A., GANDARA, R., SARTINI, M., RUBIN, E., LISANTI, M. P. & SOTGIA, F. 2013. Mitochondrial dysfunction in breast cancer cells prevents tumor growth: understanding chemoprevention with metformin. *Cell Cycle,* 12, 172-82.

SADIKOVIC, B., AL-ROMAIH, K., SQUIRE, J. A. & ZIELENSKA, M. 2008. Cause and Consequences of Genetic and Epigenetic Alterations in Human Cancer. *Current Genomics,* 9**,** 394-408.

SANDOVAL, J. & ESTELLER, M. 2012. Cancer epigenomics: beyond genomics. *Curr Opin Genet Dev,* 22, 50-5.

SANG, L., ROBERTS, J. M. & COLLER, H. A. 2010. Hijacking HES1: how tumors co-opt the anti-differentiation strategies of quiescent cells. *Trends Mol Med,* 16, 17-26.

SARRIO, D., PEREZ-MIES, B., HARDISSON, D., MORENO-BUENO, G., SUAREZ, A., CANO, A., MARTÍN-PÉREZ, J., GAMALLO, C. & PALACIOS, J. 2004. Cytoplasmic localization of p120ctn and E-cadherin loss characterize lobular breast carcinoma from preinvasive to metastatic lesions. *Oncogene,* 23**,** 3272-3283.

SAWYER, E., ROYLANCE, R., PETRIDIS, C., BROOK, M. N., NOWINSKI, S., PAPOULI, E., FLETCHER, O., PINDER, S., HANBY, A., KOHUT, K., GORMAN, P., CANEPPELE, M., PETO, J., DOS SANTOS SILVA, I., JOHNSON, N., SWANN, R., DWEK, M., PERKINS, K. A., GILLETT, C., HOULSTON, R., ROSS, G., DE IESO, P., SOUTHEY, M. C., HOPPER, J. L., PROVENZANO, E., APICELLA, C., WESSELING, J., CORNELISSEN, S., KEEMAN, R., FASCHING, P. A., JUD, S. M., EKICI, A. B., BECKMANN, M. W., KERIN, M. J., MARME, F., SCHNEEWEISS, A., SOHN, C., BURWINKEL, B., GUENEL, P., TRUONG, T., LAURENT-PUIG, P., KERBRAT, P., BOJESEN, S. E., NORDESTGAARD, B. G., NIELSEN, S. F., FLYGER, H., MILNE, R. L., PEREZ, J. I., MENENDEZ, P., BENITEZ, J., BRENNER, H., DIEFFENBACH, A. K., ARNDT, V., STEGMAIER, C., MEINDL, A., LICHTNER, P., SCHMUTZLER, R. K., LOCHMANN, M., BRAUCH, H., FISCHER, H. P., KO, Y. D., NEVANLINNA, H., MURANEN, T. A., AITTOMAKI, K., BLOMQVIST, C., BOGDANOVA, N. V., DORK, T., LINDBLOM, A., MARGOLIN, S., MANNERMAA, A., KATAJA, V., KOSMA, V. M., HARTIKAINEN, J. M., CHENEVIX-TRENCH, G., INVESTIGATORS, K., LAMBRECHTS, D., WELTENS, C., VAN LIMBERGEN, E., HATSE, S., CHANG-CLAUDE, J., RUDOLPH, A., SEIBOLD, P., FLESCH-JANYS, D., RADICE, P., PETERLONGO, P., BONANNI, B., VOLORIO, S., GILES, G. G., SEVERI, G., BAGLIETTO, L., MCLEAN, C. A., HAIMAN, C. A., HENDERSON, B. E., SCHUMACHER, F., LE MARCHAND, L., SIMARD, J., GOLDBERG, M. S.,

LABRECHE, F., DUMONT, M., KRISTENSEN, V., et al. 2014. Genetic predisposition to in situ and invasive lobular carcinoma of the breast. *PLoS Genet,* 10**,** e1004285.

SCHOTTA, G., SENGUPTA, R., KUBICEK, S., MALIN, S., KAUER, M., CALLÉN, E., CELESTE, A., PAGANI, M., OPRAVIL, S., DE LA ROSA-VELAZQUEZ, I. A., ESPEJO, A., BEDFORD, M. T., NUSSENZWEIG, A., BUSSLINGER, M. & JENUWEIN, T. 2008. A chromatin-wide transition to H4K20 monomethylation impairs genome integrity and programmed DNA rearrangements in the mouse. *Genes & Development,* 22, 2048-2061.

SCLAFANI, R. A. & HOLZEN, T. M. 2007. Cell Cycle Regulation of DNA Replication. *Annual review of genetics,* 41, 237-280.

SEEMANOVA, E., JAROLIM, P., SEEMAN, P., VARON, R., DIGWEED, M., SWIFT, M. & SPERLING, K. 2007. Cancer risk of heterozygotes with the NBN founder mutation. *J Natl Cancer Inst,* 99**,** 1875-80.

SEILERN-ASPANG, F. & KRATOCHWIL, K. 1962. Induction and differentiation of an epithelial tumour in the newt (Triturus cristatus). *J Embryol Exp Morphol,* 10, 337-56.

SEMI, K., MATSUDA, Y., OHNISHI, K. & YAMADA, Y. 2013. Cellular reprogramming and cancer development. *Int J Cancer,* 132, 1240-8.

SHAH, M. & ALLEGRUCCI, C. 2013. Stem cell plasticity in development and cancer: epigenetic origin of cancer stem cells. *Subcell Biochem,* 61, 545-65.

SHANN, Y. J., CHENG, C., CHIAO, C. H., CHEN, D. T., LI, P. H. & HSU, M. T. 2008. Genome-wide mapping and characterization of hypomethylated sites in human tissues and breast cancer cell lines. *Genome Res,* 18**,** 791-801.

SHARMA, S., KELLY, T. K. & JONES, P. A. 2010. Epigenetics in cancer. *Carcinogenesis,* 31, 27-36.

SIEGEL, P. M. & MASSAGUE, J. 2003. Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer. *Nature Rev. Cancer,* 3, 807-821.

SILVERA, D., FORMENTI, S. C. & SCHNEIDER, R. J. 2010. Translational control in cancer. *Nat Rev Cancer,* 10, 254-266.

SINGLETARY, S. E. & CONNOLLY, J. L. 2006. Breast Cancer Staging: Working With the Sixth Edition of the AJCC Cancer Staging Manual. *CA: A Cancer Journal for Clinicians,* 56, 37-47.

SMALLEY, M. & ASHWORTH, A. 2003. Stem cells and breast cancer: a field in transit. *Nature Reviews Cancer,* 3, 832-844.

SMITH, L. M., WISE, S. C., HENDRICKS, D. T., SABICHI, A. L., BOS, T., REDDY, P., BROWN, P. H. & BIRRER, M. J. 1999. cJun overexpression in MCF-7 breast cancer cells produces a tumorigenic, invasive and hormone resistant phenotype. *Oncogene,* 18, 6063-70.

SORLIE, T., TIBSHIRANI, R., PARKER, J., HASTIE, T., MARRON, J. S., NOBEL, A., DENG, S., JOHNSEN, H., PESICH, R., GEISLER, S., DEMETER, J., PEROU, C. M., LONNING, P. E., BROWN, P. O., BORRESEN-DALE, A. L. & BOTSTEIN, D. 2003. Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proc Natl Acad Sci U S A,* 100, 8418-23.

SOSA, M. S., BRAGADO, P. & AGUIRRE-GHISO, J. A. 2014. Mechanisms of disseminated cancer cell dormancy: an awakening field. *Nat Rev Cancer,* 14, 611-22.

SRIDHAR, S. S., HEDLEY, D. & SIU, L. L. 2005. Raf kinase as a target for anticancer therapeutics. *Mol Cancer Ther,* 4, 677-85.

SRIVASTAVA, R. K., CHEN, Q., SIDDIQUI, I., SARVA, K. & SHANKAR, S. 2007. Linkage of curcumin-induced cell cycle arrest and apoptosis by cyclin-dependent kinase inhibitor p21(/WAF1/CIP1). *Cell Cycle,* 6**,** 2953-61.

STEARNS, V., ZHOU, Q. & DAVIDSON, N. E. 2007. Epigenetic regulation as a new target for breast cancer therapy. *Cancer Invest,* 25.

STEELMAN, L. S., CHAPPELL, W. H., ABRAMS, S. L., KEMPF, C. R., LONG, J., LAIDLER, P., MIJATOVIC, S., MAKSIMOVIC-IVANIC, D., STIVALA, F., MAZZARINO, M. C., DONIA, M., FAGONE, P., MALAPONTE, G., NICOLETTI, F., LIBRA, M., MILELLA, M., TAFURI, A., BONATI, A., BÄSECKE, J., COCCO, L., EVANGELISTI, C., MARTELLI, A. M., MONTALTO, G., CERVELLO, M. & MCCUBREY, J. A. 2011. Roles of the Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR pathways in controlling growth and sensitivity to therapy-implications for cancer and aging. *Aging (Albany NY),* 3**,** 192-222.

STEIN, G. S., MEDINA, R., VAN WIJNEN, A. J., STEIN, J. L., LIAN, J. B. & OWEN, T. A. 2001. Cell Cycle. *eLS.* John Wiley & Sons, Ltd.

STRAHL, B. D. & ALLIS, C. D. 2000. The language of covalent histone modifications. *Nature,* 403, 41-45.

SUBRAMANIAM, M. M., CHAN, J. Y., SOONG, R., ITO, K., ITO, Y., YEOH, K. G., SALTO-TELLEZ, M. & PUTTI, T. C. 2009. RUNX3 inactivation by frequent promoter hypermethylation and protein mislocalization constitute an early event in breast cancer progression. *Breast Cancer Res Treat,* 113**,** 113-21.

SUMER, H., LIU, J. & VERMA, P. J. 2011. Cellular reprogramming of somatic cells. *Indian journal of experimental biology,* 49, 409.

SUN, S. Y., ROSENBERG, L. M., WANG, X., ZHOU, Z., YUE, P., FU, H. & KHURI, F. R. 2005. Activation of Akt and eIF4E survival pathways by rapamycin-mediated mammalian target of rapamycin inhibition. *Cancer Res,* 65, 7052-8.

SUPEK, F., BOSNJAK, M., SKUNCA, N. & SMUC, T. 2011. REVIGO summarizes and visualizes long lists of gene ontology terms. *PLoS One,* 6, e21800.

SUTER, R. & MARCUM, J. A. 2007. The molecular genetics of breast cancer and targeted therapy. *Biologics : Targets & Therapy,* 1, 241-258.

SUZUKI, M., SHIGEMATSU, H., SHAMES, D. S., SUNAGA, N., TAKAHASHI, T., SHIVAPURKAR, N., IIZASA, T., FRENKEL, E. P., MINNA, J. D., FUJISAWA, T. & GAZDAR, A. F. 2005. DNA methylation-associated inactivation of TGFbeta-related genes DRM/Gremlin, RUNX3, and HPP1 in human cancers. *Br J Cancer,* 93**,** 1029-37.

SWENSON, R. R., RIZZO, C. J., BROWN, L. K., PAYNE, N., DICLEMENTE, R. J., SALAZAR, L. F., VANABLE, P. A., CAREY, M. P., VALOIS, R. F., ROMER, D. & HENNESSY, M. 2009. Prevalence and Correlates of HIV Testing among Sexually Active African American Adolescents in Four U.S. Cities. *Sexually transmitted diseases,* 36, 584-591.

SWIERS, G., CHEN, Y. H., JOHNSON, A. D. & LOOSE, M. 2010. A conserved mechanism for vertebrate mesoderm specification in urodele amphibians and mammals. *Dev Biol,* 343, 138-52.

TABERNERO, J., ROJO, F., CALVO, E., BURRIS, H., JUDSON, I., HAZELL, K., MARTINELLI, E., RAMON Y CAJAL, S., JONES, S., VIDAL, L., SHAND, N., MACARULLA, T., RAMOS, F. J., DIMITRIJEVIC, S., ZOELLNER, U., TANG, P., STUMM, M., LANE, H. A., LEBWOHL, D. & BASELGA, J. 2008. Dose- and schedule-dependent inhibition of the mammalian target of rapamycin pathway with everolimus: a phase I tumor pharmacodynamic study in patients with advanced solid tumors. *J Clin Oncol,* 26, 1603-10.

TALMADGE, J. E. & FIDLER, I. J. 2010. AACR centennial series: the biology of cancer metastasis: historical perspective. *Cancer Res,* 70, 5649-69.

TAMADA, H., VAN THUAN, N., REED, P., NELSON, D., KATOKU-KIKYO, N., WUDEL, J., WAKAYAMA, T. & KIKYO, N. 2006. Chromatin decondensation and nuclear reprogramming by nucleoplasmin. *Mol Cell Biol,* 26, 1259-71.

TAN, J., YANG, X., ZHUANG, L., JIANG, X., CHEN, W., LEE, P. L., KARUTURI, R. K., TAN, P. B., LIU, E. T. & YU, Q. 2007. Pharmacologic disruption of Polycomb-repressive complex 2-mediated gene repression selectively induces apoptosis in cancer cells. *Genes Dev,* 21.

TAN, Z.-J., HU, X.-G., CAO, G.-S. & TANG, Y. 2003. Analysis of gene expression profile of pancreatic carcinoma using cDNA microarray. *World Journal of Gastroenterology : WJG,* 9, 818-823.

TAO, Z., SHI, A., LU, C., SONG, T., ZHANG, Z. & ZHAO, J. 2014. Breast Cancer: Epidemiology and Etiology. *Cell Biochem Biophys*.

TARANGER, C. K., NOER, A., SORENSEN, A. L., HAKELIEN, A. M., BOQUEST, A. C. & COLLAS, P. 2005. Induction of dedifferentiation, genomewide transcriptional programming, and epigenetic reprogramming by extracts of carcinoma and embryonic stem cells. *Mol Biol Cell,* 16, 5719-35.

TARCA, A. L., ROMERO, R. & DRAGHICI, S. 2006. Analysis of microarray experiments of gene expression profiling. *American journal of obstetrics and gynecology,* 195, 373-388.

TELERMAN, A. & AMSON, R. 2009. The molecular programme of tumour reversion: the steps beyond malignant transformation. *Nat Rev Cancer,* 9, 206-16.

TENGA, M. J. & LAZAR, I. M. 2013. A Proteomic Snapshot of Breast Cancer Cell Cycle: The G1/S Transition Point. *Proteomics,* 13**,** 48-60.

TENTLER, J. J., TAN, A. C., WEEKES, C. D., JIMENO, A., LEONG, S., PITTS, T. M., ARCAROLI, J. J., MESSERSMITH, W. A. & ECKHARDT, S. G. 2012. Patient-derived tumour xenografts as models for oncology drug development. *Nat Rev Clin Oncol,* 9, 338-350.

TEPEREK, M. & MIYAMOTO, K. 2013. Nuclear reprogramming of sperm and somatic nuclei in eggs and oocytes. *Reproductive medicine and biology,* 12, 10.1007/s12522-013-0155-z.

TERESI, R. E. & WAITE, K. A. 2008. PPARgamma, PTEN, and the Fight against Cancer. *PPAR Res,* 2008**,** 932632.

THACKER, J. 2005. The RAD51 gene family, genetic instability and cancer. *Cancer Lett,* 219**,** 125-35.

THE CANCER GENOME ATLAS, N. 2012. Comprehensive molecular portraits of human breast tumors. *Nature,* 490, 61-70.

THIAGALINGAM, S., LENGAUER, C., LEACH, F. S., SCHUTTE, M., HAHN, S. A., OVERHAUSER, J., WILLSON, J. K. V., MARKOWITZ, S., HAMILTON, S. R., KERN, S. E., KINZLER, K. W. & VOGELSTEIN, B. 1996. Evaluation of candidate tumour suppressor genes on chromosome 18 in colorectal cancers. *Nat Genet,* 13, 343-346.

THOMPSON, C. B. 2009. Attacking cancer at its root. *Cell,* 138, 1051-4.

THORNTON, T. M. & RINCON, M. 2009. Non-Classical P38 Map Kinase Functions: Cell Cycle Checkpoints and Survival. *International Journal of Biological Sciences,* 5, 44-52.

THURN, K. T., THOMAS, S., MOORE, A. & MUNSTER, P. N. 2011. Rational therapeutic combinations with histone deacetylase inhibitors for the treatment of cancer. *Future oncology (London, England),* 7, 263-283.

TORII, S., YAMAMOTO, T., TSUCHIYA, Y. & NISHIDA, E. 2006. ERK MAP kinase in G cell cycle progression and cancer. *Cancer Sci,* 97, 697-702.

TOYODA, T., YAMAMOTO, M., TAKASU, S., OGAWA, K., TATEMATSU, M. & TSUKAMOTO, T. 2014. Molecular Mechanism of Gastric Carcinogenesis in Helicobacter pylori-Infected Rodent Models. *Diseases,* 2, 168-186.

TREVINO, V., FALCIANI, F. & BARRERA-SALDANA, H. A. 2007. DNA microarrays: a powerful genomic tool for biomedical and clinical research. *Mol Med,* 13, 527-41.

TRICHOPOULOS, D., ADAMI, H. O., EKBOM, A., HSIEH, C. C. & LAGIOU, P. 2008. Early life events and conditions and breast cancer risk: from epidemiology to etiology. *Int J Cancer,* 122, 481-5.

TROP, I., LEBLANC, S. M., DAVID, J., LALONDE, L., TRAN-THANH, D., LABELLE, M. & EL KHOURY, M. M. 2014. Molecular classification of infiltrating breast cancer: toward personalized therapy. *Radiographics,* 34, 1178-95.

TRYNDYAK, V. P., KOVALCHUK, O. & POGRIBNY, I. P. 2006. Loss of DNA methylation and histone H4 lysine 20 trimethylation in human breast cancer cells is associated with aberrant expression of DNA methyltransferase 1, Suv4-20h2 histone methyltransferase and methyl-binding proteins. *Cancer Biol Ther,* 5.

TUYNDER, M., FIUCCI, G., PRIEUR, S., LESPAGNOL, A., GEANT, A., BEAUCOURT, S., DUFLAUT, D., BESSE, S., SUSINI, L., CAVARELLI, J., MORAS, D., AMSON, R. & TELERMAN, A. 2004. Translationally controlled tumor protein is a target of tumor reversion. *Proc Natl Acad Sci U S A,* 101, 15364-9.

TUYNDER, M., SUSINI, L., PRIEUR, S., BESSE, S., FIUCCI, G., AMSON, R. & TELERMAN, A. 2002. Biological models and genes of tumor reversion: cellular reprogramming through tpt1/TCTP and SIAH-1. *Proc Natl Acad Sci U S A,* 99, 14976-81.

UTIKAL, J., MAHERALI, N., KULALERT, W. & HOCHEDLINGER, K. 2009. Sox2 is dispensable for the reprogramming of melanocytes and melanoma cells into induced pluripotent stem cells. *Journal of Cell Science,* 122, 3502-3510.

VAKOC, C. R., SACHDEVA, M. M., WANG, H. & BLOBEL, G. A. 2006. Profile of Histone Lysine Methylation across Transcribed Mammalian Chromatin. *Molecular and Cellular Biology,* 26, 9185-9195.

VALDESPINO, V. & VALDESPINO, P. M. 2015. Potential of epigenetic therapies in the management of solid tumors. *Cancer Management and Research,* 7, 241-251.

VALK-LINGBEEK, M. E., BRUGGEMAN, S. W. M. & VAN LOHUIZEN, M. 2004. Stem Cells and Cancer: The Polycomb Connection. *Cell,* 118, 409-418.

VAN DER MEIJDEN, C. M., LAPOINTE, D. S., LUONG, M. X., PERIC-HUPKES, D., CHO, B., STEIN, J. L., VAN WIJNEN, A. J. & STEIN, G. S. 2002. Gene profiling of cell cycle progression through S-phase reveals sequential expression of genes required for DNA replication and nucleosome assembly. *Cancer Res,* 62**,** 3233-43.

VAN ROOZENDAAL, C. E., GILLIS, A. J., KLIJN, J. G., VAN OOIJEN, B., CLAASSEN, C. J., EGGERMONT, A. M., HENZEN-LOGMANS, S. C., OOSTERHUIS, J. W., FOEKENS, J. A. & LOOIJENGA, L. H. 1998. Loss of imprinting of IGF2 and not H19 in breast cancer, adjacent normal tissue and derived fibroblast cultures. *FEBS Lett,* 437**,** 107-11.

VAN PHAM, P. 2015. Direct reprogramming of somatic cells: an update. *Biomedical Research and Therapy,* 2, 231-240.

VASILAKI, E., PAPADIMITRIOU, E., TAJADURA, V., RIDLEY, A. J., STOURNARAS, C. & KARDASSIS, D. 2010. Transcriptional regulation of the small GTPase RhoB gene by TGF{beta}-induced signaling pathways. *Faseb j,* 24, 891-905.

VASKOVA, E. A., STEKLENEVA, A. E., MEDVEDEV, S. P. & ZAKIAN, S. M. 2013. "Epigenetic Memory" Phenomenon in Induced Pluripotent Stem Cells. *Acta Naturae,* 5, 15-21.

VAZQUEZ, A., LIU, J., ZHOU, Y. & OLTVAI, Z. N. 2010. Catabolic efficiency of aerobic glycolysis: The Warburg effect revisited. *BMC Systems Biology,* 4, 58-58.

VEGA, F. M., COLOMBA, A., REYMOND, N., THOMAS, M. & RIDLEY, A. J. 2012. RhoB regulates cell migration through altered focal adhesion dynamics. *Open Biol,* 2, 120076.

VEGRAN, F., BOIDOT, R., OUDIN, C., DEFRAIN, C., REBUCCI, M. & LIZARD-NACOL, S. 2007. Association of p53 gene alterations with the expression of antiapoptotic survivin splice variants in breast cancer. *Oncogene,* 26**,** 290-7.

VEINOTTE, C. J., DELLAIRE, G. & BERMAN, J. N. 2014. Hooking the big one: the potential of zebrafish xenotransplantation to reform cancer drug screening in the genomic era. *Dis Model Mech,* 7, 745-54.

VERMEULEN, K., VAN BOCKSTAELE, D. R. & BERNEMAN, Z. N. 2003. The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. *Cell Prolif,* 36.

VICIER, C., DIECI, M. V., ARNEDOS, M., DELALOGE, S., VIENS, P. & ANDRE, F. 2014. Clinical development of mTOR inhibitors in breast cancer. *Breast Cancer Res,* 16, 203.

VINCENT-SALOMON, A., GANEM-ELBAZ, C., MANIE, E., RAYNAL, V., SASTRE-GARAU, X., STOPPA-LYONNET, D., STERN, M. H. & HEARD, E. 2007. X inactive-specific transcript RNA coating and genetic instability of the X chromosome in BRCA1 breast tumors. *Cancer Res,* 67**,** 5134-40.

VLEUGEL, M. M., GREIJER, A. E., BOS, R., VAN DER WALL, E. & VAN DIEST, P. J. 2006. c-Jun activation is associated with proliferation and angiogenesis in invasive breast cancer. *Hum Pathol,* 37, 668-74.

VO, A. T. & MILLIS, R. M. 2012. Epigenetics and breast cancers. *Obstetrics and gynecology international,* 2012.

VOGELSTEIN, B., PAPADOPOULOS, N., VELCULESCU, V. E., ZHOU, S., DIAZ, L. A. & KINZLER, K. W. 2013. Cancer Genome Landscapes. *Science,* 339, 1546-1558.

VOLINIA, S., CALIN, G. A., LIU, C. G., AMBS, S., CIMMINO, A., PETROCCA, F., VISONE, R., IORIO, M., ROLDO, C., FERRACIN, M., PRUEITT, R. L., YANAIHARA, N., LANZA, G., SCARPA, A., VECCHIONE, A., NEGRINI, M., HARRIS, C. C. & CROCE, C. M. 2006. A microRNA expression signature of human solid tumors defines cancer gene targets. *Proc Natl Acad Sci U S A,* 103, 2257-61.

VOLINIA, S., GALASSO, M., SANA, M. E., WISE, T. F., PALATINI, J., HUEBNER, K. & CROCE, C. M. 2012. Breast cancer signatures for invasiveness and prognosis defined by deep sequencing of microRNA. *Proceedings of the National Academy of Sciences of the United States of America,* 109, 3024-3029.

VOSPER, J. M., MCDOWELL, G. S., HINDLEY, C. J., FIORE-HERICHE, C. S., KUCEROVA, R., HORAN, I. & PHILPOTT, A. 2009. Ubiquitylation on canonical and non-canonical sites targets the transcription factor neurogenin for ubiquitin-mediated proteolysis. *J Biol Chem,* 284, 15458-68.

VOSS, S. R. & SHAFFER, H. B. 2000. Evolutionary genetics of metamorphic failure using wild-caught vs. laboratory axolotls (Ambystoma mexicanum). *Mol Ecol,* 9, 1401-7.

VOSS, S. R., SMITH, J. J., GARDINER, D. M. & PARICHY, D. M. 2001. Conserved vertebrate chromosome segments in the large salamander genome. *Genetics,* 158, 735-46.

WAGNER, E. F. & NEBREDA, A. R. 2009. Signal integration by JNK and p38 MAPK pathways in cancer development. *Nat Rev Cancer,* 9, 537-49.

WAKAYAMA, S., JAKT, M. L., SUZUKI, M., ARAKI, R., HIKICHI, T., KISHIGAMI, S., OHTA, H., VAN THUAN, N., MIZUTANI, E., SAKAIDE, Y., SENDA, S., TANAKA, S., OKADA, M., MIYAKE, M., ABE, M., NISHIKAWA, S., SHIOTA, K. & WAKAYAMA, T. 2006. Equivalency of nuclear transfer-derived embryonic stem cells to those derived from fertilized mouse blastocysts. *Stem Cells,* 24, 2023-33.

WALTER, S. A., GUADAGNO, T. M. & FERRELL, J. E. 1997. Induction of a G(2)-Phase Arrest in Xenopus Egg Extracts by Activation of p42 Mitogen-activated Protein Kinase. *Molecular Biology of the Cell,* 8**,** 2157-2169.

WANG, Z., DAO, R., BAO, L., DONG, Y., WANG, H., HAN, P., YUE, Y. & YU, H. 2014. Epigenetic reprogramming of human lung cancer cells with the extract of bovine parthenogenetic oocytes. *J Cell Mol Med,* 18, 1807-15.

WANG, Z., GAO, H., WANG, H., REN, X., BAO, L., SA, R., WANG, J., BAI, H. & YU, H. 2013a. Specific reversal of tumor-suppressor gene promoter hypermethylation with bovine oocyte extract. *Oncol Rep,* 30, 179-84.

WANG, Z., YUE, Y., HAN, P., SA, R., REN, X., WANG, J., BAI, H. & YU, H. 2013b. Remodeling epigenetic modifications at tumor suppressor gene promoters with bovine oocyte extract. *Cytotherapy,* 15, 1164-73.

WANG, Z., ZANG, C., ROSENFELD, J. A., SCHONES, D. E., BARSKI, A., CUDDAPAH, S., CUI, K., ROH, T. Y., PENG, W., ZHANG, M. Q. & ZHAO, K. 2008. Combinatorial patterns of histone acetylations and methylations in the human genome. *Nat Genet,* 40, 897-903.

WEAVER, V. M., PETERSEN, O. W., WANG, F., LARABELL, C. A., BRIAND, P., DAMSKY, C. & BISSELL, M. J. 1997. Reversion of the malignant phenotype of human

breast cells in three-dimensional culture and in vivo by integrin blocking antibodies. *J Cell Biol,* 137, 231-45.

WEIGELT, B., MACKAY, A., A'HERN, R., NATRAJAN, R., TAN, D. S., DOWSETT, M., ASHWORTH, A. & REIS-FILHO, J. S. 2010. Breast cancer molecular profiling with single sample predictors: a retrospective analysis. *Lancet Oncol,* 11, 339-49.

WEIGELT, B., PETERSE, J. L. & VAN'T VEER, L. J. 2005. Breast cancer metastasis: markers and models. *Nat Rev Cancer,* 5, 591-602.

WEINBERG, S. E. & CHANDEL, N. S. 2015. Targeting mitochondria metabolism for cancer therapy. *Nat Chem Biol,* 11, 9-15.

WHETSTINE, J. R., NOTTKE, A., LAN, F., HUARTE, M., SMOLIKOV, S., CHEN, Z., SPOONER, E., LI, E., ZHANG, G., COLAIACOVO, M. & SHI, Y. Reversal of Histone Lysine Trimethylation by the JMJD2 Family of Histone Demethylases. *Cell,* 125, 467-481.

WHETSTINE, J. R., NOTTKE, A., LAN, F., HUARTE, M., SMOLIKOV, S., CHEN, Z., SPOONER, E., LI, E., ZHANG, G., COLAIACOVO, M. & SHI, Y. 2006. Reversal of histone lysine trimethylation by the JMJD2 family of histone demethylases. *Cell,* 125, 467-81.

WHYTE, J., BERGIN, O., BIANCHI, A., MCNALLY, S. & MARTIN, F. 2009. Key signalling nodes in mammary gland development and cancer. Mitogen-activated protein kinase signalling in experimental models of breast cancer progression and in mammary gland development. *Breast Cancer Res,* 11, 209.

WILKINSON, M. G. & MILLAR, J. B. 2000. Control of the eukaryotic cell cycle by MAP kinase signaling pathways. *FASEB J,* 14**,** 2147-57.

WISDOM, R., JOHNSON, R. S. & MOORE, C. 1999. c-Jun regulates cell cycle progression and apoptosis by distinct mechanisms. *The EMBO Journal,* 18, 188-197.

WU, S., WANG, W., KONG, X., CONGDON, L. M., YOKOMORI, K., KIRSCHNER, M. W. & RICE, J. C. 2010. Dynamic regulation of the PR-Set7 histone methyltransferase is required for normal cell cycle progression. *Genes Dev,* 24, 2531-42.

YAMANAKA, S. & BLAU, H. M. 2010. Nuclear reprogramming to a pluripotent state by three approaches. *Nature,* 465, 704-712.

YANG, C. R., WEI, Y., QI, S. T., CHEN, L., ZHANG, Q. H., MA, J. Y., LUO, Y. B., WANG, Y. P., HOU, Y., SCHATTEN, H., LIU, Z. H. & SUN, Q. Y. 2012. The G protein coupled receptor 3 is involved in cAMP and cGMP signaling and maintenance of meiotic arrest in porcine oocytes. *PLoS One,* 7, e38807.

YEE, A. S., PAULSON, E. K., MCDEVITT, M. A., RIEGER-CHRIST, K., SUMMERHAYES, I., BERASI, S. P., KIM, J., HUANG, C. Y. & ZHANG, X. 2004. The HBP1 transcriptional repressor and the p38 MAP kinase: unlikely partners in G1 regulation and tumor suppression. *Gene,* 336, 1-13.

YEH, A. C. & RAMASWAMY, S. 2015. Mechanisms of Cancer Cell Dormancy-Another Hallmark of Cancer? *Cancer Res,* 75, 5014-22.

YERSAL, O. & BARUTCA, S. 2014. Biological subtypes of breast cancer: Prognostic and therapeutic implications. *World Journal of Clinical Oncology,* 5, 412-424.

YILMAZER, A., DE LÁZARO, I. & TAHERI, H. 2015. Reprogramming cancer cells: A novel approach for cancer therapy or a tool for disease-modeling? *Cancer Letters,* 369, 1-8.

YOKOTA, J. 2000. Tumor progression and metastasis. *Carcinogenesis,* 21, 497-503.

YOKOYAMA, Y., MATSUMOTO, A., HIEDA, M., SHINCHI, Y., OGIHARA, E., HAMADA, M., NISHIOKA, Y., KIMURA, H., YOSHIDOME, K., TSUJIMOTO, M. & MATSUURA, N. 2014. Loss of histone H4K20 trimethylation predicts poor prognosis in breast cancer and is associated with invasive activity. *Breast Cancer Res,* 16, R66.

YOO, K. H. & HENNIGHAUSEN, L. 2012. EZH2 methyltransferase and H3K27 methylation in breast cancer. *Int J Biol Sci,* 8, 59-65.

YOSHIDA, Y., NAKAMURA, T., KOMODA, M., SATOH, H., SUZUKI, T., TSUZUKU, J. K., MIYASAKA, T., YOSHIDA, E. H., UMEMORI, H., KUNISAKI, R. K., TANI, K., ISHII, S., MORI, S., SUGANUMA, M., NODA, T. & YAMAMOTO, T. 2003. Mice lacking a transcriptional corepressor Tob are predisposed to cancer. *Genes Dev,* 17, 1201-6.

YOU, J. S. & JONES, P. A. 2012. Cancer Genetics and Epigenetics: Two Sides of the Same Coin? *Cancer Cell,* 22, 9-20.

YOULDEN, D. R., CRAMB, S. M., DUNN, N. A., MULLER, J. M., PYKE, C. M. & BAADE, P. D. 2012. The descriptive epidemiology of female breast cancer: an international comparison of screening, incidence, survival and mortality. *Cancer Epidemiol,* 36, 237-48.

YUAN, J., LUO, R. Z., FUJII, S., WANG, L., HU, W., ANDREEFF, M., PAN, Y., KADOTA, M., OSHIMURA, M., SAHIN, A. A., ISSA, J. P., BAST, R. C., JR. & YU, Y. 2003. Aberrant methylation and silencing of ARHI, an imprinted tumor suppressor gene in which the function is lost in breast cancers. *Cancer Res,* 63**,** 4174-80.

YUNES, M. J., NEUSCHATZ, A. C., BORNSTEIN, L. E., NABER, S. P., BAND, V. & WAZER, D. E. 2003. Loss of expression of the putative tumor suppressor NES1 gene in biopsy-proven ductal carcinoma in situ predicts for invasive carcinoma at definitive surgery. *Int J Radiat Oncol Biol Phys,* 56**,** 653-7.

ZAMANIAN-AZODI, M., REZAEI-TAVIRANI, M., RAHMATI-RAD, S., HASANZADEH, H., REZAEI TAVIRANI, M. & SEYYEDI, S. S. 2015. Protein-Protein Interaction Network could reveal the relationship between the breast and colon cancer. *Gastroenterology and Hepatology From Bed to Bench,* 8, 215-224.

ZANG, X., CHEN, M., ZHOU, Y., XIAO, G., XIE, Y. & WANG, X. 2015. Identifying CDKN3 Gene Expression as a Prognostic Biomarker in Lung Adenocarcinoma via Meta-analysis. *Cancer Informatics,* 14, 183-191.

ZARUBIN, T. & HAN, J. 2005. Activation and signaling of the p38 MAP kinase pathway. *Cell Res,* 15, 11-18.

ZHANG, X., CRUZ, F. D., TERRY, M., REMOTTI, F. & MATUSHANSKY, I. 2013. Terminal differentiation and loss of tumorigenicity of human cancers via pluripotency based reprogramming. *Oncogene,* 32, 2249-226121.

ZHANG, Y., PU, X., SHI, M., CHEN, L., QIAN, L., SONG, Y., YUAN, G., ZHANG, H., YU, M., HU, M., SHEN, B. & GUO, N. 2007. c-Jun, a crucial molecule in metastasis of breast cancer and potential target for biotherapy. *Oncol Rep,* 18, 1207-12.

ZHAO, Z.-K., YU, H.-F., WANG, D.-R., DONG, P., CHEN, L., WU, W.-G., DING, W.-J. & LIU, Y.-B. 2012. Overexpression of lysine specific demethylase 1 predicts worse prognosis in primary hepatocellular carcinoma patients. *World Journal of Gastroenterology : WJG,* 18, 6651-6656.

ZHOU, Q. M., WANG, X. F., LIU, X. J., ZHANG, H., LU, Y. Y. & SU, S. B. 2011. Curcumin enhanced antiproliferative effect of mitomycin C in human breast cancer MCF-7 cells in vitro and in vivo. *Acta Pharmacol Sin,* 32, 1402-10.

ZHOU, T., CHOU, J. W., SIMPSON, D. A., ZHOU, Y., MULLEN, T. E., MEDEIROS, M., BUSHEL, P. R., PAULES, R. S., YANG, X., HURBAN, P., LOBENHOFER, E. K. & KAUFMANN, W. K. 2006. Profiles of global gene expression in ionizing-radiation-damaged human diploid fibroblasts reveal synchronization behind the G1 checkpoint in a G0-like state of quiescence. *Environ Health Perspect,* 114, 553-9.

ZOU, P., YOSHIHARA, H., HOSOKAWA, K., TAI, I., SHINMYOZU, K., TSUKAHARA, F., MARU, Y., NAKAYAMA, K., NAKAYAMA, K. I. & SUDA, T. 2011. p57(Kip2) and p27(Kip1) cooperate to maintain hematopoietic stem cell quiescence through interactions with Hsc70. *Cell Stem Cell,* 9, 247-61.

ZOU, S., MENG, X., MENG, Y., LIU, J., LIU, B., ZHANG, S., DING, W., WU, J. & ZHOU, J. 2015. Microarray analysis of anti-cancer effects of docosahexaenoic acid on human colon cancer model in nude mice. *Int J Clin Exp Med,* 8, 5075-84.

Table S3.1: List of biological processes enriched in upregulated genes by GO analysis using REVIGO

GO term ID	Description	Frequency	log10 p-value
GO:0006396	RNA processing	1.63%	-8.0585
GO:0016071	mRNA metabolic process	1.10%	-7.1965
GO:0006397	mRNA processing	0.82%	-7.0343
GO:0015031	protein transport	3.60%	-5.5361
GO:0045184	establishment of protein localization	3.79%	-5.4248
GO:0046907	intracellular transport	3.54%	-5.4179
GO:0007049	cell cycle	3.70%	-5.3242
GO:0008380	RNA splicing	0.65%	-4.983
GO:0008104	protein localization	4.79%	-3.9101
GO:0006333	chromatin assembly or disassembly	0.28%	-3.7595
GO:0022402	cell cycle process	2.58%	-3.6737
GO:0048193	Golgi vesicle transport	0.51%	-3.6234
GO:0000278	mitotic cell cycle	1.73%	-3.5751
GO:0006334	nucleosome assembly	0.22%	-3.5436
GO:0006323	DNA packaging	0.36%	-3.52
GO:0031497	chromatin assembly	0.24%	-3.4191
GO:0065004	protein-DNA complex assembly	0.32%	-3.2612
GO:0034728	nucleosome organization	0.26%	-3.1858
GO:0051276	chromosome organization	2.33%	-3.1778
GO:0033365	protein localization to organelle	1.27%	-3.1675
GO:0006886	intracellular protein transport	2.20%	-3.1349
GO:0034613	cellular protein localization	3.13%	-2.9083
GO:0016192	vesicle-mediated transport	3.27%	-2.905
GO:0070727	cellular macromolecule localization	3.14%	-2.8677
GO:0022403	cell cycle phase	0.01%	-2.8677
GO:0032269	negative regulation of cellular protein metabolic process	1.09%	-2.7924
GO:0006261	DNA-dependent DNA replication	0.27%	-2.6695
GO:0051248	negative regulation of protein metabolic process	1.27%	-2.6374
GO:0051301	cell division	1.71%	-2.6113
GO:0043161	proteasome-mediated ubiquitin-dependent protein catabolic process	0.46%	-2.3285
GO:0010498	proteasomal protein catabolic process	0.49%	-2.3285
GO:0010605	negative regulation of macromolecule metabolic process	3.67%	-2.2977
GO:0046034	ATP metabolic process	5.50%	-2.246
GO:0006754	ATP biosynthetic process	1.28%	-2.1722
GO:0000279	M phase	0.01%	-2.1046
GO:0015986	ATP synthesis coupled proton transport	1.24%	-2.1036

GO:0015985	energy coupled proton transport, down electrochemical gradient	1.24%	-2.1036
GO:0006605	protein targeting	1.10%	-2.1019
GO:0006325	chromatin organization	1.76%	-2.0881
GO:0017038	protein import	0.59%	-2.0878
GO:0000910	cytokinesis	0.28%	-2.0582
GO:0000377	RNA splicing, via transesterification reactions with bulged adenosine as nucleophile	0.42%	-2.0564
GO:0000375	RNA splicing, via transesterification reactions	0.43%	-2.0564
GO:0000398	mRNA splicing, via spliceosome	0.42%	-2.0564
GO:0010324	membrane invagination	0.06%	-2.0185
GO:0006897	endocytosis	1.43%	-2.0185
GO:0006888	ER to Golgi vesicle-mediated transport	0.22%	-2.0142
GO:0006091	generation of precursor metabolites and energy	6.57%	-2.0032
GO:0006457	protein folding	0.72%	-2.0028
GO:0006417	regulation of translation	0.52%	-1.9577
GO:0009205	purine ribonucleoside triphosphate metabolic process	8.15%	-1.9485
GO:0051130	positive regulation of cellular component organization	1.70%	-1.9307
GO:0009206	purine ribonucleoside triphosphate biosynthetic process	1.33%	-1.9292
GO:0006633	fatty acid biosynthetic process	0.31%	-1.9279
GO:0009199	ribonucleoside triphosphate metabolic process	8.17%	-1.9258
GO:0009145	purine nucleoside triphosphate biosynthetic process	1.34%	-1.9043
GO:0009201	ribonucleoside triphosphate biosynthetic process	1.35%	-1.9043
GO:0009144	purine nucleoside triphosphate metabolic process	8.17%	-1.8379
GO:0009142	nucleoside triphosphate biosynthetic process	1.37%	-1.8316
GO:0032268	regulation of cellular protein metabolic process	3.40%	-1.829
GO:0010638	positive regulation of organelle organization	0.83%	-1.8178
GO:0010608	posttranscriptional regulation of gene expression	0.84%	-1.8032
GO:0006412	translation	1.98%	-1.7735
GO:0034220	ion transmembrane transport	6.09%	-1.7406
GO:0006606	protein import into nucleus	0.53%	-1.7401
GO:0045596	negative regulation of cell differentiation	1.43%	-1.7269
GO:0045665	negative regulation of neuron differentiation	0.17%	-1.7216
GO:0051170	nuclear import	0.53%	-1.6905
GO:0000280	nuclear division	1.12%	-1.6689
GO:0007067	mitotic nuclear division	0.69%	-1.6689
GO:0009141	nucleoside triphosphate metabolic process	8.21%	-1.656
GO:0030705	cytoskeleton-dependent intracellular transport	0.25%	-1.639

GO:0010558	negative regulation of macromolecule biosynthetic process	2.48%	-1.6371
GO:0006986	response to unfolded protein	0.19%	-1.6262
GO:0016053	organic acid biosynthetic process	0.80%	-1.6172
GO:0046394	carboxylic acid biosynthetic process	0.80%	-1.6172
GO:0000087	mitotic M phase	0.01%	-1.6127
GO:0007018	microtubule-based movement	0.72%	-1.591
GO:0030514	negative regulation of BMP signaling pathway	0.07%	-1.5729
GO:0034504	protein localization to nucleus	0.65%	-1.5515
GO:0048285	organelle fission	1.18%	-1.5441
GO:0009150	purine ribonucleotide metabolic process	8.62%	-1.5279
GO:0010604	positive regulation of macromolecule metabolic process	4.92%	-1.5239
GO:0009891	positive regulation of biosynthetic process	3.39%	-1.5173
GO:0009152	purine ribonucleotide biosynthetic process	1.74%	-1.5124
GO:0032259	methylation	1.04%	-1.4984
GO:0045787	positive regulation of cell cycle	0.25%	-1.4864
GO:0031400	negative regulation of protein modification process	0.87%	-1.4748
GO:0006119	oxidative phosphorylation	3.10%	-1.4661
GO:0006892	post-Golgi vesicle-mediated transport	0.14%	-1.4581
GO:0006260	DNA replication	0.93%	-1.4302
GO:0009890	negative regulation of biosynthetic process	2.62%	-1.42
GO:0010557	positive regulation of macromolecule biosynthetic process	3.14%	-1.4195

Table S3.2: List of biological processes enriched in downregulated genes by GO analysis using REVIGO

GO term ID	Description	Frequency	log10 p-value
GO:0006412	translation	1.98%	-14.0264
GO:0006414	translational elongation	0.31%	-10.3516
GO:0034660	ncRNA metabolic process	0.92%	-5.6271
GO:0019318	hexose metabolic process	0.69%	-4.9281
GO:0016051	carbohydrate biosynthetic process	0.51%	-4.5129
GO:0006259	DNA metabolic process	3.16%	-4.3261
GO:0005996	monosaccharide metabolic process	0.74%	-4.2111
GO:0034637	cellular carbohydrate biosynthetic process	0.21%	-3.9318
GO:0006364	rRNA processing	0.21%	-3.7033
GO:0043038	amino acid activation	0.29%	-3.6799
GO:0006418	tRNA aminoacylation for protein translation	0.28%	-3.6799
GO:0043039	tRNA aminoacylation	0.29%	-3.6799
GO:0033554	cellular response to stress	3.48%	-3.6402
GO:0016072	rRNA metabolic process	0.22%	-3.4949
GO:0006281	DNA repair	1.41%	-3.4295
GO:0042254	ribosome biogenesis	0.32%	-3.2774
GO:0006974	cellular response to DNA damage stimulus	1.97%	-3.1649
GO:0034470	ncRNA processing	0.55%	-3.0218
GO:0022613	ribonucleoprotein complex biogenesis	0.60%	-2.8641
GO:0006839	mitochondrial transport	0.33%	-2.7817
GO:0006091	generation of precursor metabolites and energy	6.57%	-2.7298
GO:0006006	glucose metabolic process	0.52%	-2.5747
GO:0009303	rRNA transcription	0.07%	-2.5689
GO:0006399	tRNA metabolic process	0.57%	-2.5556
GO:0044275	cellular carbohydrate catabolic process	0.11%	-2.523
GO:0046165	alcohol biosynthetic process	0.28%	-2.5218
GO:0006396	RNA processing	1.63%	-2.5209
GO:0055114	oxidation-reduction process	10.93%	-2.445
GO:0046364	monosaccharide biosynthetic process	0.22%	-2.3664
GO:0006007	glucose catabolic process	0.07%	-2.2897
GO:0006308	DNA catabolic process	0.25%	-2.1909
GO:0070271	protein complex biogenesis	2.59%	-2.0663
GO:0006461	protein complex assembly	2.58%	-2.0663
GO:0016052	carbohydrate catabolic process	0.48%	-2.055
GO:0044264	cellular polysaccharide metabolic process	0.26%	-2.0175

GO:0006260	DNA replication	0.93%	-1.9258
GO:0006457	protein folding	0.72%	-1.9214
GO:0006413	translational initiation	0.47%	-1.8925
GO:0000737	DNA catabolic process, endonucleolytic	0.18%	-1.8471
GO:0019320	hexose catabolic process	0.09%	-1.8033
GO:0006096	glycolytic process	0.31%	-1.7931
GO:0006261	DNA-dependent DNA replication	0.27%	-1.79
GO:0015980	energy derivation by oxidation of organic compounds	5.87%	-1.7715
GO:0043933	macromolecular complex subunit organization	5.28%	-1.7614
GO:0048548	regulation of pinocytosis	0.01%	-1.7464
GO:0006434	seryl-tRNA aminoacylation	0.02%	-1.7464
GO:0065003	macromolecular complex assembly	2.98%	-1.7439
GO:0046365	monosaccharide catabolic process	0.10%	-1.728
GO:0060627	regulation of vesicle-mediated transport	0.67%	-1.6966
GO:0042274	ribosomal small subunit biogenesis	0.03%	-1.6692
GO:0010885	regulation of cholesterol storage	0.05%	-1.6692
GO:0030100	regulation of endocytosis	0.36%	-1.6677
GO:0009311	oligosaccharide metabolic process	0.09%	-1.536
GO:0019319	hexose biosynthetic process	0.20%	-1.536
GO:0016053	organic acid biosynthetic process	0.80%	-1.5149
GO:0046394	carboxylic acid biosynthetic process	0.80%	-1.5149
GO:0006289	nucleotide-excision repair	0.21%	-1.4527

Appendix

Supplementary information for Western Blotting

1. 5X Tris-glycine electrophoresis buffer (TE buffer) in 500ml

No	Ingredient	Volume
1.	25 mM Trizma base	7.55g
2.	250 mM Glycine	47 g
3.	0.1% SDS	25ml

2. 10x TBS-Tween 20 (0.5M Tris Base, 9% NaCl, 0.5% Tween 20), pH 8.4

No	Ingredient	Volume
1.	25 mM Trizma base	61g
2.	250 mM Glycine	90g
3.	ddH2O	25ml
	Dilute 1:10 with distilled water before use and adjust pH if necessary	

3. Polyacrylamide gel

No	Ingredient	12% Resolving gel (5ml)	5% Stacking gel (2ml)
1.	H_2O	1.6	1.4
2.	30% acrylamide mix	2.0	0.33
3.	1.5M Tris (pH 8.8)	1.3	0.25
4.	10% SDS	0.05	0.02
5.	10% ammonium persulphate	0.05	0.02
6.	TEMED	0.002	0.002

4. Transfer buffer (pH 8.3)

No	Ingredient	Volume
1.	48 mM Tris	2.9g
2.	0.037% SDS	0.1g
3.	39 mM Glycine	1.45
4.	20% methanol	100ml
5.	Sterile water	500ml

5. 2x Laemmli Sample Buffer

No	Ingredient	Volume (ml)
1.	Deionized water	3.55
2.	Tris-HCl, pH 6.8	1.25
3.	Glycerol	2.5
4.	10% (w/v) SDS	2.0
5.	0.5% (w/v) bromophenol blue	0.2
Total volume		9.5

`

*Add 50 µl β-mercaptoethanol to 950 µl of sample buffer before use.