MicroRNAs Modulate the Response of p53-MDM2 Dynamics to DNA Double-strand Breaks at Single-cell Level

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Biology, Medicine and Health

2016

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List of Abbreviations

5-hmUra – 5-hydroxymethyluracil
53BP1 – p53 Binding Protein 1
8-oxodG – 7,8-dihydro-8-oxo-2′-deoxyguanosine
8-oxoGua – 7,8-dihydro-8-oxoguanine
β-GAL – β-Galactosidase
aa – Amino Acid
ADP – Adenosine Diphosphate
ALL – Acute Lymphoblastic Leukaemia
ANOVA – Analysis of Variance
APAF1 – Apoptotic Protease Activating Factor 1
APS – Ammonium Persulfate
ARF – ADP-ribosylation Factor
AT – Ataxia Telangiectasia
ATM – Ataxia Telangiectasia Mutated
ATR – Ataxia Telangiectasia and Rad3 Related
ATRIP – ATR-interacting Protein
BAC – Bacterial Artificial Chromosome
bp – Base Pair(s)
BRCA1 – Breast Cancer Type 1
BSA – Bovine Serum Albumin
CAK – CDK-activating Kinase
CBP – CREB-binding Protein

CDC25A – Cell Division Cycle 25 Homologue A

CDK – Cyclin Dependent Kinase

CHK1/2 – Checkpoint Kinase 1/2

Chlor – Chloramphenicol

CK2 – Casein Kinase 2

CLL – Chronic Lymphoblastic Leukaemia

COP1 – Constitutive Photomorphogenesis Protein 1

CREB – cAMP Response Element-binding Protein

CRISPR – Clustered Regularly Interspaced Short Palindromic Repeats

CTD – C-terminal Domain

DBD – DNA Binding Domain

DDR – DNA Damage Response

DRM – DNA Repair Machinery

DMEM – Dulbecco’s Modified Eagle’s Medium

DMSO – Dimethylsulfoxide

DNA-PK – DNA-dependent Protein Kinase

DNase – Deoxyribonuclease

DSB – Double-strand Break

DsRedXP – Discosoma sp. Red Fluorescent Protein Express

DTT – Dithiothreitol

eCFP – Enhanced Cyan Fluorescent Protein
ECL – Enhanced Chemiluminescence
EDTA – Ethylenediaminetetraacetic Acid
EF3 – Elongation Factor 3
EMT – Epithelial-mesenchymal Transition
ERK – Extracellular Signal-regulated Kinase
FACS – Fluorescence-activated Cell Sorting
FBS – Foetal Bovine Serum
FCS – Foetal Calf Serum
FCS – Fluorescence Correlation Spectroscopy
FISH – Fluorescent in situ Hybridisation
FRAP – Fluorescence Recovery after Photobleaching
FRET – Fluorescence Resonance Energy Transfer
GADD45α – Growth Arrest and DNA Damage 45α
GAPDH – Glyceraldehyde-3-phosphate Dehydrogenase
H2A.X – Histone 2A, member X
HAT – Histone Acetyltransferase
HEAT – Huntingtin, EF3, PP2A and TOR1
HIF – Hypoxia-inducible Factors
HRP – Horse Radish Peroxidase
IR – Ionising Radiation
JNK – c-Jun N-terminal Kinase
kb – Kilo Base Pairs
KD – Kinase Domain
KLF4 – Kruppel-like Factor 4
IncRNA – Long Non-coding RNA
Lys – Lysine
M1dG – 3-(2'-deoxy-beta-D-erythro-pentofuranosyl)-pyrimido[1,2-a]-purin-10(3H)-one
MAPK – Mitosis-activated Protein Kinases
MDC1 – Mediator of DNA Damage Checkpoint Protein 1
MDM2 – Murine Double Minute 2
MET – Mesenchymal-epithelial Transition
miRNA – microRNA
miRNP – microRNA Ribonucleoprotein Complex
MOF – Males Absent on the First
MRN – MRE11/RAD50/NBS1
NCS – Neocarzinostatin
NCTC – National Collection of Type Cultures
NEAA – Non-essential Amino Acid
Neo – Neomycin
NLS – Nuclear Localisation Sequence
OD – Oligomerisation Domain
PAH – Polycyclic Aromatic Hydrocarbons
PARP – Poly (ADP-ribose) Polymerase
PBS – Phosphate Buffered Saline
PCD – Programmed Cell Death

pCMV – Cytomegalovirus Promoter

PEI40 – Polyethylenimine 40

PFGE – Pulse-field Gel Electrophoresis

PI3K – Phosphatidylinositol-3-kinases or Phosphatidylinositol-3-kinases

PIKK – Phosphatidylinositol-3-kinases-related Kinases or Phosphatidylinositol-3-kinases-related Kinases

PIRH2 – p53-induced Protein with a RING-H2 Domain

PKB (AKT) – Protein Kinase B

PRD – PIKK-regulatory Domain

PRP19 – Pre-RNA Processing 19

pMT – Metallothionein Promoter

PP2A – Protein Phosphatase 2A

PTM – Post-translational Modification

PUMA – p53 Upregulated Modulator of Apoptosis

Rb – Retinoblastoma Protein

RCS – Reactive Carbonyl Species

RE – Response Element

RFP – Red Fluorescent Protein

RING – Really Interesting New Gene

RISC – RNA-induced Silencing Complex

RNase – Ribonuclease

RNS – Reactive Nitrogen Species
ROS – Reactive Oxygen Species

RPA – Replication Protein A

SDS – Sodium Dodecyl Sulphate

Ser – Serine

SIRT1 – Silent Information Regulator 1

SOC – Super Optimal Broth with Catabolite Repression

SSB – Single-strand Break

SUMO – Small Ubiquitin-like Modifier

SWI/SNF – SWItch/Sucrose Non-fermentable

TAD – Trans-activation Domain

Thr – Threonine

TIP60 – TATA-interactive Protein 60

TIPIN – TIMELESS-interacting Protein

TOPBP1 – Topoisomerase II Binding Protein 1

TOR – Target of Rapamycin

TRRAP – Transformation/Transcription Domain-associated Protein

USP7 – Ubiquitin-specific-processing Protease 7

UTR – Untranslated Region

UV – Ultraviolet

WIP1 – Wild-type p53-induced phosphatase 1

WT – Wild-type

XRCC1 – X-ray Cross-complementing 1
YFP – Yellow Fluorescent Protein

ZEB1/2 – Zinc Finger E-box Binding Homeobox 1

Word count: 58704
Abstract

The tumour suppressor protein p53 plays a pivotal role in the response to various cellular stresses and thus undergoes complicated patterns of regulation, which has been well-studied. However, recent research exploring this topic at single-cell level has revealed that the dynamical behaviour of p53 and its regulator MDM2 in response to DNA damage was distinct from the general trends seen at population level. Moreover, these studies demonstrated that p53-MDM2 dynamics at single-cell level is correlated with cell fate determination.

However, the original conclusions were obtained from the simple plasmid-based cell models, which cannot fully recreate the native behaviour of p53-MDM2 in response to stress signals. To better understand this question, we adapted and improved the previous model by introducing a BAC vector containing native p53 gene labelled with a fluorescent protein. This model has also allowed us to investigate whether miRNAs can modulate p53 dynamics and follow cell fate at the single-cell level.

By using time-lapse fluorescent microscopy, our results indicated that p53-MDM2 dynamics in response to DNA double-strand breaks was indeed different in single cells when compared with the population-based results obtained in parallel in both models. Furthermore, the dynamics of p53 signalling in individual cells showed the following characteristics: 1) It was variable from cell to cell, but the mean value of some parameters remained fixed; 2) It was able to be fine-tuned by miRNAs; 3) It is involved in shaping cell fate determination with the regulation by miRNAs leading to distinct. This work identified discrepancies in different cell models, suggesting the relative importance of transgenic tools used for single-cell research.

In conclusion, our research not only provided a new approach to study the interaction between miRNAs and p53, but also suggested a new dimension of p53 regulatory mechanism and cell fate determination, which further demonstrates the complexity of biological systems that respond to cellular stress in mammalian cells.
Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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Acknowledgement

First of all, I would like to thank my supervisor Professor Dean Jackson, who accepted my PhD application and provided me with loads of helpful guidance and support for my study in the last four and half years; I particularly want to express my gratitude to Dean for his great sympathy and patience while I was difficulty in writing the thesis for the psychological reason. Then I want to say thank you to Professor Mark Muldoon, for his massive help to do huge amount of advanced data analysis for me, which I definitely cannot achieve on my own. I also want to thank my advisor Professor Mike White, who kindly provided me with Lahav’s fluorescent-labelled stable MCF7 cell line, original p53-DsRedXP BAC and LaminB1-eCFP plasmid to use, all of which are key materials in my project. He, together with my tutor Dr. Lisa Swanton, gave me key suggestions for my project and thesis as well; many thanks for both of your kind help. Another huge thank you is to give Dr. Antony Adamson who offered me great guidance for all BAC and plasmid manipulation, which is one of the most important assays in my project. Dr. James Boyd and Dr. Dave Spiller, I can guarantee that you are the very guys who have invested the longest time to help me familiarise with time-lapse microscopy and CellTracker®; I would not get so much key data without your kind help. Dr. Apolinar Maya-Mendoza and Dr. James Bagnall, thank you very much for giving me useful guidance for Western Blot, immunofluorescence and transfection. Dr. Angela Pisco, Dr. Emanuela Monteiro and Dr. Amro Ahmed-Ebbiary, thank you very much for providing me with loads of your thoughts, ideas, experiences and technical tips during lab work. And also, to Mrs. Lorraine Schmidt, who has been investing a lot on making our lab one of the best places to do cancer research across the world, I would definitely say thank you so much for your wonderful contributions. Finally, to all of the past and present members of Jackson, White and Paszek groups I did not mention above, thank you very much for your help and making my entire PhD career enjoyable and unforgettable.
To all of my friends in China and UK, I sincerely say thank you so much for always being there with me to make me live happily, either physically or psychologically; particularly, I would like to show my special gratitude to two of my friends, Miss Xiaoni Wen and Dr. Zhan Gao, for their kind help to arrange the format of my thesis and statistical data.

Last but not the least, to my family, especially my parents, I really don’t know how to express my gratitude to you, for your eternal and enormous support and love to me. Even though it is from thousands miles away, I can still feel it clearly. What I definitely know is that, I will not be right here without you; thank you very much indeed.
Chapter 1  Introduction

The first chapter of the thesis aims to provide the detailed introduction of the background knowledge involved in this project. It is necessary to do so because it helps to understand what the project sets out to achieve, why these objectives are important, how hypotheses are proposed and tested, as well as how results are obtained and analysed. According to the overall conclusion of the study, elucidated in the abstract, the following aspects of background information and development are included: 1) The sources of DNA damage and the types of damage that result; 2) The reaction of cells to various stress signals and detailed responsive mechanisms, such as cell fate, the architecture of responsive signalling pathways and key components of pathways; 3) The comprehensive introduction of p53, ranging from the gene structure, biological function and modulation to dynamics in individual cells; 4) A brief introduction of microRNAs (miRNAs), including the process of biogenesis, general function and the crosstalk with p53; 5) The complexity of p53 regulatory system, focusing on the heterogeneity and stochasticity of various cellular processes. Following the introduction of these topics, the last part of this chapter will describe the overall aim of the project by detailing specific points in logical order, with a general indication of the approaches and potential expectations of each point.

1.1  DNA Damage

In our project, p53 is investigated in the scenario of DNA damage events and thus it is definitely necessary to learn the nature of DNA damage, how damage arises and the consequences for a cell.

As we know, DNA is responsible for storage and transmission of genetic information. Therefore, maintaining the integrity of DNA molecules is one of the most essential cellular function. Unfortunately, DNA integrity is always threatened by a variety of endogenous and exogenous factors, which can lead to DNA damage. Fundamentally, DNA damage is an abnormal change in the chemical structure of DNA molecules, which
has multiple types, such as DNA single-strand breaks (SSBs), DNA double-strand breaks (DSBs), base deletions, base pair mismatch and chemically modified bases (De Bont and van Larebeke, 2004).

DNA damage is directly triggered either by hydrolytic processes or reactive metabolites. With DNase catalysis, hydrolytic reaction cleaves phosphodiester bonds between phosphate groups, generating both DSBs and SSBs on the backbone of DNA molecules; this reaction is involved in DNA replication and recombination process and is the predominant way to correct replication errors (Lindahl and Barnes, 2000). Reactive metabolic by-products, such as reactive oxygen species (ROS), reactive nitrogen species (RNS), reactive carbonyl species (RCS) and lipid peroxides, are also able to result in DNA breaks. Moreover, the active metabolites can add, remove or change chemical groups on bases, forming 7,8-dihydro-8-oxo-2’-deoxyguanosine (8-oxodG), 7,8-dihydro-8-oxoguanine (8-oxoGua), 5-hydroxymethyluracil (5-hmUra) or 3-(2’-deoxy-beta-D-erythro-pentofuranosyl)-pyrimido[1,2-a]-purin-10(3H)-one (M1dG) that can lead to gene dysfunction at worst (De Bont and van Larebeke, 2004, Jackson and Bartek, 2009).

DNA damage occurs spontaneously as well. For example, during DNA replication, the probability of base pair mismatch or loss is $10^{-10}$, which is approximately equal to 1 base pair (bp) per chromosome per cell cycle, although DNA polymerase can automatically correct them by 3’-5’ exonuclease activity (Lindahl and Barnes, 2000). DNA molecules might also lose their chemical groups, such as amino (deamidisation), pyrimidine (depyrimidination) and purines (depurination), hence form a series of isoforms of bases on their backbones as a consequence (Lindahl and Nyberg, 1972).

Although the origins of DNA damage listed above are endogenous, many exogenous factors are able to massively boost these effects (Jackson and Bartek, 2009). These factors are divided into three categories. The first one is physical factors, such as ultraviolet (UV), ionising radiation (IR) and X-ray damage; the second category is chemical factors, including mutagenic polycyclic aromatic hydrocarbons (PAH), dioxin, nitrosamines, antibiotics and anti-cancer drugs with DNA damage effect like alkylating agents; the third group is biological factors, such as viruses and microorganism-derived toxins (Jackson and Bartek, 2009). In addition, there is concrete evidence to show that with exposure to different damaging agents, different types of DNA damage take place
A typical example is that IR predominantly generates DSBs while UV causes single-strand lesions; however, most sources result in multiple types of damage (Marteijn et al., 2014, Thompson, 2012).

1.2 Major Signalling Pathways in Response to DNA Damage

1.2.1 Cell Fate

Since one of the most innovative ideas in our project is to better understand the correlation between p53 dynamics in single cells and cell fate using an improved cell model, it is necessary to define exactly what the cell fate is, and elucidate the background knowledge of cell fate and how cell fate is determined.

In this thesis cell fate is defined as the final state of a cell in response to stress signals, which generally includes cell cycle arrest, DNA repair, cell senescence (permanent cell cycle arrest) and programmed cell death (PCD, also known as cell apoptosis).

How cell fate is determined depends on three major factors. The first one is how intense the level of damage is. It is evident that the DNA repair machinery (DRM) has a limited maximum capability so if it is overloaded due to high levels of damage, it is unable to repair damage effectively and hence cell death, rather than any other types of cell fate, occurs (Kastan and Bartek, 2004). In other words, the uncertainty of cell fate tends to arise when cells are exposed to a level of damage below the maximum capability of DRM.

Secondly, cell state is another key point to determine cell fate. For example, the accumulation of DNA damage in non-replicating cells is prone to resulting in cell senescence; however, if the comparable level of damage occurs in replicating cells, cell death is much more likely to happen than in non-replicating cells (Kastan and Bartek, 2004). A reasonable explanation is that cell death in this case reduces replication stress and protects daughter cells from replication errors and epigenetic aberration caused by DNA damage, thus decreasing the probability of malignant gene mutations contribute to cell transformation and carcinogenesis (Kastan and Bartek, 2004). This is why some anti-cancer drugs, especially alkylating agents, are much more effective on proliferating cells than on non-growing cells.
The last and the most important factor is how cells respond to DNA damage. As previously described, due to the great importance of genome integrity, organisms have develop a complicated network of DNA damage responses (DDR) in order to process a variety of damage types that underpin genotoxic stress signalling (Jackson and Bartek, 2009). Different types of stress activate different DDR pathways, followed by different cell fate (Purvis et al., 2012). Notably, although the DDR network is very sophisticated, there are some key proteins that act as critical nodes, which are common to different modules of repair network. Some of these critical proteins are sensors, such as poly (ADP-ribose) polymerase (PARP), DNA-dependent protein kinases (DNA-PKs), ataxia telangiectasia and Rad3 related (ATR) and ataxia telangiectasia mutated (ATM); some are mediators, like checkpoint kinase 1/2 (CHK1/2) and p53; while others, such as p21, cyclin-dependent kinases (CDKs), p53 up-regulated modulator of apoptosis (PUMA) and Caspases, are components responsible for carrying out orders issued by upstream elements and thus cell fate is finally determined (Polo and Jackson, 2011). For instance, DSBs are predominantly detected by DNA-PKs and ATM, followed by the transmission of DSBs signals to p53. p53 is responsible for integrating and analysing upstream stress signals and making decisions that how cells react to them on the basis of the properties of signals, such as intensity, duration and the number of lesions on DNA strands, etc.; once the decision is made, the corresponding machineries will be activated and hence p53’s decision on cell fate is achieved eventually. However, if the damage causes SSBs, instead of DNA-PKs and ATM, PARP and ATR will take over the detection task and these proteins will then transmit SSBs signals to p53, where another cell fate decision may be made, so perhaps a different set of downstream components is activated to follow p53’s command (Harms et al., 2004).

1.2.2 ATM Signalling Pathway

As one of the most important members of the DRM, ATM is a key protein in detection of lesions on DNA strands and transmission of damage signals to downstream components for further processing by activating p53 and other mediators. This fact makes the ATM-p53 signalling pathway essential in response to stress signals. Therefore, it is necessary
to introduce ATM and ATM-dependent pathways as the activation of p53 following DNA damage is the key theme of our project.

Containing 3056 amino acids (aa), ATM is a 350 kilo Dalton (kDa) nuclear serine/threonine (Ser/Thr) protein kinase, which consists of five functional domains. Originally found to be mutated in ataxia telangiectasia patients, ATM is a key protein in monitoring DNA integrity, especially DSBs, and transmitting DNA damage signals to downstream elements to trigger DDR (Canman et al., 1998). As Figure 1.1 shows, ATM plays a major role in response to DSBs, initiating various downstream signalling pathways that lead to different cell fate.

![Figure 1.1: The Basic Architecture of ATM Pathways](Khalil et al., 2012). ATM dominates the initiation of DSBs response pathways by activating a series of downstream key components or co-activators such as p53, p-CHK2, H2AX, BRCA-1 and MRN complex, leading to various signalling-dependent biological outcomes, which are identified (coloured boxes).
The transmission of stress signals to downstream targets by ATM is activated by damage-induced post-translational modifications (PTMs), due to the protein kinase activity of ATM. Basically, when DNA damage is absent, ATM is constitutively dephosphorylated by protein phosphatase 2A (PP2A) and present in a cell as dimers or multimers (Goodarzi et al., 2004). In the presence of DSBs, ATM molecules react by undergoing conformational changes, followed by the dissociation with PP2A and autophosphorylation at Ser367, Ser1893 and Ser1981, which further break down non-functional dimers or multimers into the active monomers with kinase activity. Then activated ATM is recruited to damaged sites, followed by a series of changes achieved with the help of the interaction with mediator of DNA damage checkpoint protein 1 (MDC1) and MRE11/RAD50/NBS1 (MRN) complex (Goodarzi et al., 2004, Lee and Paull, 2007). Together with the acetylation at lysine (Lys) 3016, which is induced by TATA-interactive protein 60 (TIP60), a histone Acetyltransferase (HAT), ATM phosphorylates γ-H2A.X (Histone 2A, member X) at Ser139 and thus leads to the proper formation and preservation of specific DNA structure, which is good for the recruitment of key mediators like CHK2 and p53 at damaged sites (Huang et al., 2004, Sun et al., 2007). The binding of CHK2 and p53 gives rise to the phosphorylation of CHK2 at Thr68 and p53 at Ser15, followed by the initiation of a dual-step response (Canman et al., 1998). On one hand, CHK2 rapidly phosphorylates the phosphatase cell division cycle protein 25 homologue A (CDC25A) to abolish the dephosphorylation of cyclin/CDK complexes. This leads to cell cycle arrest, which allows DSBs to be repaired under conditions where no DNA replication is taking place; this link between cell cycle arrest and repair is crucial to genetic stability as replicating a damaged template delivers a high probability of DNA mutations. If the extent of DNA damage is too severe to be substantially repaired, CHK2 takes further action to phosphorylate p53 at Ser20, which stabilises p53 and triggers p53 trans-activities to up-regulate the expression of p53 downstream target genes such as CDKs inhibitor p21 or PCD inducer PUMA (Kern et al., 1991b, Canman et al., 1998). Depending on the trans-activation of different set of p53 downstream targets, cell fate is eventually determined. For example, if p21 is activated, the cell cycle tends to be permanently arrested and cells senesce; whereas the up-regulation of PUMA initiates caspases-induced cell apoptosis (Valerie and Povirk, 2003).
1.2.3 ATR Signalling Pathway

Mammalian cells are so complex that multiple pathways often work in parallel to combat their most dangerous threats. This is simply because the environment is so complicated that an organism must develop alternative and compensatory mechanisms to increase survival. DDR is a typical example due to its great importance. It has been found that apart from the ATM pathway, a related ATR pathway provides an alternative p53-dependent mechanism for organisms to process stress signals. Although our project puts more focus on ATM-p53 pathway, it is important to understand the synergy between ATM and ATR in combating genotoxic stress. Like ATM, ATR is a key DNA damage sensor protein and nuclear Ser/Thr protein kinase belonging to PI3K family. ATR gene is located at 3q23 and spans 129.5 kilo base pairs (kb). Consisting of 2644aa, the molecular weight of ATR is 301kDa. Similar to ATM, ATR also has five domains and the only difference is that the proline-rich domain of ATM is substituted by another HEAT repeat domain.

Though two proteins are related, ATR plays a different role from ATM in DDR. ATR is one of the key components involved in detection of SSBs induced by UV, hydrolysis, metabolites and chemicals that can block or destroy replication fork, such as aphidicolin and hydroxyurea (Nam and Cortez, 2011). When SSBs occur, ATR targets and binds to the sites of SSBs with the help of PARP1/2, ATR-interacting protein (ATRIP), replication protein A (RPA), TIMELESS, TIMELESS-interacting protein (TIPIN), Claspin, topoisomerase II binding protein 1 (TOPBP1), cell cycle checkpoint proteins RAD17 and 9-1-1 (RAD9, RAD 1 and HUS1) clamp complex. Activation of ATR results through autophosphorylation at Thr1989 and the recruitment of a series of major components involved in DNA repair and cell fate determination, including BRCA1, p53 binding protein 1 (53BP1), X-ray cross-complementing 1 (XRCC1), γ-H2AX, CHK1, p53, DNA ligase III and DNA polymerase β (Zou and Elledge, 2003, Sancar et al., 2004, Wu et al., 2014). The interaction between phosphorylated ATR and some of these proteins at damaged sites causes changes of PTMs in the interacting molecules, most of which are able to affect their biological activity. Due to their critical roles in multiple signalling pathways, the transition of their steady state levels leads to different biological outcomes. For example, activated ATR in response to UV-induced SSBs phosphorylates
γ-H2A.X at Ser139 and CHK1 at Ser317 and Ser345, which then gives rise to the activation of p53 by phosphorylating Ser15, Ser20 and Ser37 of p53; activated p53 is capable of determining cell fate by regulating the expression of its downstream target genes, which can specifically induce each type of cell fate (Liu et al., 2011b).

1.3 p53

The ATM and ATR pathways are the critical pathways required to transmit damage in DNA to downstream signalling pathways that will ultimately define the appropriate cell fate. The ATM and ATR signalling modules transmit information – as proteins that are regulated by post-translational modifications - to the core part of DDR and also the theme of our study - p53. This protein was discovered in 1979 and ten years later it was found that p53 can act as a tumour suppressor, playing a central role in cellular processes and cell growth because it is the most essential cell fate regulator during genomic stress. These findings coincide with many facts that most cancers are associated with abnormal changes of p53 function, including TP53 gene mutations, aberrant p53 expression and defects in the regulation of p53 expression and function (Vogelstein et al., 2000, Kruse and Gu, 2009).

Although the importance of p53 is widely accepted and p53 has become one of the most intensively studied proteins, many questions are still unclear, especially those related to its highly complicated modes of regulation. Thousands of studies, which investigated this topic from different aspects by various approaches, have addressed the established fact that p53 regulation is a highly coordinated system in which almost all biological processes and elements are involved, ranging from protein-protein interactions, PTMs to regulation conducted by non-coding RNAs and spatial and temporal factors. These facts not only provide us with a better understanding of p53 regulation, but also indicate the challenges faced and ignite new thoughts. My project is inevitably inspired by the established literature, so it is necessary to introduce general knowledge of p53, including its basic structure, function, regulatory mechanism, as well as specific molecular detail that is more directly related to the specific questions raised in our work.
1.3.1 Structure of p53

Human p53 is encoded by the TP53 gene, which is located at the short arm of chromosome 17 (17p13.1). TP53 gene spans around 20 kb and consists of eleven exons and ten introns. The transcription of TP53 gene is driven by two promoters. The P1 promoter constitutively controls the transcription of wild-type (WT) p53 while the internal P2 promoter initiates the transcription of several p53 isoforms (Bourdon et al., 2005). The mRNA of human WT p53 spans around 3.0kb and its open reading frame has 1179 base pairs, indicating that p53 protein has 393 amino acids.

As Figure 1.2 illustrates, WT human p53 protein consists of seven domains, which are two transcriptional activation domains (TAD1&2), a proline-rich domain containing 5 PXXP repeats (P stands for a proline residue and XX represents a random residue), a DNA binding domain (DBD), an oligomerisation domain (OD), a nuclear localisation sequence (NLS) and a C-terminal domain (CTD) (Cho et al., 1994). Two TADs are catalytic domains, which activate the transcription of downstream target genes with the help of the DBD; some studies further reported that their function was context-dependent (Garcia and Attardi, 2014, Joerger and Fersht, 2010, Johnson et al., 2005). The function of the proline-rich domain is controversial, as some studies indicated that it is indispensible in p53-induced cell apoptosis and tumour suppression whereas others demonstrated that its tumour suppression activity is context-dependent (Baptiste et al., 2002, Sakamuro et al., 1997, Toledo et al., 2007). The DBD is responsible for recognising and binding to p53 response elements (REs) in the promoters of p53 target genes and is the most frequently mutated domain in various cancers (Ludwig et al., 1996). The OD mediates p53 tetramerisation, which is required for p53 activation in response to stimulation. The CTD is the regulatory subunit of p53, in which many residues can be modified to manipulate p53 stability, trans-activity and DNA-binding ability (Kruse and Gu, 2009).
1.3.2 p53 Plays a Pivotal Role in Processing Stimulation Signals

After learning the basic structure of p53 gene and protein, it is necessary to understand why p53 is such an essential protein in higher eukaryotes. As mentioned above, p53 is a tumour suppressor involved in cell fate determination. It acts as a key transcription factor to regulate the expression of target genes, most of which are major components of cellular machineries that generate different cell fate outcomes in response to a range of cellular stresses. Due to its great importance and powerful capability to control cell fate, p53 is maintained in an inert state in the absence of an activating level of cellular stress. However, once an activating level of stress occurs, p53 activation is induced very efficiently.

In non-stressed cells, p53 is kept at a very low level by the association with its inhibitor MDM2; when a stress signal is detected and transmitted to p53, PTMs and conformational changes take place among p53 protein molecules, rendering them much higher stability and transcriptional activity (Li et al., 2003). As a result, intra-cellular p53 level increases, leading to the alteration of expression level of p53 target genes. These changes give rise to different cellular outcomes, such as cell cycle arrest, DNA repair, cell senescence, apoptosis and so on, all of which aim to protect cells from the pathological consequences (such as malignancy) result from the gradual accumulation of abnormal gene mutations (Figure 1.3). Specifically, once activated, p53 induces temporary cell cycle arrest at both G1/S and G2/M cell cycle checkpoints as soon as possible, creating a window period for DRM to fix DNA damage before cells enter mitosis. Meanwhile, the level of DNA damage is recurrently inspected by damage detectors like ATM, ATR and PARP, which work in coordination with DRM. If damage sensors find that DRM is not able to cope with those lesions, they will ask p53 signaling
to take over DRM's role to determine the most appropriate cell fate under the current condition (Harris and Levine, 2005).

For example, from Figure 1.3 it can be seen that Cyclins/CDKs inhibitor p21 is one of the most important p53 downstream target genes responsible for inducing G1/S cell cycle arrest in the presence of DNA damage or permanent cell cycle arrest when DNA damage cannot be repaired (el-Deiry et al., 1993, Harper et al., 1993). Particularly, the DBD of p53 binds to the specific RE in p21 promoter to drive p21 transcription. Subsequent increases in p21 protein expression then represses the activity of CDK1/2/4/6 to block G1/S transition during the cell cycle (el-Deiry et al., 1993). p53 with a mutated DBD loses the ability to interact with the RE in p21 promoter and thus cannot induce p21 expression to act as the "stop signal" to cell division, leading to the disorder of cell cycle checkpoint regulation and higher risk of malignant carcinogenesis (Harper et al., 1993).
Figure 1.3: p53-centred Signalling Network Is Responsible for Receiving and Processing Stress Signals and Leads to Various Cellular Outcomes by Trans-activating Different p53 Downstream Target Genes (Hunten et al., 2013).

In addition, p53 induces the expression of growth arrest and DNA damage 45α (GADD45α) and 14-3-3σ at transcription level, leading to G2/M cell cycle arrest by suppressing kinase activity of Cyclin B1/CDK1 complex (also known as Cyclin B1/CDC2) (Zhan et al., 1999, Cann and Hicks, 2007). This process coordinates with p21-induced G1/S cell cycle arrest to consolidate the expression of stop signals in cell cycles, allowing sufficient time for DRM to fix genetic errors.

Apart from inducing cell cycle arrest and DNA repair, p53 is also able to induce PCD, if the level of DNA damage suffered cannot be repaired. PUMA and BAX are two key targets of p53 to achieve this goal, which are responsible for facilitating cell apoptosis
(Figure 1.3) (Harms and Chen, 2005). Basically, both of these proteins lead to the release of cytochrome C from mitochondria and with the help of apoptotic protease activating factor 1 (APAF1), further activate Caspase signalling, which is the most common pathway to result in cell apoptosis.

### 1.3.3 The PTMs of p53 in Response to Stimulation Signals

Since p53 plays such an essential role in stress response and subsequent cell fate determination, there is no surprise that it is subject to complicated regulation, which covers three aspects: stability, trans-activity and anti-repression. As these features are achieved by changing the status of PTMs, it is essential to explore how PTMs of p53 affect p53 function (Figure 1.4) (Brooks and Gu, 2003, Kruse and Gu, 2009).

![Figure 1.4: Stabilisation, Anti-repression and Promoter-specific Trans-activation Are Main Aspects Involved in p53 Activation in vivo](image)

(Kruse and Gu, 2009). 1: p53 level is maintained at a very low basal level by E3-ligase MDM2-induced ubiquitination. p53 becomes stabilised by the abrogation of MDM2 inhibition, which is required for stress signals and the involvement of other components. 2: The dissociation with p53 inhibitors such as MDM2 and MDMX (also known as MDM4) and PTMs at specific
residues are the premise that p53 can act as a transcription factor. p53 still needs to interact with many other components to regulate the expression of target genes at transcription level and cell fate determination.

Abbreviations: Ac-acetylation; P-phosphorylation; Me-methylation; N8-neddylation; S-SUMOylation

Figure 1.5: A General Landscape of PTMs of p53. More than 36 amino acids in a molecule p53 protein are reported that can be modified. The major sites of phosphorylation (P), ubiquitination (Ub), acetylation (Ac), mono-methylatlon (Me1) and dimethylation (Me2) are shown in the graph, accompanied with corresponding enzymes and stress signals required for those modifications.

As Figure 1.4 and 1.5 show, p53 stability is highly dependent on its interaction with the critical regulator MDM2. The MDM2 protein is p53-specific E3-ligase that regulates the status of p53 ubiquitination, and subsequent turnover of p53 by proteasome-mediated proteolysis. This process enables a very low basal level of p53 in unstressed cells. However, when DNA damage occurs, the interaction between p53 and MDM2 is destabilised, MDM2 degradation and p53 stability are boosted simultaneously.
Subsequent changes in p53 include sequence-specific DNA binding ability and trans-activity, which are determined by the status of PTMs and protein-protein interaction (Brooks and Gu, 2003). All of these alterations occur as p53 receives the activation signals transmitted from its upstream components in response to cellular stress. In addition, tetramerisation takes place among p53 molecules in coordination with those changes, which also aims to maximise p53 function in the shortest period in the presence of DNA damage (Kruse and Gu, 2009). The following paragraphs will mainly explore how PTMs of p53 contribute to p53 expression and function, as the status of p53 PTMs is one of the most critical factors in regulating p53 function.

1.3.3.1 Ubiquitination

As mentioned above, p53 is kept inactive and unstable by MDM2. The central mechanism to maintain p53 steady-state is MDM2-induced ubiquitination and subsequent proteasomal degradation (Haupt et al., 1997, Honda et al., 1997). As Figure 1.5 illustrates, six lysines in the CTD of p53, which are Lys370, Lys372, Lys373, Lys381, Lys382 and Lys386, are the predominant sites of MDM2-induced p53 ubiquitination (Lohrum et al., 2001).

Due to the critical role of MDM2 in the regulation of p53 stability, this process is highly regulated as well. Many p53 regulators perform their duties by modulating MDM2 status. One of the most important regulators is tumour suppressor ADP-ribosylation factor (ARF), which is a major mediator of p53 activation in response to cellular stresses (Sherr, 2001). ARF can be found in both nucleoplasma and nucleoli. Nucleolar ARF interacts with MDM2 and sequesters it in nucleoli, preventing nucleoplasmic p53 from degradation (Weber et al., 1999); nucleoplasmic ARF can directly repress E3-ligase activity of MDM2 (Honda and Yasuda, 1999, Llanos et al., 2001). Moreover, ARF inhibits other E3 ligases such as ARF binding protein 1 (ARF-BP1), constitutive photomorphogenesis protein 1 (COP1) and p53-induced protein with a RING-H2 domain (PIRH2), which mediate MDM2-independent p53 degradation (Chen et al., 2005, Dornan et al., 2004, Leng et al., 2003). MDMX stabilises MDM2 and promotes its E3-ligase activity by interacting with its RING finger domains (Marine and Jochemsen,
2005); some studies also indicated that MDMX interacts with p53’s TADs to abolish p53 trans-activity (Finch et al., 2002, Migliorini et al., 2002). In addition, ribosomal proteins L5, L11 and L23 are associated with MDM2 and thus prevent p53 from being inhibited, playing a crucial role in p53 activation during translational stress, such as declining amino acid pools (Dai et al., 2004, Dai and Lu, 2004, Zhang et al., 2003, Lohrum et al., 2003). Furthermore, ubiquitin-specific-processing protease 7 (USP7), known as a direct MDM2 inhibitor, cleaves ubiquitin from p53, and also results in p53 stabilisation (Itahana et al., 2007, Fang et al., 2000).

The PTMs of MDM2 also affect p53 stability (Meulmeester et al., 2005, Meek, 2004, Mayo and Donner, 2002). For instance, the phosphorylation of Ser395 and Tyr394 mediated by ATM and c-ABL, respectively, leads to MDM2 auto-ubiquitination and subsequent proteasomal degradation (Goldberg et al., 2002). Similarly, ATR-induced Ser407 phosphorylation inhibits E3-ligase activity of MDM2 as well (Shinozaki et al., 2003). All of these modifications abrogate MDM2-mediated p53 degradation and result in p53 accumulation in cells. In contrast, Ser166 and Ser186 phosphorylation mediated by PI3K family member protein kinase B (PKB) promotes MDM2 translocation from cytoplasm to nuclei and thus inhibits MDM2-ARF interaction, which strengthen MDM2-induced p53 degradation (Zhou and Hung, 2002). Besides, WT p53-induced phosphatase (WIP1) mediates the dephosphorylation of MDM2 Ser395, which also consolidates MDM2-induced p53 degradation (Lu et al., 2007). In a nucleus, transcription co-activator and HAT CBP/p300 mediates MDM2 acetylation at Lys466/467 and Lys469/470 in its RING finger domain in vitro and in vivo, leading to MDM2 disassociation with p53 and subsequent p53 stabilization (Wang et al., 2004).

### 1.3.3.2 Phosphorylation

Phosphorylation is required for p53 activation as well. As Figure 1.5 shows, p53 is phosphorylated by a broad range of kinases including ATM/CHK2, ATR/CHK1 and DNA-PKs (Brooks and Gu, 2003). In human cells, phosphorylation at Ser15 and Ser37 mediated by ATM, ATR and DNA-PKs stabilises p53 by decreasing the interaction with its suppressor MDM2. CHK1- and CHK2-induced Ser20 phosphorylation in response to
DNA damage has the same effect (Figure 1.5) (Tibbetts et al., 1999, Shieh et al., 1997). In addition, Thr18 phosphorylation by casein kinase 2 (CK2) can also disrupt p53-MDM2 interaction (Figure 1.5) (Sakaguchi et al., 1998). Dephosphorylation of p53 decreases p53 stability. For example, the phosphatase WIP1 induces Ser15 dephosphorylation, which promotes p53 turnover by enhancing its interaction with MDM2 (Lu et al., 2005). However, some studies based on knock-in mice with Ser18 and Ser23 (corresponding to Ser15 and Ser20 in humans respectively) mutations demonstrated that only a slight drop of p53 level occurs in response to DNA damage compared to the mice express WT p53, arguing against the central role of phosphorylation in the regulation of p53-MDM2 interaction in vivo (Chao et al., 2003, Sluss et al., 2004, MacPherson et al., 2004, Wu et al., 2002). Other research in transgenic mice further showed that the correlation between p53 stability and its phosphorylation status is cell- and tissue-dependent (Ashcroft et al., 2000, Blattner et al., 1999). Furthermore, phosphorylation-independent p53 activation has been discovered (Ashcroft et al., 2000, Blattner et al., 1999, Wu et al., 2002). These facts suggest that p53 phosphorylation might be context-specific, arguing that every PTM site may not be an essential or indispensable requirement for p53 function in all cells. However, Tang et al pointed out that p53 acetylation is essential in stabilising p53 because it prevents p53 from interacting with MDM2. This observation is in agreement with the fact that p53 level can be up-regulated in the presence of deacetylase inhibitor in unstressed cells, suggesting that p53 acetylation alone can be sufficient to activate p53 (Li et al., 2002). This conclusion may partly explain the mechanism of phosphorylation-independent p53 activation.

### 1.3.3.3 Other Types of Modification

p53 was described as a sequence-specific DNA binding protein (Bargonetti et al., 1991, Kern et al., 1991b, Kern et al., 1991a). The DBD of p53 can recognise and bind to the specific motif - 5′-RRRCA/T and A/TGYYY-3′- which is a double inverted pentamer, in the promoters of p53 target genes, followed by the regulation of gene expression at transcription level (Wei et al., 2006). Early studies suggested that p53 regulates the expression of target genes simply by interacting with general transcription factors and co-regulators such as transcription factor II D (TFIID) complex, SRB/mediator complex...
and SWItch/sucrose non-fermentable (SWI/SNF) complex (Thut et al., 1995, Gu et al., 1999). This model masks the specificity in the process of p53-dominated regulation of gene expression. In fact, it is achieved mainly by various PTMs in p53 molecules (Kruse and Gu, 2009). Particularly, acetylation and methylation at p53 CTD play a central role in the regulation of p53 target gene transcription (Appella and Anderson, 2001, Brooks and Gu, 2003).

Acetylation is a common approach to fine-tune trans-activity of transcription factors, which is achieved in two ways. The first one is to modulate DNA binding ability of transcription factors. Acetylation, conducted by acetyltransferase, can facilitate conformation changes in the molecules of transcription factors, which helps to expose their DBDs to recognise and bind to specific sequence on DNA strands (Gu and Roeder, 1997). p53 is the first non-histone protein shown to be regulated by acetylation in terms of trans-activity and protein-protein interaction (Gu and Roeder, 1997, Luo et al., 2000). For instance, as Figure 1.5 shows, six lysines in p53’s CTD are acetylated by CBP/p300 in response to stress signals and then DNA binding ability is activated; acetylation also occurs at Lys120 and Lys164 in p53’s DBD, mediated by TIP6 and CBP/p300 respectively (Tang et al., 2006, Tang et al., 2008, Mellert et al., 2007). The second way is to alter molecular conformation of histones in nucleosomes. This process is mediated by HAT and intended to make chromosomes more accessible to transcription factors (Jenuwein and Allis, 2001). For example, HAT complex CBP/p300 delivers acetyl groups to specific residues of histones around p53 target genes, switching the closed conformation of particular chromosomal regions in which p53 target genes are located to open mode and thus making DNA exposed to transcription machinery (Goodman and Smolik, 2000). Notably, p53 acetylation can be abrogated by deacetylases such as Sir2α/Silent information regulator 1 (SIRT1), leading to the inactivation of p53 trans-activity (Li et al., 2002).

Contrary to acetylation, in most cases p53 methylation influences p53 function in a negative way. Methylation often occurs at specific lysines in p53’s CTD as well, which is induced by methyltransferases. Some typical examples include SET9-mediated mono-methylation at Lys372, PR-SET7-induced mono-methylation at Lys382 and SMYD2-dependent mono-methylation at Lys370, all of which represses p53 activity (Figure 1.5) (Huang et al., 2006, Shi et al., 2007, Chuikov et al., 2004). However, it has been reported
that not all of methylation events play a negative role in the regulation of p53 activity. For instance, di-methylation at Lys370 promotes p53 trans-activity, especially to p21, which can be abolished by lysine-specific demethylase LSD1 (Huang et al., 2006).

1.3.4 p53 Dynamics at Single-cell Level

As mentioned in the abstract, one of the ultimate goals of our project is to search for the potential causal relationship between p53 dynamics in single cells and cell fate determination in the scenario of DNA damage. So the introduction of this thesis aims to provide general background knowledge necessary for us to understand this topic, which should include: 1. The nature of DNA damage and cell fate; 2. The signalling pathways of DDR and subsequent cell fate determination; 3. The structure, function and regulation of p53; 4. p53 dynamics and single-cell research. Below I consider the importance of analysing the dynamics of p53 signalling network within single cells.

In the field of biology, the general concept of the dynamics of a specific protein is changes in its intra-cellular level or subcellular localisation over time. Conventionally, such changes were investigated using biochemical techniques with cell populations. However, the past 20 years has seen new emphasis placed on the analysis of single cells using a range of recent innovations. As key techniques such as microscopy and fluorescence labelling have developed, a number of specialised single-cell studies have become possible. Hence, the dynamics of a protein can be investigated at two levels: cell population level and single-cell level. Our project focuses on the investigation of p53 dynamics, in terms of intra-cellular localisation and behaviour at single-cell level. Published studies have indicated that the dynamical changes in p53 expression within individual cells can be visualised as oscillations in terms of intra-cellular concentration following induction of DDR using DSBs. Such oscillations reflect the fact that DNA damage activates the stress response and increase p53 expression as a result of MDM2 degradation. As MDM2 expression falls and p53 is stabilised, p53 expression increases and the expression of p53 target genes is induced. However, MDM2 itself is a strong p53 target gene and the accumulation of p53 leads to the up-regulation of MDM2 expression, which in turn promotes p53 degradation. Consequently, a sub-lethal levels of DNA
damage results in a series of periodic pulses of p53 expression with relatively fixed mean amplitude, period and width – so called p53 oscillations (Lahav et al., 2004).

Oscillations are wide-spread in cell signalling pathways in organisms, which generally results from feedback loops producing limit-cycles of expression of components involved in those circuits (Figure 1.6) (Spiller et al., 2010). Compared with the on-off mode of signal control system, systems with oscillatory dynamics are based on feedback loops that are induced when the signalling is switched on so that the activation of genes in downstream pathways also serves to turn the pathway off. This signalling motif sets up oscillations, which are not only more adaptive to complicated regulation but their intrinsic structure also makes them more tolerant to a variety of exogenous stresses (Tyson et al., 2003).

Oscillations can be investigated at the cell population level and single-cell level as well. Studies conducted at cell population level use canonical biochemical approaches, such as Western blots and real-time PCR. While single-cell research employs time-lapse microscopy and fluorescent labelling technique to continuously observe the dynamics of the protein-of-interest. Specifically, it is achieved by measuring the intensity of fluorescence emitted from a fluorescent fusion protein that is ectopically expressed following transient or stable (Spiller et al., 2010). Recently, genome editing approaches using CRISPR/Cas9 have been used to generate in frame fusion proteins that are expressed from the endogenous loci.

Initially, due to technical constraints and unawareness of cell heterogeneity, studies about p53 dynamics were conducted at cell population level (Haupt et al., 1997, Lev Bar-Or et al., 2000). However, scientists were soon aware of significant limitations at cell population level. The most significant issue is that studies performed using population level can merely produce averaged data of an entire population, not allowing scientists to learn about dynamical changes occur in individual cells. Hence, cellular heterogeneity cannot be investigated by these approaches (Loewer and Lahav, 2011). Another disadvantage is that studies based on cell populations cannot take account of the cell to cell heterogeneity, an innate feature of multi-cell biological systems, so data obtained in this way may be significantly different to the same value measured within individual cells (Lahav et al., 2004).
It was not until the techniques for single-cell research became well-developed that scientists started to recognise that population-based studies and data generated by analysis of single cells could be quite different and how important these differences might be (Figure 1.6). As a result, the range of cellular heterogeneity in different cell signalling networks has been demonstrated and the differences shown in this way help to reveal pathogenic mechanisms of many diseases. Importantly, the study of cellular heterogeneity further strengthens our awareness of organismal complexity and emphasises that we must continue to develop innovative tools to build our research knowledge.

Figure 1.6: The Pattern of p53 Dynamics in Response to DNA Double-strand Breaks at Single-cell Level Is Different from Its Counterpart Obtained at Cell Population Level (Batchelor et al., 2011, Lahav et al., 2004). A: The triple-negative feedback loop architecture of core p53 signalling pathway in response to DSBs. A dashed arrow represents trans-activation; a solid arrow means activation by PTMs and/or protein-protein interaction; a solid line represents inhibition based on PTMs and/or protein-protein interaction. B and C: The Western Blots result demonstrates damped p53-MDM2 oscillations in response to DSBs in 9 hours, detected at cell population level. D: The result from time-lapse fluorescent microscopy shows periodic p53 oscillations in response to DSBs in 20 hours, detected at single-cell level.
This project sets out to probe the question that if manipulating the dynamic behaviour in p53 signalling downstream of DSBs is able to influence cell fate determination. Investigation of p53 dynamics in response to DNA damage began in 1997, when p53-MDM2 negative feedback loop was discovered (Haupt et al., 1997). Then, the initial study was performed at cell population level, indicating that p53 dynamics were present as damped oscillations in response to DSBs (Figure 1.6 B and C) (Lev Bar-Or et al., 2000).

Later work published by Lahav et al employed both fluorescently conjugated p53 and MDM2 expression plasmids to observe their dynamics in individual cells by fluorescent time-lapse microscopy (Geva-Zatorsky et al., 2006, Lahav et al., 2004). In these studies, the real p53-MDM2 dynamics were illustrated for the first time, which was a great breakthrough in p53 research. Contrary to earlier results obtained at cell population level, this work showed that in response to DSBs, p53-MDM2 dynamics were present as continuous and undamped oscillations over 20 hours, with their averaged amplitude and period fixed, independently of the extent of damage (Figure 1.6 D) (Lahav et al., 2004, Geva-Zatorsky et al., 2006). In addition, genetically identical cells in the same population showed different patterns of p53-MDM2 oscillations in terms of width, height and timing of pulses (Lahav et al., 2004, Geva-Zatorsky et al., 2006).

Geva-Zatorsky et al did further research on the long-term dynamics of p53-MDM2 oscillations in individual cells in combination with computational modelling analysis, which aimed to describe the variability of the dynamics and explore potential sources of this variability (Geva-Zatorsky et al., 2006). They found that the pulse amplitude of p53 and MDM2 is the most variable value among isogenic cells in response to DSBs, which is much greater than peak width and oscillatory period. Some cells can sustain undamped oscillations for at least 3 days (more than 10 consecutive periods), while others only show low-frequency fluctuations that cannot be considered as oscillations. By computational modelling analysis, the authors indicated that the stochasticity in protein production rates is the main source of background noise in p53-MDM2 oscillations. They also predicted that there is another negative feedback loop in the system apart from p53-MDM2 negative feedback loop, in which ATM may be involved (Geva-Zatorsky et al., 2006). And in comparison with Lahav’s short-term results (16 hours), the authors
demonstrated that p53-MDM2 oscillations in most cells are long-lasting and most oscillating cells show multi-pulse dynamics in response to DSBs (Geva-Zatorsky et al., 2006, Lahav et al., 2004).

Geva-Zatorsky's research was extended by Batchelor et al, who found that in response to DSBs, phosphorylated ATM/CHK2 shows similar dynamics to p53-MDM2 oscillations both at the cell population and single-cell level (Batchelor et al., 2008). This study further indicated that phosphorylated ATM/CHK2 oscillations were required for p53-MDM2 oscillations and that p53-MDM2 oscillations are excitable, which means that a transient stimulation can trigger a complete response as long as the intensity of the stimulation reaches the required threshold. Furthermore, the phosphatase WIP1 was found to be the mediator in ATM/CHK2-p53 negative feedback loop and the knock-down of WIP1 gene by RNAi was shown to disrupt p53 dynamics (Batchelor et al., 2008). Therefore the authors concluded that p53-MDM2 oscillations are driven by a recurrent initiation mechanism. According to this model (Figure 1.6 A), p53 becomes active and p53 level increases when DSBs are detected by ATM/CHK2; meanwhile, p53 up-regulates the transcription of WIP1 leading to the dephosphorylation and suppression of ATM and p53 by WIP1. At the same time, increasing levels of activated p53 leads to the up-regulation of MDM2 expression, because MDM2 is a target gene of p53. Hence, as the level of MDM2 increases, p53 degradation speeds up so that p53 levels return to the basal steady state. Therefore, there are three negative feedback loops in this recurrent model, suggesting that ATM, MDM2 and WIP1 may have similar oscillatory behaviour to p53. The recurrent model provides enough time for DRM to fix DSBs, monitor the efficiency of DNA damage repair repeatedly and determine cell fate as DDR proceeds (Batchelor et al., 2008).

In their latest research, Batchelor et al found that p53 dynamics is stimulus-dependent in response to UV-damage, with changes in p53 expression appearing as a single continuous pulse whose amplitude and duration were dose-dependent in contrast to the periods oscillations of fixed amplitude, which were seen in response to γ-irradiation (Batchelor et al., 2011). Based on their data and in silico models, one explanation for stimulus-dependent p53 oscillations could be that WIP1 acts to switch off signalling through ATM but does not dephosphorylate and inhibit active ATR, hence ATR, p53 and
WIP1 cannot form a negative feedback loop to ATR and thus p53-MDM2 oscillations will be less pronounced following UV-induced DNA damage (Cimprich and Cortez, 2008).

In order to explore the connection between p53 dynamics in single cells and cell fate determination, Purvis et al developed a method to transform pulsatile p53 dynamics into a sustained mode of activation in the presence of DSBs, which was achieved by adding different amount of MDM2 inhibitor Nutlin-3 into live cells at a series of particular timepoints worked out by computational simulation (Purvis et al., 2012). The authors further showed that different types of p53 dynamics can lead to different cell fates because p53 oscillations tended to trans-activate the expression of p53 target genes that correlated with cycle arrest and DNA repair, whereas sustained p53 expression preferentially trans-activate the expression of p53 target genes involved in apoptosis or senescence (Purvis et al., 2012). The expression level of p53 target genes was measured by real-time PCR.

In addition, Toettcher et al developed a synthetic biology approach to manipulate p53-MDM2 oscillations (Toettcher et al., 2010). In this study an artificially switchable p53 oscillator, comprised of both natural and synthetic elements in mammalian cells, was created. In this case, the expression of p53 was driven by Zn$^{2+}$ instead of DNA damage to avoid the activation of upstream kinases. The dynamics of this artificial p53 oscillator are damped, with fixed oscillatory period and Zn$^{2+}$-dependent amplitude. The authors also introduced an artificial negative and an artificial positive feedback loop into the previous system, respectively, and found that an additional positive feedback loop can improve the robustness of the artificial p53 oscillator by decreasing its damping rate, while an additional negative feedback loop had opposite effect (Toettcher et al., 2010).

At the same time, Loewer et al looked at basal p53 dynamics in non-stressed single cells (Loewer et al., 2010). They found that basal p53 dynamics was activated by transient DNA damage during cell growth and had a causal relationship with cell cycle progression; although basal the p53 level was considerably higher and the dynamical behaviour in single cells was similar to the damage-responsive in some examples; in the majority of cells signalling appears as a series of irregular, asynchronous and low-frequency fluctuations. However, an important point is that basal p53 dynamics was not functional because the necessary PTMs of p53 are not induced (Loewer et al., 2010).
The studies introduced above include all features of p53 dynamics that have been verified experimentally at the single-cell level so far. Furthermore, more research on p53 dynamics was only conducted in silico. For example, a model suggested that the reason why peak number has causal relationship to cell fates is due to the accumulation of cell apoptosis mediator PUMA (Sun et al., 2009). The authors proposed that cell survival is determined within two pulses in response to DNA damage. If DSBs cannot be repaired during this period, subsequent peaks will activate transcription of Puma, which is responsible for cell apoptosis (Sun et al., 2009, Ma et al., 2005). MDMX and ARF have also been computationally suggested as regulators of p53 dynamics (Liu et al., 2007, Proctor and Gray, 2008). p53-MDM2 oscillations were also investigated in vivo using transgenic mice. A plasmid combined p53 with firefly luciferase and driven by p53-responsive mdm2 promoter was transfected and expressed in transgenic mice. Luciferase report assays showed that bioluminescent intensity was dependent on p53 expression level. p53 dynamics was activated in response to DSBs and the changes of luciferase intensity were observed. However, because the resolution of luminescence was low in live animals, this study demonstrated that the profile of p53 expression looked like damped oscillations that were frequently seen in cell populations using Western blots, further consolidating the previous studies (Hamstra et al., 2006).

### 1.4 Cellular Heterogeneity

Recent advances in single-cell technologies have emphasised that the surprising levels of cellular heterogeneity exists in mammalian cells and is revealed in almost every aspect of cellular processes (Altschuler and Wu, 2010). For example, even across a population of isogenic cells grown in a flask, the basic rate of metabolism and doubling time varies; how cells respond to a particular stimulus may differ from one to another, i.e. the extraordinary variability of p53-MDM2 dynamical pattern in terms of peak numbers, amplitude, width and timing; and more specifically, the degradation and production rate of a certain protein, such as p53, is also variable from one cell to the other (Loewer and Lahav, 2011). Moreover, previous studies concluded a series of factors that contribute to cellular heterogeneity. The first one is micro-environment of growth, which includes the distribution of growth nutrients and stimuli, the number
and frequency of cell-cell contacts, and local cell density, etc (Marusyk and Polyak, 2010). For a particular tumour tissue, different areas have different numbers, proportion, types and density of normal cells and cancer cells, a different volume of blood supply and different composition of extracellular matrix (Marusyk and Polyak, 2010). For example, cellular differentiation preference exists in normal cells of blastocysts when teratocarcinoma cells are injected, which demonstrates the important contributions of the interaction between cells and microenvironment to cellular heterogeneity and tumour progression (Hiley and Swanton, 2014, Marusyk and Polyak, 2010).

Another factor that influences heterogeneity is the innate stochasticity of almost all cellular processes, generating a series of irregular fluctuations that can be seen in dynamical data between individual cells (Paulsson, 2004). A very typical example is transcriptional bursting among a population of cells (Loewer and Lahav, 2011, Golding et al., 2005). Many studies have revealed that gene transcription is not a linear and continuous process; instead, chromatin state and the transcriptional machinery have periods with high transcription efficiency and periods with low transcription efficiency, leading to a pulsatile or stochastic pattern of the entire process (Golding et al., 2005, Bahar Halpern et al., 2015, Raj et al., 2006). This phenomenon is observed in a variety of organisms from bacteria to mammals and can be seen at the single-cell level by RNA fluorescence in situ hybridisation (FISH) or MS2 tagging (Golding et al., 2005, Corrigan and Chubb, 2015). Even two genetically identical cells have different pattern of transcriptional bursting, and this may correlate with changing epigenetic properties such as chromatin conformation, PTMs and activity of the transcription machinery (Raj et al., 2006). Corrigan and Chubb found that in Dictyostelium, although gene transcription behaviour was coupled with extra-cellular cAMP signalling pulses and transcriptional bursting shows oscillations which approach antiphase to cAMP waves at the population level, considerable heterogeneity was clearly observed in individual Dictyostelium cells and transcriptional bursting was variable within the same population; the authors suggested that this phenomenon comes from the local effects of every single cell and is one of the sources of cellular heterogeneity and transcription noise (Corrigan and Chubb, 2015).
Another origin of heterogeneity is the potential distinction of multi-dimensional chromatin conformation, which is one of the major causes of discrepancy of transcriptional bursting across a population of cells (Voss and Hager, 2014). Last but not the least, some recent reports demonstrated that non-genetic elements, including different phenotypic and/or epigenetic states, asymmetric cell division and probabilistic processes associated with low copy number of reactants, resulting in cellular heterogeneity and stochasticity within a population of cells (Altschuler and Wu, 2010, Hunziker et al., 2010).

Why do cells or organisms show heterogeneity? One reasonable explanation is that biological individuals are able to generate a range of responses or gradual, inter-convertible reactions to environmental changes, which massively increase their survivability (Loewer and Lahav, 2011). It is not hard to imagine how an organism can live normally if it only has a single-mode response to a specific stimulus. Because every single cell is a heterogeneous individual and each one of them reacts to a certain stimulus differently and stochastically, thus variable responses take place to provide flexible solutions to cope with variable natural environment. Therefore, cellular heterogeneity is a consequence of Darwinian evolution.

1.5 miRNAs

As mentioned above, one of the ultimate goals of this project is to search for the potential relationship between p53 dynamics in single cells and cell fate determination under stressed conditions. In addition, a specific focus of my interests in p53 regulation is to understand whether miRNAs are able to fine-tune p53 dynamics at single-cell level and how this modulation is present. Taking these questions together, the major aim would be, using miRNAs as a regulatory tool to manipulate p53 dynamics at the single-cell level and investigate if these changes in p53 dynamics are able to further influence cell fate determination. Therefore, it is necessary to introduce the background knowledge of miRNAs, ranging from their biogenesis, function to interaction with p53.

miRNAs are a group of short non-coding RNA molecules that are found in eukaryotic cells and viruses. They are only 20-25 nucleotide-long (22 nucleotides on average) and originated from endogenous short hairpin transcripts. Although their size is very small
and they are not able to encode protein as well, they play a very important role in the regulation of gene expression at post-transcription level. Specifically, as Figure 1.8 indicates, miRNAs bind to fully or partially complementary target sequences in 3’-untranslated regions (3’-UTRs) of target mRNAs and then recruit several proteins to form RNA-induced silencing complex (RISC), leading to mRNAs degradation or translational repression. At the current time, it has been estimated that more than 1000 miRNAs have been discovered in different types of human cells and over 60% of human protein-coding genes are subject to miRNAs (Friedman et al., 2009, Bentwich et al., 2005). Therefore, scientists have always been trying to use them as targeted therapeutic tools to suppress intra-cellular levels of key proteins in many diseases (Bartel, 2004, Lujambio and Lowe, 2012). Interestingly, miRNAs do not always act as repressors, some miRNAs also strengthen the expression of target genes (Bartel, 2009). It is also noteworthy that one miRNA can target to more than one mRNA and vice versa (Hermeking, 2012). Both facts render the role of miRNAs in cell regulation much more complicated. As a result, targets and function of most miRNAs have not been fully validated, let alone the regulation of their biogenesis, maturation and degradation (Bartel, 2009). These questions, together with complicated crosstalk with other signalling pathways, will be the main focuses of miRNA research in the future.

1.5.1 miRNA Biogenesis

There are canonical and alternative miRNAs biogenesis pathways (Figure 1.7). As Figure 1.7 A demonstrates, the canonical pathway is dominated by two double-stranded RNA-specific RNase III endonucleases DROSHA/DGCR8/PASHA and DICER. In this pathway, miRNA genes are transcribed by RNA polymerase II to generate primary transcripts known as primary miRNAs (pri-miRNA) (Cai et al., 2004, Lee et al., 2004). A pri-miRNAs has a double-stranded hairpin loop, which is then recognised and associated with DROSHA/DGCR8/PASHA, resulting in the cleavage of the proximal eleven nucleotides from the hairpin loop (Gregory et al., 2004, Denli et al., 2004). After this enzymatic processing, a pri-miRNA is transformed into a precursor-miRNA (pre-miRNA) consisting of a new hairpin structure with 55-70 nucleotides and a 2-nucleotide long overhang at 3’-end (Gregory et al., 2004). Then a pre-miRNA is transported from a
nucleus into the cytoplasm by Exportin-5, where another cleavage event, catalysed by DICER, takes place (Gregory et al., 2004). Consequently, a mature miRNA-miRNA* (* represents the strand that is much less than the other and will be degraded thereafter) asymmetric duplex is formed, followed by the degradation of the miRNA* strand and incorporation into a RISC (Bohnsack et al., 2004, Yi et al., 2003, Lund et al., 2004).

There are several alternative pathways of miRNAs biogenesis, whose most distinctive feature from the canonical pathway is either DROSHA/DGCR8/PASHA- or DICER-independent (Figure 1.7 B and 1.7 C) (Yang and Lai, 2011). For example, the Mirtron pathways, as well as snoRNA-, tRNA-, endo-shRNA- and tRNase Z-derived pathways, are DROSHA/DGCR8/PASHA-independent but DICER-dependent; these pathways have their own nuclear machineries to process primary transcripts but share DICER with the canonical pathway (Figure 1.7 B) (Yang and Lai, 2011). Interestingly, the maturation of some miRNAs, such as miR-451 and miR-144, can be achieved in the absence of DICER (Figure 1.7 C) (Yang et al., 2010). Particularly, as Figure 1.7 C shows, premature miR-451 is directly incorporated into RISC and sliced by a key component of RISC called AGO2 without Dicer participation (Yang et al., 2010). The existence of non-canonical pathways demonstrates the diversity and complexity of miRNAs biogenesis machinery.
**Figure 1.7: The Canonical and Alternative Pathways of miRNAs Biogenesis in Animal Cells** (Yang and Lai, 2011). **A:** In the canonical pathway mature miRNAs are generated through DROSHA/DGCR8 and DICER cleavages from hairpin-like precursors pri-miRNAs and pre-miRNAs, respectively. **B:** Some alternative miRNA biogenesis pathways include Mirtron pathways, snoRNA-, tRNA-, endo-shRNA- and tRNase Z-derived pathways, are DROSHA/DGCR8-independent but DICER-dependent. They share the identical process to the canonical pathway in cytoplasm. **C:** Pri-miR-451 is cut by DROSHA/DGCR8 into an 18bp product in the nucleus and exported to cytoplasm, where it is directly incorporated into Ago2 and subject to Ago2-induced cleavage to become functional.

### 1.5.2 How miRNAs Work as Gene Silencers

Mature miRNAs suppress the translation of target genes by binding to specific sites in 3’-UTRs of target mRNAs based on complementary bases pairing. The interaction between miRNAs and target mRNAs can be categorised into two types. The first type is that the miRNA sequence is perfectly matched with the sequence of the 3’-UTR of the target mRNA, which leads to rapid degradation of the target mRNA. This case is only seen in plants cells and because of this fact, plant miRNAs usually have only one or two target gene(s) (Lelandais-Briere et al., 2010).
Figure 1.8: Mature miRNAs Are Incorporated into a RISC to Exert Function at Post-transcription Level. A matured miRNA binds to fully (in plants) or partially (in animals) complementary target sequences in 3’-UTRs of target mRNAs and then recruits several proteins to form RISC, leading to mRNAs cleavage (in plants) as well as mRNAs degradation or translational repression (in animals).

The other situation is that miRNA sequence is partially complementary to the target mRNAs, forming a bulge structure in miRNA-mRNA duplexes (Figure 1.8). This case is widespread across animal cells, leading to the translational repression of target mRNAs or mRNAs degradation by deadenylation (Eulalio et al., 2009, Williams, 2008). In this scenario, 2-8 nucleotides in the miRNA sequence, known as the “seed” sequence, is required to be perfectly complementary to target sequence in the 3’-UTRs of the target mRNAs (Lewis et al., 2005). Therefore, miRNAs in animal cells can target to multiple mRNAs as long as their “seed” sequences is completely complementary to target sequences. However, in most scenarios a miRNA actually targets to multiple mRNAs which share similar biological function or are highly coordinated to achieve a common biological outcome (Bartel, 2009). In addition, mRNAs of housekeeping genes are less affected due to fewer miRNA-targeted sites in their 3’-UTRs, which is thought to be important for keeping cells in at a steady-state (Stark et al., 2005). Currently, how the binding of a RISC represses mRNA translation is still controversial (Bartel, 2009). Some studies showed that miRNAs disrupt translation initiation (Humphreys et al., 2005, Pillai et al., 2005), while others indicate that peptide chain elongation was suppressed after RISC binds to a mRNA (Maroney et al., 2006, Nottrott et al., 2006).
1.5.3 Interaction with p53

In 2007, seven groups reported that the genes of miR-34a, b and c were the direct downstream targets of p53 and involved in cell apoptosis, cell cycle arrest and senescence. These studies provided the first evidence to show the interaction between p53 and miRNAs (Figure 1.9) (Hermeking, 2007). This fact is very important because it reveals a completely new mechanism of p53 regulation, which further increases the complexity, flexibility and diversity of p53 regulatory network (Figure 1.3) (Hermeking, 2012). Since then, more and more studies have demonstrated that miRNAs play a critical role in p53 regulatory network (Figure 1.9).

Figure 1.9: p53 Can Repress the Expression of Downstream Components and Control Cell Fate by Trans-activating Target miRNA Genes (Hermeking, 2012). The regulation of miRNA expression by p53 plays a key role in negative regulation of p53 downstream components, leading to a dual-mode regulation apart from p53’s activity as a transcription factor.
The interaction between p53 and miRNAs can be categorised into three classes. The first one is that p53 performs as a transcription factor to regulate the expression of miRNA genes (Figure 1.9). Take miR-34a as an example. miR-34a is a multi-functional miRNA: 1. miR-34a leads to cell cycle arrest at G1/S checkpoint by repressing cell cycle activators cyclin E2, CDK4, CDK6, E2F3, MYC and NMYC (He et al., 2007, Bommer et al., 2007, Chang et al., 2007); 2. miR-34a is required for the induction of cell apoptosis as anti-apoptosis components Bcl2 and Survivin are direct targets of miR-34a (Ravert-Shapira et al., 2007, Bommer et al., 2007); 3. miR-34a is also involved in other key processes such as the regulation of EMT/MET (Siemens et al., 2011, Kim et al., 2011a), cellular stemness (Choi et al., 2011, Liu et al., 2011a) and metabolism (Rottiers and Naar, 2012). Moreover, these studies also reported that both up- or down-regulation of miR-34a expression were almost able to affect all p53-mediated processes, suggesting the importance of the interaction between p53 and miR-34a. Not surprisingly, down-regulation of miR-34a expression or miR-34a gene deletion has been frequently observed in various tumours, indicating that miR-34a is a p53-dependent tumour suppressor (Bommer et al., 2007, Chang et al., 2007).

p53 can not only trans-activate the expression of miR-34a, but also facilitate other miRNAs expression, such as miR-145, miR-200a/b/c, miR-15a/16-1, miR-107, miR-29a/b/c, miR-192 and so on (Figure 1.9) (Feng et al., 2011). For example, p53 trans-activates miR-145, which targets to cell cycle promoter CDK4, CDK6 and MYC, thus leading to the induction of G1/S cell cycle arrest (Sachdeva et al., 2009, Zhu et al., 2011). The trans-activation of miR-145 also gives rise to the inhibition of OCT4, SOX2, KLF4 and NOTCH so that cell pluripotency is repressed (Xu et al., 2009). The miR-200 family is another essential downstream target of p53, which plays a pivotal role in blocking EMT by repressing the EMT-inducing transcription factor ZEB1/2 (Chang et al., 2011, Kim et al., 2011b, Burk et al., 2008, Gregory et al., 2008). Furthermore, miR-200 is able to repress BMI, Kruppel-like factor (KLF4), as well as the key components of NOTCH signalling Jagged-1 and MAML2/3, to down-regulate cell pluripotency (Shimono et al., 2009, Brabletz et al., 2011). Encoded by an intron of p53 transcriptional target gene dleu2, both miR-15a and 16-1 are the key components in p53-centered cell fate determination in B cells, because they decrease the expression level of cell cycle activators Cyclin D/E and CDK4/6, which is in agreement with the fact that frequent
down-regulation or even deletion of dleu2 occurs in human chronic lymphoblastic leukaemia (CLL) (Calin et al., 2002, Klein et al., 2010).

The second class of miRNAs-p53 interaction is miRNA-induced repression of p53 expression, which can be further categorised into direct regulation and indirect regulation (Figure 1.10). miR-125b is a typical example of direct regulation, which has been reported as a direct p53 suppressor by binding to the “seed” sequence in the 3’-UTR of p53 mRNA (Le et al., 2009, Le et al., 2011). Ectopic expression of miR-125b represses p53-induced cell apoptosis, which is commonly seen in some cancers, such as acute lymphoblastic leukaemia (ALL) and colorectal cancer (Le et al., 2009, Nishida et al., 2011, Enomoto et al., 2011). In contrast, down-regulation or knock-out of miR-125b gene causes p53 accumulation and helps to induce p53-dependent apoptosis (Le et al., 2009). Moreover, it has been revealed that miR-125b can even target to some of p53 downstream components such as PUMA and insulin-like growth factor-binding protein 3 (IGFBP3), which suggests that it may potentially be a p53-independent anti-apoptotic component (Le et al., 2011). However, in certain types of human breast cancer cell lines miR-125b was found to act as a tumour suppressor, implying its context-dependent manner (Zhang et al., 2011). Apart from miR-125b, miR-504, miR-33, miR-380 and miR-25 were also reported to directly target to p53 mRNA, which may result in the down-regulation of cell apoptosis, enhanced cell pluripotency and so on (Figure 1.10) (Hu et al., 2010, Herrera-Merchan et al., 2010, Swarbrick et al., 2010, Kumar et al., 2011).

Figure 1.10: p53 Can Be Either Positively or Negatively Regulated by Many miRNAs (Hermeking, 2012).
Figure 1.10 also demonstrates that some miRNAs can affect p53 expression and activity by manipulating the expression of p53 regulators (Hermeking, 2012). For example, miR-34a and miR-449 target to deacetylase and p53 repressor SIRT1 to strengthen p53 trans-activity (Lize et al., 2011, Yamakuchi et al., 2008); miR-29a/b/c improve p53 function by suppressing the expression of p53 negative regulators CDC42, WIP1, as well as PI3K subunits p85α and p110 (Park et al., 2009, Ugalde et al., 2011); miR-192 family members miR-192, miR-194 and miR-215 are also p53 enhancers as all of them can target to MDM2 (Pichierri et al., 2010).

The third class of miRNAs-p53 interaction is involved in the regulation of miRNA biogenesis. It has been shown that WT p53 can promote the interaction between DROSHA/DGCR8 and DEAD-box RNA helicase p68/p72 (also known as DDX5) so as to elevate intra-cellular level of some mature miRNAs such as miR-143 and miR-145 (Suzuki et al., 2009). Surprisingly, the authors also found that DBD-mutated p53 repressed this interaction in certain types of cancer cell lines. These findings suggest that p53 mutation is able to impair miRNAs biogenesis and also support the importance of miRNAs in carcinogenesis.

The examples listed in this part are only a small part of interaction between p53 and miRNAs, but it is still sufficient to conclude that p53-miRNAs interaction is very widespread and complicated in organisms, shaping a dense network in which various types of crosstalk ranging from positive feedback loops, negative feedback loops to feed-forward signal control system can be found (Hermeking, 2012). Through the precise control of this regulatory network, organisms are capable of coping with various stress signals in a proper way.

### 1.6 The Objectives of the Study

The tumour suppressor p53 is one of the most intensively studied proteins because of its great importance in DDR. Apart from the fundamental research topics, including transcription activity, the role of PTMs and protein-protein interactions, investigation of p53 behaviour in individual cells has become a new topic as the following facts have been demonstrated: 1) p53 dynamics in response to DNA damage at single-cell level has provided a unique insight into the behaviour of p53 signalling that is not seen in
population-based results. Population studies are only able to reflect the average level of the entire population, masking individual behaviours; 2) p53 dynamics shows multiple patterns depending on the type and level of stress, such as DSBs and SSBs, due to the different architecture of responsive signalling pathways; 3) There is a causal relationship between p53 dynamics and biological outcomes, such as the expression level of downstream target genes and cell fate determination, suggesting that manipulating p53 behaviour in individual cells might be a potential mechanism to regulate cellular output when stress response signalling is induced.

However, previous studies have either used a simple and artificial plasmid-based cell model, or merely conducted computational simulation. Because of these approaches, the value of the published data may not precisely reflect the natural behaviour of p53 signalling and the correlation with biological outcomes might also be artificial. To address these concerns, we made the following improvements to the published studies:

1) Develop a new BAC-based vector, in which the native p53 gene, including the entire coding sequence and proximal regulatory sequences, was fused with a fluorescent protein in order to monitor dynamic changes in p53 expression; 2) Transient and stable transfection systems would be used to test if this model is competent enough in terms of transfection efficiency, gene expression level and fluorescent intensity, etc; 3) Demonstrate p53 dynamics in response to DSBs at single-cell level by time-lapse microscopy and make comparison with plasmid-based data; 4) On the basis of the fact that many miRNAs are involved in modulating p53 expression and the results obtained from step 3, investigate that whether miRNAs can fine-tune the pattern of p53 dynamics at single-cell level; 5) Further explore whether the change induced by miRNAs are able to affect cell fate determination and the feasibility of manipulating cell fate through this approach. In conclusion, by conducting this plan of work, the ultimate goals of this project are: 1) To create a new dimension to look at miRNAs regulation; 2) To better understand the complexity of p53 regulation in terms of cellular heterogeneity, dynamical pattern at single-cell level and their links to cell fate.
Chapter 2  Materials and Methods

2.1  The Cell Lines

2.1.1  Cell Culture

Human breast adenocarcinoma cell line MCF7 was obtained from National Collection of Type Cultures (NCTC). MCF7 cells were grown in Dulbecco's modified Eagle’s medium (DMEM) contains 4.5mM glutamine, 10% (v/v) foetal bovine serum (FBS) or foetal calf serum (FCS), 100IU/ml penicillin, 100µg/ml streptomycin and 1% (v/v) non-essential amino acid (NEAA) (All items from Sigma).

p53-Cyan Fluorescent Protein (CFP) and MDM2-Yellow Fluorescent Protein (CFP) stably transfected MCF7 cells were supplied [to Professor Mike White] by courtesy of Professor Galit Lahav's lab and previously described (Lahav et al., 2004). The growing conditions of these transfected MCF7 cells were exactly the same as that of normal MCF7 cells.

WT human colorectal cancer cell line HCT116 was also obtained from NCTC. HCT116 p53⁻/⁻ cell line was a kind gift of Professor Bert Vogelstein's lab and had already been published (Bunz et al., 1998). This cell line was generated by modifying the second exon of p53 gene in WT HCT116 cells, leading to the knock-out of WT p53 gene expression (Bunz et al., 1998). Both cell lines were maintained in DMEM contains 4.5mM glutamine, 10% (v/v) FBS or FCS, 100IU/ml penicillin, 100µg/ml streptomycin and 1% (v/v) NEAA.

Human non-small cell lung cancer cell line H1299 was acquired from the American Type Culture Collection (ATCC). This p53 negative cell line was grown in RPMI 1640 medium supplemented with 2mM glutamine, 10% (v/v) FBS or FCS, 100IU/ml penicillin, 100µg/ml streptomycin and 1% (v/v) NEAA.
All cell lines were grown in an incubator where 37°C and 5% CO₂ was constantly maintained. Cells were split every 2-3 days once they reached 80%-90% of confluence. The protocol of cell passaging was as follows:

Medium was removed and cells were rinsed with 1× phosphate buffered saline (PBS) (Sigma). Then cells were detached by being incubated in 1ml 0.25% (m/v) trypsin-PBS solution for 1 minute. Flasks were gently tapped until cells were completely detached before 3-5ml fresh medium was added into each flask. The cell suspension was gently pipetted and 0.5-1ml of the cell suspension was transferred to a new flask where appropriate amount of fresh medium had already been added. The newly added cell suspension was gently mixed with fresh medium and then the new flasks were placed immediately in an incubator where 37°C and 5% CO₂ was constantly maintained (New Brunswick).

2.1.2 Cell Cryopreservation

At least 5×10⁵ cells were required for cryopreservation to guarantee sufficient numbers of cells can survive during freezing and subsequent thawing process. Cells were washed, detached and then re-suspended in 1ml FBS or FCS supplemented with 10% (v/v) dimethylsulfoxide (DMSO) (Sigma). The cell suspension was then quickly transferred into a cryogenic vial and placed at -80°C as soon as possible. When needed, frozen cells were thawed by being immersed into a 37°C water bath and the cell suspension was seeded in a flask where growing medium had been added. The cell suspension was gently mixed and placed in an incubator where 37°C and 5% CO₂ was constantly maintained.
### 2.2 Western Blotting

#### 2.2.1 Protein Extraction and Quantification

Cells were harvested for protein extraction once they reached 80%-90% confluent. Medium was removed and then cells were washed with cold 1×PBS and placed on ice. Cells were scraped from dishes with 1.5ml 1×PBS and collected in Eppendorf tubes. Centrifugated at 3000rpm, 4°C for 3 minutes, cell pellets were re-suspended in 30μl 1×PBS and 10μl 4× sodium dodecyl sulphate (SDS) lysis/loading buffer (200mM pH6.8 Tris-HCl and 8% SDS (v/v)) (All components from Calbiochem). Then 4μl DTT (Sigma), 3μl 25× protease inhibitor cocktail (Roche) and 1μl 100× phosphatase inhibitor cocktail (Roche) were added immediately into the cell lysates to prevent them from being degraded. Each tube of sample was boiled at 100°C for 10 minutes, followed by 30-second sonication at maximum power twice and 10-minute centrifugation at 12000rpm and 4°C. The supernatants, which contained protein samples, were collected in fresh tubes and placed on ice.

Protein samples were quantified using Bradford reagent (Bio-Rad). To precisely measure the protein concentration of each sample, producing standard curves was required. The first step was to prepare a series of bovine serum albumin (BSA) (Cell Signalling Technology) solution as the standard protein samples, whose concentration was as follows:

<table>
<thead>
<tr>
<th>Tube</th>
<th>Tube</th>
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<td>1</td>
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<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>ddH$_2$O</td>
<td>20</td>
<td>19.375</td>
<td>18.75</td>
<td>17.5</td>
<td>15</td>
<td>12.5</td>
<td>10</td>
</tr>
<tr>
<td>2μg/μl BSA</td>
<td>0</td>
<td>0.625</td>
<td>1.25</td>
<td>2.5</td>
<td>5</td>
<td>7.5</td>
<td>10</td>
</tr>
<tr>
<td>1×SDS lysis/loading buffer</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
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</tr>
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Unit: μl

| **Table 2.1: The Preparation of BSA Solution as the Standard Protein Samples for Producing Standard Curves.** The unit of volume is μl. |
For each standard sample, 779μl ddH$_2$O was added to reach 800μl of total volume. In parallel, 1μl protein sample was added to 799μl ddH$_2$O to reach 800μl of total volume. Next, 200μl Bradford reagent was added to each tube to reach 1ml and mixed properly. Finally, 300μl of mixture was taken from each sample and then transferred to a corresponding well of a 96-well plate. This step was repeated two times to have triplicate samples measured by a micro-plate spectrophotometer (Bio-Tek) at 600nm wavelength.

Taking the volume of 2μg/μl BSA solution as the X axis and the absorbance of the samples as the Y axis, the mean absorbance of each triplicate standard sample was calculated and plotted, thus the standard curve was created. The concentration of each protein sample was able to be obtained from the standard curve since the absorbance had been measured. For each lane on a gel, 20μg protein sample in 10μl solution was set to be the universal loading amount. Therefore, if the concentration of the sample was very high, the loading sample needed to be diluted by 1× lysis/loading buffer with 5× loading dye (0.5% bromophenol blue (w/v) and 50% glycerol (v/v)) (both components from Sigma); if the concentration of the sample was too low to meet 20μg protein in 10μl solution, the re-preparation of the sample was required to meet the universal loading amount. The remaining samples were stored at -70°C for future use.

### 2.2.2 Gel Preparation, Electrophoresis and Transfer

Gels were made using Mini-PROTEAN 3 kit and glass plates with 0.75mm integrated spacers (All items from Bio-Rad). To make two 10% (v/v) running gels, 3.3ml 30% acrylamide/bis-acrylamide (v/v) (Sigma), 2.5ml 1.5 M Tris–HCl (pH 8.8), 4ml ddH$_2$O, 100μl 10% SDS (w/v), 100μl 10% APS (w/v) (Sigma) and 4μl TEMED (Sigma) were mixed and pipetted into the space between two special glass plates, leaving 3 cm at the top for stacking gels. Gels were covered with ddH$_2$O at the top and left to solidifying. To make two stacking gels, 830μl 30% acrylamide/bis-acrylamide (v/v), 630μl 1M Tris-HCl (pH 6.8), 3.4ml ddH$_2$O, 50μl 10% (w/v) SDS, 50μl 10% (w/v) APS and 5μl TEMED were mixed and pipetted into the space between two glass plates after the running gels had solidified and ddH$_2$O removed. Then 10-well combs were mounted at the top of the stacking gels to create wells.
When stacking gels had solidified, combs were removed and two gels (with glass plates) were inserted into a Mini-PROTEAN 3 clamping frame and electrode assembly, creating two chambers that were used for holding running buffer (25mM Tris, 192mM glycine (Calbiochem) and 3.4mM SDS). It was noteworthy that the inner chamber must be filled with fresh running buffer while the running buffer for outer chamber can be used a couple of times. Followed by the inoculation of running buffer, each sample was loaded into a corresponding well, respectively, and the pre-stained coloured protein ladder (Bio-Rad), which consists of a series of standard protein molecular weight indicators, was loaded in the first well from the left. Electrophoresis was performed at 100V, room temperature until the bromophenol blue approached the bottom end of the gel.

The separated protein samples on gels were then transferred onto nitrocellulose membranes (Whatman) using a Mini Trans-Blot Cell kit (Bio-Rad). Specifically, when electrophoresis was completed, every gel was taken from the space between two glass plates, cut off the blank parts on top and bottom and immersed into water, followed by placing onto a wet nitrocellulose membranes lying on top of a blotting paper soaked with transfer buffer (20% methanol (v/v) (Calbiochem), 0.1% SDS (w/v), 25mM Tris and 200mM glycine). Before being covered by another pre-soaked blotting paper, air bubbles between the gel and the membrane must be driven off by gently rolling the gel with a glass stick. This “sandwich” was then placed within a clamp with a rinsed sponge on the inner surface of each side. Notably, the gel must face the black side of the clamp whereas the membrane has to be on the transparent side. Otherwise, protein samples on the gel will be transferred to the buffer instead of the membrane, leading to sample loss and failure of the transfer. Next, the clamp and the “sandwich” inside were inserted into a cell and then put into a tank filled with transfer buffer. Because transfer generates heat, cooling is required or else gels may melt; an ice block was put into the tank. Transfer was run at 300mA constant current for 1.5h.

### 2.2.3 Detection

After transfer, the membranes were gently washed with water and temporarily stained with Ponceau S (Sigma) to monitor transfer efficiency. Then Ponceau S was washed and
membranes were blocked with 5% non-fat milk (w/v) (Fluka) (5% BSA (w/v) is used for phosphorylated primary antibody) in 1×PBST (0.1% Tween-20 (v/v) (Sigma) and 1×PBS) for 30 minutes at room temperature. Membranes were then washed with 1×PBST for 3 times, 5 minutes per time at room temperature and incubated overnight at 4°C with a primary antibody in 1×PBST with 1% non-fat milk (w/v) (1% BSA (w/v) is used for phosphorylated primary antibody). Primary antibodies were diluted as required for optimal signal quality, but typically within the range 500 to 1000 times. The next day the membranes were washed again and incubated with anti-mouse or anti-rabbit secondary antibodies conjugated with horse radish peroxidase (HRP) (Bio-Rad) for 1 hour in 1×PBST with 1% non-fat milk (w/v) (1% BSA (w/v) is used for phosphorylated primary antibody) at room temperature. Next, membranes were washed three times, 5 minutes per time and enhanced chemiluminescence (ECL) reaction mixture (Pierce) was added onto the surface of the membrane attached with protein samples, followed by final detection in a dark room by exposing the blot to film (Kodak) – different exposure times were used depending on signal intensity.

The following antibodies were used in this project: p53 DO-1 (Santa Cruz), phosphorylated p53 (Ser15) (Cell signalling technology), phosphorylated γ-H2A.X (Ser139) (Cell signalling technology), p21 (Cell signalling technology), CHK2 (Cell signalling technology), phosphorylated CHK2 (Thr68) (Cell signalling technology) and β-actin (Sigma).

2.3 Reverse Transcription Quantitative PCR

2.3.1 RNA Extraction and Quantification

miRCURY™ RNA isolation kit (Exiqon) was used for extracting total RNA, including miRNAs, from MCF7 and H1299 cells. All extraction procedures were based on the manufacturer's instruction. Purified RNA was dissolved in 50µl RNase-free water and quantified by a NanoDrop spectrometer (Thermo Fisher). RNA samples were aliquoted and stored at -80°C for future use.
2.3.2 cDNA Synthesis

cDNA was obtained from extracted RNA samples by using Universal cDNA synthesis kit (Exiqon) according to the manufacturer’s manual. 200ng freshly extracted total RNA was used in each cDNA synthetic reaction. cDNA samples were than aliquoted and stored at -20°C for future use.

2.3.3 Quantitative PCR

ExiLENT SYBR® Green Master Mix kit (Exiqon) and LightCycler® 480 (Roche) were employed for quantitative PCR (qPCR) assays. The sequence of primers for miR-34a and miR-125b (Both items from Exiqon) is as follows:

Homo sapiens miR-34a-5p primer set:
Forward: 5’-TGACTCTGTAGTACCTCGGT-3’;
Reverse: 5’-CGATTAGGCATAGCTAGGCA-3’

Homo sapiens miR-125b-5p primer set:
Forward: 5’-CTAACGAGACGCTAACTTGT-3’;
Reverse: 5’-TTCGTTATGCCACGGATTCA-3’

qPCR assays were conducted in 96-well plates (Roche). Each well contained 20 µl reaction mixture, which is comprised of 8µl cDNA templates, 2µl primer mixture (1µl forward primer and 1µl reverse primer) and 10µl 2× SYBR® Green Master Mix (Exiqon). The qPCR program was set according to the manufacturer’s instructions. Relative miRNAs expression levels were demonstrated as comparative Ct values (ΔCt), which were normalised by the primers of the reference gene SNORD 48. The comparison was made either between miRNA mimics non-transfected cells and transfected cells, or between non-stressed cells and stressed cells. Non-transfected cells and non-stressed cells were the controls, respectively, whose ΔCt values were used for another normalisation applied between the control groups and experimental groups (ΔΔCt). Then by using the formula $2^{-\Delta\Delta C_t}$, the fold change between the control groups and the
experimental groups was obtained and bar graphs were produced by Excel. Each sample was measured in triplicate for one biological replicate and three biological replicates were conducted in total. The dataset shown in Chapter 3 is representative of three biological replicates. The mean value and standard deviation were calculated because triplicate was performed for each sample.

2.4 Immunofluorescence

Each glass cover-slip was placed in a well of a 12-well plate before 1ml normal growth medium was inoculated into each well. Then 2.5×10⁴ MCF7 cells were seeded in each well. To treat cells, 200, 300, 500, 800, 1000 and 1200ng/ml (final concentration) neocarzinostatin (NCS) was added into six wells 2 hours prior to the experiment, respectively; and 400ng/ml (final concentration) NCS was added into another five wells of cells respectively, but the timing of addition was different: 0.5, 1, 2, 4 and 6 hour(s) before the experiment. The last well was for untreated cells as a negative control. Once the period for NCS treatment ends, medium was removed, cells were washed with 1×PBS, immersed into 4% (v/v) formaldehyde (Sigma) diluted in 1×PBS and fixed for 15 minutes at room temperature. Then cells were washed three times with 1×PBS, 5 minutes per time and cell membrane was permeated by immersing in 0.5% Triton X-100 (v/v) (Sigma) diluted in 1×PBS for 20 minutes at room temperature. Then cells were washed with 1×PBS in the same way again and blocked with 5% BSA (v/v) in 1×PBST for 1 hour at room temperature. The primary antibody against phosphorylated γ-H2A.X (1000-fold dilution by 1×PBST) was incubated overnight with permeated cells at 4°C. The next day, the primary antibody solution was removed (can be used a couple of times), cells were washed with 1×PBS and placed in a dark box before the fluorescence-conjugated secondary antibody was used for 1-hour incubation with cells at room temperature. The dark box was used for preventing the secondary antibody from being exposed to light, which can lead to the inactivation of the fluorescent group conjugated with the secondary antibody. Therefore, light protection steps must be taken in the following procedures as well. Once the incubation was completed, the secondary antibody solution was removed and cells were washed three times with 1×PBS in the same way again. Then cover slips where cells were grown were taken out of the 12-well
plate and placed on a piece of filter paper to dry. Meanwhile, 12 glass microscope slides were prepared and a small drop of DAPI applied to each. Each cover slip was placed on each drop of DAPI to stain DNA (hence the cell nuclei) for 5 minutes at room temperature. Excess fluid around the edge of each cover slip was removed by filter paper. These specimens were investigated by bright-field and confocal microscopes, respectively, and the images were analysed and quantified by CellTracker®, which will be illustrated in chapter 4.

2.5 BAC Retrofitting

p53-DsRedXP BAC was generated by Rachel Nelson and Antony Adamson and kindly provided (courtesy of Michael White) as glycerol stocks of E.coli SW102. The plasmid pL451, with cytomegalovirus promoter (pCMV)-Lamin B1-enhanced CFP (eCFP) fusion cassette integrated (Figure 2.1), was made by Antony Adamson and available in the glycerol stocks of E.coli DH5α. This series of experiments aim to insert pCMV-Lamin B1-eCFP cassette and kanamycin/neomycin-resistant gene, both derived from the plasmid pL451, into p53-DsRedXP BAC to generate a BAC recombinant p53-DsRedXP-Lamin B1-eCFP, which can serve as a positive transfection marker while indicating real-time p53 dynamics in single cells. With a eukaryotic antibiotics selection marker integrated in the retrofitted BAC, BAC stable transfection was able to be achieved as antibiotics-based cell screening was allowed to be conducted (the original chloramphenicol-resistant gene in p53-DsRedXP BAC is driven by a prokaryotic promoter). BAC retrofitting was a multi-step manipulation and achieved by the following procedures.

2.5.1 Bacterial Cell Culture

Both LB-broth and LB-agar (both from Sigma) medium were prepared and autoclaved before use. Autoclaved medium had to be cooled down to approximate 40°C before appropriate antibiotics can be added. The plasmid pL451 is kanamycin/neomycin-resistant (the kanamycin-resistant gene only works in prokaryotes while the neomycin-
resistant gene merely takes effect in eukaryotes due to the different properties of their promoters) so kanamycin (Sigma) was added into the medium for plasmid extraction. Chloramphenicol (Sigma) was added into the medium for BAC extraction because p53-DsRedXP BAC contains anti-chloramphenicol gene. To prepare LB-agar plates, LB-agar medium that contained antibiotics was poured into dishes. It would become solid after cooling off to room temperature. Spare LB-agar plates were covered with lids and sealed in order for long storage at 4°C. Notably, LB-agar plates must be kept upside down all the time to prevent medium from being contaminated by vapourised water collected on the lids of the dishes. To streak a LB-agar plate for BAC (*E.coli SW102*) or plasmid (*E.coli DH5α*) extraction, a sterile loop was employed to dip bacteria from a glycerol stock and to streak on a plate three times. Then the plate was incubated at 37°C (*E.coli DH5α*) or 32°C (*E.coli SW102*) for 24-48 hours to let bacteria grow until many small white round colonies appeared. Note that growth should not be performed for >48 hours as this will result in smearing and potential contamination between individual colonies. As a result, single colonies were not able to be picked from a plate. To speed up bacteria growth, a single colony on a plate was picked by a sterile loop and transferred to a tube containing 5ml LB-broth with appropriate antibiotics, followed by incubation on a rotary platform in an incubator set at 230rpm to grow for 6-8 hours at 37°C (*E.coli DH5α*) or 32°C (*E.coli SW102*) until the medium became cloudy. If bacterial glycerol stocks need to be prepared for future use, remove 400µl bacterial cell suspension from the tube, mix with 400µl 40% (v/v) glycerol (Sigma) and store the mixture at -70°C as soon as possible. To expand bacterial culture, 5ml cloudy bacterial cell suspension was transferred to 300ml LB-broth with appropriate antibiotics for further incubation under the same condition, which lasted 12-16 hours until the medium became cloudy again. This turbid bacterial cell suspension was ready for plasmid or BAC maxi-prep. The plate with bacterial colonies can be stored upside down at 4°C for 1-2 month(s). It was noteworthy that all steps of bacterial culture operation must be conducted next to a Benson light to avoid cross contamination.
2.5.2 BAC Maxi-prep

BAC maxi-prep aims to produce highly purified circular BAC DNA for transfection, transformation and recombination assays. By following the manufacturer's instructions, *E.coli* SW102 cell suspension with appropriate antibiotics obtained from the last step was treated by NucleoBond® BAC 100 kit (Macherey-Nagel) for BAC maxi-prep. Purified BAC DNA was dissolve in 300µl pre-warmed water (65°C). Given that BAC molecules are large (~200kbp) and prone to shearing, pipetting the solution up and down must be avoided; gently flick the bottom of the tube to help BAC dissolve instead. The solution can be placed in a 65°C water bath for 20-30 minutes to speed up dissolving. Eventually, the concentration of BAC solution was measured using a NanoDrop spectrometer.

2.5.3 Plasmid Mini-prep

As described in 2.5.1, a single colony of *E.coli* DH5α was picked and dipped into 5ml LB-broth with kanamycin. The bacterial cell suspension was incubated on a rotary platform in an incubator set at 230rpm, 37°C overnight, until the suspension became cloudy. Then the plasmid DNA was extracted and purified by QIAprep Spin Miniprep Kit (Qiagen) from the bacterial cell suspension, followed by being dissolved in 200µl pre-warmed water (65°C). The concentration of the purified plasmid DNA solution was measured using a NanoDrop spectrometer.

2.5.4 Isolation of the Specific Fragment from the Plasmid pL451

pCMV-Lamin B1-eCFP (6219bp, Figure 2.1) fusion cassette was isolated from the purified plasmid pL451 through hydrolysis reaction catalysed by restricted endonucleases. To achieve this goal, the first step was to analyse the sequence of pL451 to identify appropriate restricted sites to release pCMV-Lamin B1-eCFP cassette from the plasmid pL451.
Figure 2.1: The Isolation of pCMV-Lamin B1-eCFP Cassette and Kanamycin/Neomycin-resistant Gene from the Plasmid pL451 by Endonuclease Digestion. According to pL451’s map, Pci I and Pvu I
were chosen because all key sequence, including 5’ and 3’ homologous arms required for recombination process, was able to be conserved after their digestion.

We took account of several factors in selecting appropriate restricted endonucleases, which will be addressed in detailed in chapter 5, and eventually chose Pvu I HF and Pci I HF (both from New England Biolabs) to isolate pCMV-Lamin B1-eCFP cassette from the plasmid pL451. Then a 20µl reaction system was set up as follows: 5µl (2-3µg) plasmid DNA, 2µl 10× reaction buffer 3.1 (New England Biolabs), 1µl Pvu I HF, 1µl Pci I HF and 11µl distilled water. The reaction mixture was placed in a water bath at 37°C and incubated overnight. When the reaction was completed, 2µl digested plasmid DNA was loaded on a 1% agarose gel to look at the effectiveness of enzymatic digestion, which was evaluated in terms of the intensity of the band at ~6.2kb. If a proper result was shown, then the rest of digested plasmid DNA was loaded on a 1% low-melt agarose gel, followed by gel extraction. QIAEX II gel extraction kit (Qiagen) was employed to extract 6.2kb bands, which contained pCMV-Lamin B1-eCFP cassette, from the gel. Note that using distilled water for DNA elusion and dissolution instead of elusion buffer as salt and EDTA in the elusion buffer can inhibit recombination process. The purified sample extracted in this way can be directly used in the following recombination assay. Finally, the concentration of purified DNA was measured by a NanoDrop spectrometer.

**2.5.5 Homologous Recombination**

The recombination assay aims to incorporate pCMV-Lamin B1-eCFP fusion cassette with circular p53-DsRedXP BAC to create the retrofitted BAC - p53-DsRedXP-Lamin B1-eCFP, where p53-DsRedXP expression was controlled by p53 native promoter while Lamin B1-eCFP expression was initiated by the constitutive eukaryotic promoter pCMV. Thus the retrofitted BAC can serve as a transfection marker in addition to an indicator of p53 dynamics in single cells. Moreover, the original chloramphenicol-resistant gene driven by a prokaryotic promoter in p53-DsRedXP BAC was replaced by a complete kanamycin/neomycin-resistant gene during recombination process, which made BAC stable transfection feasible in eukaryotic cells.
2.5.5.1 The Preparation of Electrocompetent (Recombination-efficient) Bacteria

The first step of recombination assay was to treat bacteria to make them suitable for DNA recombination process. In our project electroporation was employed to achieve this goal. First, picked up a freshly streaked single colony of *E.coli* SW102 and let it grow in 5ml LB-broth (contains chloramphenicol) overnight at 230rpm and 32°C until the culture became cloudy. Then removed 1ml from the suspension and diluted into 50ml LB-broth with chloramphenicol, followed by growing at the same condition until OD_{600} value of the bacterial cell suspension reached 0.6, which was detected by an Ultralp 10 Cell Density Metre (GE Healthcare). 50ml suspension was split into three samples: a 10ml control sample and two 15ml induced samples. The control sample was incubated in a 32°C water bath while two induced samples were put in a 42°C water bath. All samples were incubated for 15 minutes and kept swirling during incubation. Next, each sample was quickly transferred to three pre-chilled tubes and left on ice/water slurry for 2 minutes, followed by 10-second centrifugation at 4°C, 6000rpm. Supernatant was removed and bacterial pellet of each sample was re-suspended in 10ml cold distilled water. Repeated this spinning-re-suspending step and re-suspended bacteria of each sample in 1ml cold distilled water in pre-chilled Eppendorf tubes. Three samples were centrifuged at 4°C, 12000rpm and supernatant was removed again. Repeated this spinning-re-suspending step and eventually re-suspended bacteria of each sample in 40µl cold distilled water. The bacteria in this suspension were electrocompetent and ready for recombination.

2.5.5.2 Recombination by Electroporation

20µl *E.coli* SW102 bacterial suspension of each sample was mixed with 100ng isolated pCMV-Lamin B1-eCFP cassette and left on ice for 5 minutes. Avoid pipetting up and down but flicking the tubes occasionally instead to gently mix the reaction mixtures during incubation. Then each mixture was transferred to a pre-chilled 0.1cm silex cuvette, followed by electroporation using a Gene Pulser II Electroporation System (Bio-Rad) with the following settings: 1.8kV, 25µF and 200Ω. After electroporation, each
mixture was immediately transferred to a new Eppendorf tube and re-suspended in 1ml super optimal broth with catabolite repression (SOC) medium. Then each bacterial suspension was incubated for 90 minutes at 32°C and 230rpm, which aimed to rescue bacteria from damage caused by electroporation. Next, each bacterial suspension was streaked on a LB-agar plate with kanamycin and incubated at 32°C for screening. If bacterial colonies appeared, it suggested that BACs in these bacteria were worth being characterised to see if recombination was successful, which will be detailed in the following paragraphs. The bacteria containing correctly retrofitted BAC can be used for BAC maxi-prep to prepare highly purified BACs for subsequent transfection.

2.5.6 BAC Mini-prep

BAC mini-prep can quickly produce small amount of BAC DNA. However, the samples prepared in this way are not suitable for tranfection, transformation, recombination and other DNA manipulation assays because they are not highly purified. Therefore, these samples are frequently used for characterisation. Both original and retrofitted BACs were extracted as follows:

A single colony of *E.coli* SW102 was picked from a LB-agar plate with appropriate antibiotics and transferred to 5ml LB-broth medium with appropriate antibiotics. This bacterial cell suspension was placed on a rotary platform in an incubator set at 230rpm, 32°C and incubated overnight, followed by 1500rpm centrifugation to spin down bacterial pellet at room temperature. 200μl resuspension buffer (Qiagen) was added to re-suspend the bacterial pellet after the supernatant was removed. The suspension was then transferred to an Eppendorf tube before 200μl lysis buffer (Qiagen) was added, followed by gently inverting the tube 6-8 times to lyse bacteria and release the BAC DNA. This step had to be done very carefully to avoid BAC shearing. Next, the tube was left at room temperature for 3 minutes for lysis before 320μl neutralisation buffer (Qiagen) was added. Again, the tube was carefully inverted 6-8 times, followed by 1500rpm centrifugation at 4°C. The supernatant was transferred to a new tube and the pellet discarded, followed by adding 600μl isopropanol (Sigma). The tube was gently inverted again before being spun down at 15000rpm, 4°C for 30 minutes. The supernatant was
discarded and the pellet was washed with 1ml 70% ethanol. Then the pellet was air
dried at room temperature before being dissolved in 65°C pre-heated distilled water.
DNA concentration was measured using a NanoDrop spectrometer.

2.5.7 BAC Characterisation

To confirm if BAC retrofitting had been achieved, the following characterisation steps
were taken by means of restriction endonucleases digestion and PFGE. Basically, the
primary principle to identify the retrofitted BAC was to look at the size and number of
main fragments cut from the backbone of the retrofitted BAC by particular restricted
endonucleases and to compare them with the size and number of their counterparts
derived from the original BAC. Any significant discrepancy shown by PFGE, such as
different number of major bands [of the predicted size] or position-shifting of the
relevant fragments, indicated that BAC retrofitting was successful.

2.5.7.1 Enzymatic Digestion

Both retrofitted and original BACs were prepared by mini-prep mentioned in 2.5.6.
According to the principle of BAC characterisation addressed in last paragraph, all
restriction sites on DNA of both BAC were studied. As a consequence, Mlu I HF and Sal I
HF (both from New England Biolabs) were selected. Again, a 20µl reaction system was
used: 1µl Mlu I HF, 1µl Sal I HF, 2µl 10× reaction buffer 3.1, 8µl BAC DNA and 8µl
distilled water. Two reaction mixtures were incubated in a 37°C water bath overnight.

2.5.7.2 Pulse-field Gel Electrophoresis

When the enzymatic digestion was completed, PFGE was employed to separate digested
products due to their high molecular weight. PFGE was performed using CHEF DR I
system (Bio-Rad). 3L 0.5×TBE (54g Tris Base, 27.5g boric acid and 4.65g
Ethylenediaminetetraacetic Acid (EDTA) (All from Calbiochem) were all added in 1L
distilled water) was poured in an electrophoresis tray and chilled by the cooling and pumping systems of CHEF DR II PFGE apparatus before use. A 1% low melting point agarose gel (Bio-Rad) was made, where 20µl enzymatic-treated BAC samples pre-mixed with 4µl 6× loading buffer (New England Biolabs) were loaded (Bio-Rad). Mid-range PFGE marker I and II (Both from New England Biolabs) were also required to indicate the size of bands. The cooling and pumping systems were switched off before the gel was placed at the centre of the tray, preventing the samples from being washed off. Samples ran 30 minutes at 6V/cm to let them completely enter the gel before the cooling and pumping systems were switched on again. Then PFGE underwent 18 hours at 14°C and 6V/cm. Once completed, gels were stained in 0.5×TBE with SYBR safe dye (Invitrogen) for 1 hour and photographed using a Gel Doc bioimaging system (Bio-Rad).

2.6 Transfection

2.6.1 BAC Transient Transfection

For transfection of human cells in culture, two transfection reagent, ExGen500 (Fermentas) and polyethylenimine 40 (PEI40) (Sigma) were used. Notably, normal medium must be replaced by antibiotic- and serum-free medium one hour before transfection assays.

When ExGen500 was used, the following steps were taken: 1µg BAC DNA and 200µl 150mM NaCl were mixed and then 9.9µl ExGen500 was added. The mixture was vortexed for 15 seconds and incubated at room temperature for 15 minutes. This solution was added directly into antibiotic- and serum-free medium and cells were incubated at 37°C and 5% CO₂ for 6 hours. Then antibiotic- and serum-free medium was replaced by fresh normal growth medium, followed by 24-48 hours culture before time-lapse microscopy.

When PEI40 was used, the procedures were as follows: 1µg BAC DNA and 200µl 150mM NaCl were mixed and then 10µl PEI40 was added. Then the solution was vortexed for 15 seconds, incubated at room temperature for 20 minutes and added into antibiotic- and
serum-free medium, which was replaced by normal growth medium 4 hours later. Then cells were grown at 37°C and 5% CO₂ for 24-48 hours before imaging.

2.6.2 BAC Stable Transfection

2.6.2.1 Geneticin Dose Response

To achieve BAC stable transfection, antibiotics screening is required after BAC transient transfection to collect the cells in which BAC vectors can stably exist and the genes they contain can be normally expressed. As previously described, the retrofitted BAC contains a kanamycin- and a neomycin-resistant gene but only the neomycin-resistant gene works in eukaryotes. However, neomycin cannot kill eukaryotes; in fact, eukaryotes are impaired neither by neomycin nor kanamycin at all. So in this case, an analogue of neomycin called geneticin (Melford) was used for cell screening as cells that express neomycin-resistant gene are geneticin-proof as well. After deciding what antibiotics we were going to use, we had to work out the concentration of geneticin used for cell screening. Therefore a geneticin dose response curve must be produced. 1×10⁶ Lahav's modified MCF7 cells were diluted in normal growth medium to make the cell suspension where 1ml contained 10000 cells. Each well of a 24-well plate was inoculated by 0.5 ml of cell suspension and grown for 24 hours at normal condition before geneticin was added. The final concentration of geneticin was set as follows: 0, 0.2, 0.4, 0.6, 0.8, 1, 1.2, 1.4, 1.6, 1.8, 2 and 2.5 mg/ml. Every concentration had a repeat in the same 24-well plate and totally 3 24-well plates were prepared. Then growth medium with different final concentration of geneticin was refreshed every 48 hours. This step was repeated for 10-14 days. The best screening concentration of geneticin was the lowest concentration that can cause death of all cells in a single well. When the proper concentration was determined, a bit higher level of the proper concentration on the dose response curve was used as the initial screening concentration. When single colonies appeared, screening concentration was reduced by 50% to maintain the stable cells for a couple of months or else cells were highly likely to get rid of BACs before the cassettes in the BAC incorporated with cell genome by homologous recombination.
2.6.2.2 BAC Stable Transfection

Lahav's modified MCF7 cells were transiently transfected with p53-DsRedXP-Lamin B1-eCFP BAC using either ExGen500 or PEI40 as previously described. 0.5mg/ml geneticin applied 24 hours after transient transfection. Growth medium was replaced by fresh growth medium with 0.5mg/ml geneticin every 48 hours until single-cell clones were formed, which generally took 2-3 weeks. Each transfected single-cell clone was isolated by a cloning cylinder (Sigma) and detached through trypsinisation. Specifically, cells in every cylinder were incubated in 0.1ml trypsin for 2 minutes at room temperature before being transferred to a well of a 48-well plate. Cells were grown in normal growth medium for 24 hours before 0.5mg/ml geneticin was added. As cells proliferated they were transferred to bigger wells, such as a well of 24-well plate, 12-well plate, 6-well plate and eventually 35mm glass-bottom Greiner dishes. All single-cell clones were checked by a confocal microscope to see if the retrofitted BAC worked properly, which was marked by the constitutive expression of Lamin B1-eCFP gene and conditional expression of p53-DsRedXP gene in response to stress induction, such as NCS addition.

2.6.3 miRNA Transfection

miR-34a, miR-125b and control miRNAs used in our experiments were human miRNA mimics (Ambion). The sequence of human miR-34a-5p and miR-125b-5p is as follows:

miR-34a-5p:
GGCCAGCUGUGAGUUUCUUUGCGACUGUCUUGCCGUGGAGCAUAGUAAAGG
AAGCAACGACAGUAUACUUGCCCUAGAAGUGCGACGUUGUGGGG;

miR-125b-5p:
UGCGCUCUCUCAGUCUCCUCAGACCCUAACUUGUGAUUGUUACGUUAAUCCACGGGU
AGGCUCUUGGGGAGCUGCGAGUCGUCU.

Lipofectamine RNAiMAX (Invitrogen) transfection reagent was used for miRNA transfection. The manufacturers’ instructions of miRNA mimics and Lipofectamine
RNAiMAX were strictly followed during miRNAs transfection, including the ratio between miRNA mimics and transfection reagent under different conditions. 20pmol or 60pmol miRNA mimics applied in each 35mm Greiner glass-bottom dish or normal dish; for each compartment of a 35mm four-compartment Greiner glass-bottom dish, 5pmol or 15pmol miRNA mimics applied; for each 60mm dish, 50pmol miRNA mimic was applied. Again, serum- and antibiotic-free medium was required for transfection assays.

2.7 Live-cell microscopy

2.7.1 Cell Culture

For time-lapse imaging, $1 \times 10^5$ cells were seeded in a 35mm Greiner glass-bottom dish 72 hours before imaging and grown under the same condition as normal cell culture described previously. If needed, BAC DNA was transfected into cells 48 hours before imaging and miRNAs were transiently transfected 24 hours prior to microscopy assays.

2.7.2 Time-lapse Microscopy

Carl Zeiss LSM 510, 710 or Pascal confocal microscopes and 40× oil immersion objectives were used for live-cell imaging. Cells were maintained in a Greiner glass-bottom dish, which was fixed on an electronically controlled platform. This platform was mounted in a closed cabinet, where 37°C, 5% CO₂ and appropriate humidity were maintained to keep cells growing during imaging. Zen 2010 software (Carl Zeiss) was used to setup the imaging parameters, control the microscope and change the position of the platform. Particularly, this software allows users to monitor the behaviour of live cells from a computer screen, which simplifies the operation of time-lapse microscopy. Furthermore, when a particular position is selected, Zen also allows users to mark this position, which means that this position is automatically memorised by the system. As a consequence, time-lapse imaging becomes a fully automatic process once all positions are selected and marked. In addition, by changing the imaging parameters such as the laser wavelength (including excitation and emission wavelength), the power of laser
sources, the number of imaging repetitions for each position and the imaging interval between two positions, researchers are able to optimise their imaging conditions.

2.7.3 Imaging Data Analysis

The raw data acquired from time-lapse microscopy was a series of video clips, the number of which is equal to the number of positions marked before the program started. Consisting of certain numbers of imaging repetitions of a specific position, each video clip recorded the real-time behaviour of all live cells within the vision field during the experiment. The length of time course of each video clip, which was determined by the number of imaging repetitions and the time interval between two positions that had already been setup before the experiment, was fixed and identical. These video clips were input into a cell imaging quantification software called CellTracker® (version 0.1) to calculate the fluorescent intensity of each type of fluorescent protein and the results were exported to Excel. Notably, we had to mark the contour of the nucleus of every single cell we wanted to quantify in every repetition of every single position, which was vastly time-consuming, before actual values of fluorescent intensity can be produced automatically. Further advanced data analysis, such as plotting, normalisation and classification, was performed using Excel and Prism. The approaches used to classify individual time-lapse traces will be discussed in detail in the relevant results chapters.
Chapter 3  The Response of p53 Signalling to DNA Double-strand Breaks at Cell Population Level

As the first chapter that shows our experimental results in this thesis, the following content aims to validate the basic properties of the cell lines and miRNAs used in our project at cell population level, which was achieved by Western Blotting and real-time PCR under both stressed and normal conditions. The data helps us learn that if the cell lines and miRNAs are suitable for the project and that if their performance is reliable and reproducible. Specifically, we will use Western Blotting to probe the expression level of p53 and some key components of p53 signalling in response to DSBs in different cell lines and time series. Meanwhile, it is also necessary to look at the endogenous level and transfection efficiency of miRNAs mimics and how their level reacts to DSBs by qPCR. Furthermore, miRNAs effect on the chosen components of p53 signalling at cell-population level needs to be validated before doing single-cell assays. The results shown in this chapter are expected to build a solid foundation for the subsequent in-depth investigations in individual cells.

3.1 Introduction

Encoded by TP53 gene in human cells, tumour suppressor p53 plays a crucial role in DDR and subsequent cell fate determination. Therefore, p53 is well-known as "the guardian of the genome", referring to its great importance in maintaining genome integrity (Kruse and Gu, 2009). It has been widely reported that most cancers are caused by and accompanied with p53 catastrophe including TP53 mutations, aberrant p53 trans-activity and expression level, as well as the disorders of p53 regulatory machineries (Brady and Attardi, 2010).
Mainly induced by upstream kinases ATM and ATR in response to various types of stress signal, p53 activation embodies two aspects: up-regulation of stability and trans-activity. p53 stability is largely determined by E3-ligase MDM2. At normal growth condition, MDM2 is tightly associated with p53 and gives rise to constant ubiquitination at specific lysine residues in p53 CTD, followed by rapid p53 degradation in proteasomes. Thus the basal level of p53 is very low in unstressed cells, despite a good basal level of mRNA synthesis and protein expression. However, in the presence of stress, p53 is phosphorylated by upstream kinases at some specific residues like Ser15 and Ser37, leading to stabilisation by PTM-induced conformational changes and dissociation with MDM2, which is then degraded to reduce p53 turnover.

The elevation of trans-activity also attributes to changes in PTM status and molecular conformation. Phosphorylation status is one of the most important changes in PTMs. Once DNA damage is detected, kinases ATM and ATR are subject to auto-phosphorylation very quickly at Ser1981 and Ser428 respectively and this leads to the phosphorylation of γ-H2A.X at Ser139, which is a very sensitive and typical biomarker of DNA damage (Canman et al., 1998, Liu et al., 2011b). Phosphorylated ATM, mainly induced by DSBs signal, results in CHK2 phosphorylation at threonine 68; whereas ATR predominantly processes SSBs signal and induces CHK1 phosphorylation at Ser317 (Lee and Paull, 2007). The phosphorylation of these key upstream components subsequently causes p53 phosphorylation at multiple sites of its N-terminal domain, such as Ser15 and Ser20 (Canman et al., 1998). Accompanied with the structural transition from polymers to monomers, p53 becomes sufficiently stable and active to drive its downstream targets both at transcription and post-translation level, most of which are involved in the process of cell fate determination, such as inducing cell apoptosis, cell cycle arrest and DNA damage repair. For example, p53-dependent activation of CDKs inhibitor p21 at the transcription level represses the activity of CDK2/4 and leads to cell cycle arrest at G1/S checkpoint as a consequence (el-Deiry et al., 1993). Another direct trans-activated target of p53, PUMA, can augment Caspase-dependent PCD pathway and thus promote cell apoptosis (Polo and Jackson, 2011). It is also not surprising to see that many phosphatases, like WIP1, are able to impair p53 stability and trans-activity by abrogating phosphorylation at specific sites, rendering p53 inactive (Lu et al., 2007).
Apart from phosphorylation, p53 acetylation in its CTD has great impact on p53 stability and trans-activity as well. Acetyltransferases such as CBP/p300 bring acetyl groups to particular lysine residues and give rise to a massive increase of p53 trans-activation (Wang et al., 2004). In contrast, there are various deacetylates, like NAD-dependent deacetylase SIRT1, that can down-regulate p53 trans-activity (Wang et al., 2006).

3.2 The Response of p53 to DNA Double-strand Breaks Induced by 4-hour NCS Treatment in Different Cell Lines

First, we want to learn what p53 behaviour looks like in response to DSBs in the cell lines we used, as p53 plays a essential role in processing stress signals. In this project we are going to use five cell lines: WT MCF7, Lahav’s modified MCF7, H1299, WT HCT116 and HCT116 p53−/−. WT MCF7 and HCT116 cell lines express WT p53, while H1299 and HCT116 p53−/− cell lines are p53 negative. However, we are not able to rule out the expression of p53 isoforms, which may have different properties and function from WT p53 and thus may become a consideration of the experiment, in both cell lines. Therefore, at the beginning total p53 expression of those cell lines in response to DSBs needs to be characterised by Western Blotting. In this experiment, DSBs were generated by NCS treatment. As an anti-tumour, anti-proliferative protein-small molecule complex and a rapidly active radiomimetic drug as well, NCS can cause γ-irradiation-like DSBs, DNA synthesis inhibition and lead to G2/M cell cycle arrest or cell apoptosis. On the basis of previous studies, these cell lines were treated with 400ng/ml NCS (final concentration) for 4 hours before being harvested (Lahav et al., 2004). For each cell line, non-treated samples were also prepared in parallel as negative controls. As Figure 3.1 shows, the basal level of WT p53 was very low but can be dramatically elevated by NCS treatment in WT HCT116 and MCF7 cells; whereas both H1299 and HCT116 p53−/− cell lines did not show p53 expression under either stressed or non-stressed conditions. In addition, minor p53 isoforms were not detected in these cell lines, although we used p53 DO-1 antibody (Santa Cruz), which can react to most of p53 isoforms. The level of phosphorylated γ-H2A.X was also investigated to show that the DNA damage response was activated in these cells. Finally, in order to normalise the intensity of each blot, β-actin blots need to be produced as the internal control of the experiment, due to its
constantly stable expression level in the cells under study. So if the amount of the protein sample loaded on each lane is identical, the intensity of every β-actin blot on that gel would keep the same, making it feasible to compare intensity of blots among different samples.

**Figure 3.1:** The Expression Level of p53 in Different Cell Lines Either Treated with DSBs Inducer NCS for 4 Hours or at Normal Condition. **A:** The expression level of p53, phosphorylated γ-H2A.X and β-actin in p53 positive cell line WT HCT116 and its p53 knocked-out variant at normal condition or both treated with 400 ng/ml (final concentration) NCS for 4 hours; **B:** The expression level of p53, phosphorylated γ-H2A.X and β-actin in p53 positive cell line WT MCF7 and p53-null cell line H1299 at normal condition or both treated with 400 ng/ml (final concentration) NCS for 4 hours. This experiment was repeated twice and this figure is a representative.
3.3 The Long-term Behaviour of p53 Signalling in Response to DNA Double-strand Breaks in Wild-type MCF7 Cells

After visualising p53 expression in different cell lines at a specific time point under both stressed and non-stressed conditions, next we wanted to know how p53 signalling responds to DSBs over extended periods of time. Given the complexity of p53 signalling, we only selected a couple of typical components, ranging from upstream to downstream of p53 signalling, as reporter proteins, which included phosphorylated and total CHK2, phosphorylated and total p53, p21 and phosphorylated γ-H2AX. The expression of these proteins was measured in the hope of partially demonstrating the behaviour of p53 signalling in response to DSBs. ATM was not selected because its molecular weight is 350kDa, making it difficult to run on classical polyacrylamide gels and measure accurately by Western Blotting. CHK2 is an upstream kinase in p53 signalling, which is largely activated by ATM-dependent phosphorylation in the presence of stress signals and leads to p53 phosphorylation and activation. p21 is one of the most important early downstream target of p53 signalling and determinants of cell fate, which induces DNA repair, cell cycle arrest or cell senescence depending on the level of damage (Harper et al., 1993). In this experiment, WT MCF7 cells were treated with 400ng/ml NCS from 1 hour to 10 hours before being harvested. The untreated protein samples were prepared in parallel as the negative control, which was prepared as 0 hour NCS treatment in Figure 3.2. As seen from the result, the level of phosphorylated CHK2 increased quickly in response to NCS treatment but the activated period only lasted for around an hour before dropping back to basal levels of expression. Total CHK2 expression remained unchanged over the 10 hour time-course. Phosphorylated and total p53 were both activated by DSBs but it took 3-4 hours, longer than phosphorylated CHK2, to reach the highest expression level. In this case a further period of more than 4 hours was needed to return to basal level from the peak level. In comparison with phosphorylated CHK2 dynamics, the lag of p53 and phosphorylated p53 expression coincides with the fact that CHK2 is considered as a component of DNA damage early responsive machinery that leads to p53 activation (Kruse and Gu, 2009). Similarly, the rate of p21 activation was slower than p53 and phosphorylated p53, reaching the highest level of expression around 4 hours after NCS addition. The pattern of p21 expression was as expected because p21 is a downstream target of p53.
The level of phosphorylated γ-H2A.X expression peaked as quickly as phosphorylated CHK2, which reinforces the role of γ-H2A.X as one of the primary responses seen during the early DDR; this provides a sensitive biomarker of DNA damage. Therefore it was not surprising to see that phosphorylated γ-H2A.X decayed very slowly because it is strongly correlated with DNA damage level (Kuo and Yang, 2008); that is to say, as long as cells do not fully recover from DNA damage, phosphorylated γ-H2A.X still remains. In this series of experiments, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. Like β-actin, GAPDH is a house-keeping gene and therefore always keeps very stable intra-cellular level. Because of this property, both β-actin and GAPDH provide a standard for normalising the intensity of blots, which makes it possible to compare intensity of blots among different samples. For example, all values in Figure 3.2 B, Figure 3.1 were relative because they were obtained by being normalised against GAPDH. Specifically, every blot in Figure 3.2 A was input and analysed respectively by Image J (National Institutes of Health), scientific image processing software, followed by the output of the intensity of each blot showed in Figure 3.2 B.
Figure 3.2: The Expression Level of Some Key Components of p53 Signalling in Response to NCS-induced DSBs within 10 Hours. A: The blots of some typical components in p53 signalling in response to NCS-induced DSBs within 10 hours; B: The plotted graphs sketching the alteration of expression level of each component. The intensity of each blot was quantified by Image J; all data was analysed by Excel; the intensity of blots of internal control GAPDH was set as a standard and other values were normalised against GAPDH. This experiment was repeated twice.

3.4 The Quantification of Endogenous Expression or Over-expression Level of miR-34a and miR-125b in H1299 and Wild-type MCF7 Cells

In our project, miR-34a and miR-125b were chosen as miRNA regulators of p53 dynamics at single-cell level because they both interact with p53 but in completely
different ways. Specifically, miR-125b is a direct p53 suppressor that can target to the seed sequence in the 3'-UTR of p53 mRNA, impairing mRNA stability and disrupting the translation process of p53 gene (Le et al., 2009). miR-34a is not able to directly target to p53 mRNA, however, as a p53 downstream target genes, p53-dependent trans-activation increases miR-34a intra-cellular level and then results in the repression of deacetylase SIRT1 that can impair p53 trans-activity; as a consequence, p53 function is enhanced indirectly by miR-34a, which in turn facilitates its transcription (Raver-Shapira et al., 2007). These facts indicate that miR-34a enhances p53 activity via a positive feedback loop mediated by SIRT1. More importantly, previous studies revealed that different combination of multiple feedback loops gave rise to different patterns of protein dynamics at the single-cell level (Toettcher et al., 2010, Moore et al., 2015). On the basis of this conclusion, we hypothesise that miR-34a and miR-125b can make different impact on p53 dynamics in single cells. Therefore, the first thing to do with miR-34a and miR-125b was to validate their endogenous expression level in WT MCF7 and H1299 cell lines, which was achieved by reverse transcription qPCR (RT-qPCR).

Total RNA, including miRNAs, was isolated from WT MCF7 and H1299 cell lines, respectively. Both cell lines were treated with 400ng/ml NCS (final concentration) for 1 hour and 4 hours, respectively. Meanwhile, non-treated MCF7 and H1299 cells were also prepared as negative controls. All purified RNA samples were then transformed into cDNA by reverse transcriptase. Similar to Western Blotting assays, an internal control was required to set a standard for normalisation of gene expression level. Here we chose a classical reference gene called SNORD 48, a stably expressed small nucleolar RNA gene, as the internal control of the qPCR assay. Therefore, miR-34a and miR-125b specific primers, as well as the internal control primers SNORD 48, were employed for the qPCR assay. Our raw data (not shown) demonstrated that the expression level of miR-34a and miR-125b in both cell lines was relatively low under non-stressed condition, with all Ct values higher than 25. However, the bar graphs below show that in both cell lines, miR-34a expression level increases in a time-dependent manner in response to NCS treatment; and the up-regulation of miR-34a expression is much more significant in cells that express WT p53 protein (e.g. WT MCF7) than in cells that do not (e.g. H1299). Combined with the results we showed in Figure 3.3A, the qPCR data indicates that the elevation of miR-34a expression coincides with p53 activation in WT
MCF7 cells in response to NCS-induced DSBs (Figure 3.2A and 3.3A). In contrast, due to lack of p53 expression, miR-34a level only increased by 20% in H1299 cells, which may arise from other p53-independent factors. Therefore, the qPCR data further consolidates the fact that there is a positive feedback loop between p53 and miR-34a.

Meanwhile, Figure 3.3A also illustrates that miR-125b expression was down-regulated in both cell lines in the presence of NCS, although the extent of reduction was not as drastic as the increase of miR-34a level. Interestingly, in both cell lines longer exposure to NCS mitigated the down-regulation of miR-125b expression compared to 1 hour NCS treatment; and this phenomenon was more obvious in p53 negative H1299 cells than in p53 positive WT MCF7 cells. So on one hand, this data suggests that DSBs-induced p53 activation in WT MCF7 cells is accompanied with the down-regulation of p53 repressor miR-125b. On the other hand, we are not able to properly explain why DSBs-responsive down-regulation of miR-125b expression is alleviated after 4-hour exposure to NCS when compared with 1 hour NCS treatment, despite higher level of p53 expression in cells exposed to NCS for 4 hours than in cells with only 1 hour NCS treatment (Figure 3.2A).
Figure 3.3: The Endogenous Expression Level of miR-34a and miR-125b in Response to NCS-induced DSBs and the Total Expression Level after miR-34a and miR-125b Transfection. A: The endogenous expression level of miR-34a and miR-125b was responsive to NCS-induced DSBs and was time-dependent, respectively. SNORD 48 was used as the reference gene (internal control) and its $2^{-\Delta\Delta C_t}$ values were set as 1; all fold-change of $2^{-\Delta\Delta C_t}$ values of miR-34a and miR-125b genes are normalised against the reference gene SNORD 48. B: The total expression level of miR-34a and miR-125b is vastly up-regulated after transient transfection. 20pmol miR-34a or miR-125b mimic was transfected into WT MCF7 cells grown in a 35mm dish, respectively; 24 hours later total RNA was isolated and RT-qPCR was performed to evaluate the transfection efficiency in terms of the total expression level of each miRNA mimic.
In addition, we transfected the mimics of miR-34a and miR-125b into WT MCF7 cells to probe the transfection efficiency. It can be seen from Figure 3.3B that the expression level of each miRNA gene increased more than 100 folds when 20pmol of corresponding miRNA mimic was transfected into WT MCF7 cells, respectively, indicating high transfection efficiency of both miRNA mimics.

### 3.5 miRNAs Fine-tune the Dynamics of p53 Signalling in Response to DNA Double-strand Breaks at Cell Population Level

After learning the basic dynamics of p53 signalling and miRNAs expression in response to DSBs at cell population level, next we wanted to know how miR-34a and miR-125b modulate p53 signalling in the presence of DSBs. As concluded in previous studies, miR-125b directly targets to the 3’-UTR of p53 mRNA and leads to down-regulation of p53 level (Le et al., 2009); whereas miR-34a promotes p53 trans-activity by disrupting the expression of the deacetylase SIRT1 (Yamakuchi et al., 2008). Therefore, in order to show that these results can be reproduced, WT MCF7 cells were transfected with miR-34a or miR-125b 24 hours ahead of the experiments. Meanwhile, two negative controls were set: the first one was that WT MCF7 cells were transfected with the same amount of scrambled mimics while in the second control group cells were not transfected at all. Moreover, these four groups of cells were either treated with 400ng/ml NCS (final concentration) for 1 hour prior to the experiment or not treated with NCS. Similar to Figure 3.2, selected key components of p53 signalling were investigated (Figure 3.4). The reason why we treated the cells for 1 hour was that based on the results of Figure 3.2, the level of phosphorylated CHK2 was maximal at 1 hour after NCS addition but then decayed quickly over the following 1 hour period. Here, at 1 hour following NCS treatment all components measured had been activated in response to NCS.

At first, we looked at how miR-34a and miR-125b modulated p53 activation in response to NCS treatment. As seen from Figure 3.4, compared to non-treated cells, phosphorylated p53, total p53 and phosphorylated γ-H2A.X were all significantly activated in response to 1 hour NCS treatment. Furthermore, when miR-34a mimics
were transfected into NCS-treated WT MCF7 cells 24 hours beforehand, both phosphorylated p53 and total p53 level was up-regulated in comparison with that in NCS-treated, scrambled mimics transfected or non-transfected cells. Figure 3.4 also illustrates that transfection of miR-125b mimics for 24 hours prior to NCS treatment of MCF7 cells leads to down-regulation of phosphorylated p53 and total p53 level compared to that in NCS-treated, scrambled mimics transfected or non-transfected cells. In addition, both miRNA mimics had no effect on γ-H2A.X activation. In non-treated groups, except the slight increase of total p53 level in the cells transfected with miR-34a mimics, the over-expression of both mimics did not take effect compared to two negative controls. Combined with previous qPCR results, this experiment suggests that the regulator roles of miR-34a and miR-125b on p53 expression are most obvious under conditions of the DNA damage response.

**Figure 3.4: miR-34a and miR-125b Modulate p53 Activation in Response to NCS-induced DSBs.**

400ng/ml NCS (final concentration) was added 1 hour prior to protein extraction; WT MCF7 cells were transfected with 20pmol of miR-34a, miR-125b and the negative control mimics 24 hours before the experiment, respectively; samples extracted from non-transfected cells were also prepared in parallel as the negative control. This experiment was repeated twice and this figure is a representative.

Next, we were interested in probing the effect of miR-34a and miR-125b on the expression of key p53 downstream targets. As mentioned above, here we chose p21 and PUMA as representatives due to their important roles in cell fate determination. From
Figure 3.5, we can see that under stressed condition, p21 and PUMA were both activated while in the cells growing in normal condition, p21 and PUMA levels remained very low. Although both miRNA mimics do not directly target either p21 or PUMA, their over-expression still can affect the expression of p21 and PUMA in response to NCS treatment as a result of their differential effects on p53 activity. Figure 3.5 shows that in comparison with non-transfected cells and cells transfected with scrambled mimics, miR-125b over-expression vastly inhibits the activation of p21 and PUMA in response to DSBs, whereas miR-34a transfection up-regulates p21 and PUMA level. Again, in non-stressed MCF7 cells both miR-125b and miR-34a over-expression did not influence the basal level of p21 and PUMA.

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**Figure 3.5:** miR-34a and miR-125b Modulate the Expression of p21 and PUMA in Response to NCS-induced DSBs. 400ng/ml NCS (final concentration) was added 1 hour prior to protein extraction; WT MCF7 cells were transfected with 20pmol of miR-34a, miR-125b and the negative control mimics 24 hours before the experiment, respectively; samples extracted from non-transfected cells were also prepared in parallel as the negative control. This experiment was repeated twice and this figure is a representative.

Finally, we wanted to see if miRNAs altered or fine-tuned p53 upstream components under stressed condition, which could provide insights into the direct effect of both miRNA mimics on the cellular stress response machinery. Total CHK2, phosphorylated CHK2 and γ-H2A.X were investigated. Similar to Figure 3.4, Figure 3.6 also demonstrates that as a direct responsive component at the early phase of DDR, phosphorylated γ-H2A.X was a very sensitive and reliable biomarker for activation of
DDR downstream of genotoxic stress signals. The transfection of both miRNA mimics had no impact on its activation. Regarding to phosphorylated CHK2, 1 hour NCS treatment did not activate it as significantly as what we had shown in 3.2, although it was higher than the basal level (Figure 3.6). However, the level of phosphorylated CHK2 was responsive to the over-expression of both miRNA mimics: miR-34a significantly increased the expression p-CHK2 (greater than two-fold) whereas miR-125b appeared to suppress the expression of p-CHK2. These observations are consistent with the changes in p53 behaviour in response to both miRNAs transfection. In contrast, the increased expression of these two miRNAs had no significant impact on the constitutive levels of total CHK2.

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![Figure 3.6: miR-34a and miR-125b Modulate the Expression of Upstream Components CHK2, Phosphorylated CHK2 and γ-H2A.X in p53 Signalling in Response to NCS-induced DSBs.](image)

400ng/ml NCS (final concentration) was added 1 hour prior to protein extraction; WT MCF7 cells were transfected with 20pmol of miR-34a, miR-125b and the negative control mimics 24 hours before the experiment, respectively; samples extracted from non-transfected cells were also prepared in parallel as the negative control. This experiment was repeated twice and this figure is a representative.

### 3.6 Discussion

In this chapter we did a series of assays at cell population level to validate the basic properties of p53 signalling in response to DSBs in different cell lines. We showed how the over-expression of miR-125b and miR-34a regulated p53 signalling in the presence
of DSBs. These population-based results depicted a general profile of how p53 signalling processed DNA damage and induced subsequent expression of p53 target genes that contribute to cell fate decisions.

Basically, as Figure 3.1 shows, the basal level of p53 expression is very low in the absence of DNA damage; when cells suffered NCS-induced DSBs, p53 was activated within an hour in p53 positive cells while in p53 negative cells, p53 activation did not occur. However, by detecting a sensitive and specific biomarker of DDR, γ-H2A.X, we were still able to know that DSBs were indeed present. Notably, 400ng/ml NCS (final concentration) we used for inducing DSBs, as well as WT MCF7 cell line selected as the cell model, were both sourced from Lahav’s previous studies (Lahav et al., 2004, Batchelor et al., 2008). According to our preliminary test (data not shown), 400ng/ml NCS (final concentration) caused moderate DNA damage, inducing approximately 30% of WT MCF7 cells to die in the following 24 hours; most cells were able to recover from the level of damaged caused. Hence, except cell apoptosis (represented by PUMA expression), we were able to look at other cell fates such as cell cycle arrest, DNA repair and cell senescence (represented by p21 expression) as well, compared to high DNA damage dose.

In addition, we can also see from this experiment that no p53 isoform were revealed on the blots with the reagents used; the p53 antibody that we employed is known to detect most of them. p53 isoforms originate from variable splicing during p53 gene expression and potentially have different properties and function from full-length p53. For example, some p53 isoforms have their special downstream targets; some of them are capable of processing stress signals via p53-independent pathways; some are not responsive to DNA damage or even inhibit the activity of full length p53 in some context (Khoury and Bourdon, 2010, Aoubala et al., 2011). Therefore, it is necessary to learn if any of these p53 isoforms are expressed in selected cell lines beforehand.

Figure 3.2 concisely demonstrated how p53 signalling worked in response to DSBs, including the behaviour of some very important upstream kinases and downstream targets subjected to p53 trans-activation. It was noteworthy that ATM (includes its active counterpart phosphorylated ATM (Ser1981)), which is one of major members in DSBs responsive machinery and also plays an essential role in p53 signalling initiation,
was supposed to be investigated at the beginning. However, due to its high molecular weight (around 350kDa), we failed to produce blots of total ATM and phosphorylated ATM (Ser1981) by Western Blot assays. Fortunately, because the phosphorylation of another important protein kinase CHK2 (Thr68), one of the direct downstream targets of activated ATM, is induced by ATM self-phosphorylation at Ser1981 in response to DSBs, we can look at the pattern of CHK2 and its active counterpart phosphorylated CHK2 (Thr68) in response to DSBs to represent the behaviour of ATM (Batchelor et al., 2008). Similarly, we investigated p21 to see how p53 downstream targets reacted to DSBs and whether activated p53 was able to effectively trans-activate its target genes.

Regarding to the result shown in Figure 3.2, there are two points that need to be mentioned. The first point is that compared to phosphorylated CHK2 and γ-H2A.X, the peak of p53 activation lagged for a couple of hours and p21 was activated approximately 1 hour later than p53. Similarly, phosphorylated CHK2 was the first component that returned to basal level, followed by (phosphorylated) p53 and p21. Combined with the structural model of p53 signalling (Figure 1.3 and 1.6 A), this data clearly indicates how the pathway of induction of the stress response and the dynamic changes in the activation pathway. Notably, the fact that phosphorylated γ-H2A.X expression did not decline substantially over 10 hours was consistent with the role of γ-H2A.X in DNA damage response and repair machineries because it is one of the key components in maintaining the proper DNA conformation at the damage sites (Huang et al., 2004, Kuo and Yang, 2008). Therefore, γ-H2A.X phosphorylation is always present as long as DNA damage factors remain in the vicinity of the DNA breaks.

The second point is that total p53, p21, phosphorylated CHK2 and p53 in WT MCF7 cells were all shown to be activated and then decline in a simple fashion – activated as one-peak – over the time course under study. Over longer periods, Lahav et al were able to show damped oscillations of phosphorylated ATM, CHK2, p53, total p53 and p21 using the same dose of NCS-induced DSBs presented at cell population level (Lahav et al., 2004, Batchelor et al., 2008, Batchelor et al., 2011). However, our result was not the only one that did not conform to the results shown in Lahav's studies, suggesting that many factors, such as the variability of cellular micro-environment and heterogeneity, could all contribute to contradiction between different studies (Al-Ejeh et al., 2010, Brady and Attardi, 2010).
Figure 3.3 shows how endogenous miR-34a and miR-125b reacts to DNA damage and that both miRNA mimics are good for transfection. Both miRNAs endogenous level was responsive to NCS treatment and time-dependent, although neither the basal level nor activated level was relatively high. In the p53 positive cell line WT MCF7, miR-34a level was up-regulated in response to NCS-induced DSBs because of the activation of its trans-activator p53. miR-125b expression was suppressed in MCF7 cells treated with NCS, which might be in synergy with p53 activation via a potential yet unknown positive feedback loop. Interestingly, according to Figure 3.2, although p53 level was higher at the 4th hour than at the first hour after NCS addition, the repression of miR-125b level alleviated at the 4th hour after NCS addition, compared to the level measured at the first hour following NCS addition. We speculated that this may correlate to the property of the unknown positive feedback loop we mentioned above, which achieved bilateral communication between miR-125b and p53. If this speculation is correct, the previous conclusion that miR-125b represses p53 expression but its level is not affected by stress signal would have been modified (Le et al., 2009).

We can also see from Figure 3.3A that in p53 negative cell line H1299, the time-dependent pattern of endogenous expression of miR-34a and miR-125b was similar to that in MCF7 cells; a couple of minor differences included the relative endogenous expression level of both miRNAs and the amplitude of variation in their expression level against time course. Why the behaviour of expression of both p53-related miRNAs in response to DSBs in p53 negative cell line H1299 was similar to that in p53 positive cell line WT MCF7? According to previous studies, miR-125b and miR-34a can respond to stress signals via p53-independent pathways apart from p53 signalling, such as p38-MAPK, PI3K/Akt and NF-κB signalling that either directly or indirectly triggered by ATM (Tan et al., 2012, Wu et al., 2013, Liu et al., 2014). Since both cell lines were subject to the same dose of NCS-induced DSBs, the damage signal detected and transmitted by ATM, it was reasonable to obtain similar pattern of endogenous expression of both miRNAs in response to DSBs in p53 positive cell line and in p53 negative cell line regardless of p53-dependent pathway or p53-independent pathways because they were almost all activated by DSBs-induced phosphorylated ATM.

Figure 3.3B indicates great transfection efficiency of both miRNA mimics. It is not surprisingly to see that miRNAs transfection efficiency is generally much higher than
plasmids or other vectors because their size is very small (18-30nt). Although this in vitro approach suggested that the MiRNA mimics are present at ~100-fold greater concentration than the endogenous levels, previous studies reported that this result was usual especially for miRNA with very low basal levels of endogenous expression (Li et al., 2014).

Figure 3.4, 3.5 and 3.6 demonstrate how miR-34a and miR-125b over-expression affects activated p53 signalling pathway. Figure 3.4 depicts that miR-34a over-expression can boost the level of both total p53 and phosphorylated p53 while miR-125b performed the opposite effect under stressed condition. And due to the low basal level of p53 expression, both of them did not take much effect.

Figure 3.5 shows that despite p21 and PUMA are not the direct targets of both miRNAs, their expression in response to DSBs is still affected by the over-expression of both miRNAs. Interestingly, at stressed condition both p21 and PUMA expression were significantly impaired by miR-125b transfection, whereas their levels increased slightly during miR-34a over-expression. We speculated that the modulation of p53 activation by miR-34a and miR-125b underlay the change of p21 and PUMA expression in response to both miRNAs over-expression at stressed condition (Yamakuchi et al., 2008). And given the fact that miR-125b directly targets to p53 mRNA while miR-34a interacts with p53 via a positive feedback loop, it was not surprisingly to see that the impact of miR-125b on p21 and PUMA expression was more significant than that of miR-34a.

Figure 3.6 looks at how p53 upstream component CHK2 (includes its active counterpart phosphorylated CHK2) expression was regulated by miR-34a and miR-125b at stressed condition. When it comes to phosphorylated CHK2 (Thr68), in 1 hour NCS treated WT MCF7 cells transfected with miR-34a or miR-125b mimics, our result shows very similar pattern to phosphorylated p53 (Ser15) and total p53 (Figure 3.4). Surprisingly, at the same condition, total CHK2 expression level in miR-34a or miR-125b transfected cells was both lower than that in non-transfected cells and in cells transfected with scramble miRNA mimics. According to previous studies and our own results (Figure 3.2), total CHK2 levels are relatively stable in MCF7 cells at both stressed and non-stressed conditions (Batchelor et al., 2011, Batchelor et al., 2008). Moreover, currently there is
no evidence to indicate the direct interaction between p53 upstream components (like ATM, ATR and CHK1/2) and miR-34a or miR-125b. Some studies only revealed that both miRNAs can target to p53 downstream components, such as Bcl-W (miR-125b), Mcl-2 (miR-125b) and SIRT1 (miR-34a), to regulate cell apoptosis (Gong et al., 2013, Yamakuchi et al., 2008).
In chapter 3, we have grasped the basic dynamical profile of p53 signalling and how miR-125b and miR-34a modulate the behaviour of p53 signalling within cell populations. For this chapter, we began to conduct a series of single-cell assays to look at p53 dynamics in individual cells and the impact of miRNAs over-expression on p53 dynamics. As Galit Lahav’s group has put a lot of effort into understanding this topic, we decided to start our own research by using their validated cell model, which not only get easier access to this cutting-edge field, but also test the reproducibility of their result and the reliability of this cell model. Moreover, we hoped to find something new in this field by doing so. So in this chapter, we will demonstrate the use of the Lahav’s p53 reporter cell line and undertake a detailed analysis of the regulatory principles involving micro RNAs.

4.1 Introduction

Discovered in 1979, p53 has been widely studied. As a result, more and more facts about p53 function and regulation have been revealed. Notably, key conclusions have been obtained from population-based experiments such as Western Blotting, PCR, co-IP and ChIP. However, a paper published on Nature Genetics in 2004 by Galit Lahav and her colleagues, demonstrated that when DSBs was induced by γ-irradiation, the behaviour of p53 and its main regulator MDM2 show temporal variability when individual cells were compared (Lahav et al., 2004). Although at that moment there were studies that had computationally simulated and predicted the dynamics of p53-MDM2 negative feedback loop in single cells at various conditions, this was the first
paper that revealed the heterogeneity of p53-induced DDR by practical experiments, which further suggested the vast complexity of the p53 signalling network.

To some extent, this study partially reproduced some properties of p53-MDM2 dynamics in individual cells, which was present by previous computational simulation. For example, using fluorescent-tagged vectors and time-lapse microscopy, the authors indicated that in response to DSBs in DNA, p53 dynamics in every single cell depicted continuous and periodic oscillations, which was completely different from the pattern shown at cell population level under the same condition (Lahav et al., 2004). However, Lahav and her colleagues published more in-depth data to further develop their theories, most of which were revealed and demonstrated for the first time. The key conclusions they have drawn include: 1) The mean amplitude and period of p53-MDM2 oscillations are fixed and dose-independent; 2) The number of pulses in p53-MDM2 oscillations and the fraction of oscillatory cells increase as damage level elevates; 3) p53-MDM2 dynamics inherit oscillatory behaviour from an important p53 upstream component ATM, which is activated in a recurrent fashion in response to DNA damage; 4) The oscillations of phosphorylated ATM/CHK2, together with other potential feedback loops in p53 signalling, are required for maintaining p53-MDM2 oscillations; 5) DSBs and SSBs lead to different patterns of p53-MDM2 dynamics because they trigger different branches of p53 signalling, such as ATM- or ATR-dependent pathways; 6) Different patterns of p53-MDM2 dynamics result in different cell fate by inducing different sets of downstream target gene expression, such as p21 and PUMA; 7) Under normal growth condition, p53-MDM2 oscillations are still present in a minority of cells, presumably as a result of transient intrinsic DNA damage that occurs during cell growth (Batchelor et al., 2009, Purvis et al., 2012).

Interestingly, from these studies it can be seen that even if p53-MDM2 oscillations had a fixed range of period and mean amplitude, individual cells in the same population were not able to maintain their synchrony with others over significant periods of time, although they were reasonably well synchronised at the beginning of the experiments by the induction of DNA damage signals. This finding further leads us to focus on an even bigger topic, which is, cellular heterogeneity. In their studies, Lahav and her colleagues indeed suggested that the source of asynchrony across the same population of cells came from cellular heterogeneity (Loewer and Lahav, 2011).
Currently, more and more research has revealed that cellular heterogeneity permanently exists and is present in every aspect of cellular behaviour (Altschuler and Wu, 2010). Even among isogenic cells in the same population, every cell is distinct from another in terms of the basic rate of metabolism, doubling time, the response to various stimuli, the degradation and production rate of proteins and so on (Altschuler and Wu, 2010). Moreover, a series of factors that contribute to cellular heterogeneity was demonstrated. The first one is the growing micro-environment, which includes the distribution of growth nutrients and stimuli, the number and frequency of cell-cell contacts, local cell density and so on. The second factor is the stochasticity of gene transcription bursting in different individual cells. Another origin is the potential distinction of multi-dimensional chromatin conformation, which is also one of the major sources of discrepancy of transcriptional bursting in different individual cells (Raj et al., 2006). The discovery of cellular heterogeneity is very important as many biological outcomes can be explained more precisely and reasonably by this aspect of fundamental biology. For example, why a specific stimulus can lead to different cellular behaviour and biological outcomes between isogenic cells could properly attribute to the activation of different branches of p53 signalling and different sets of p53 downstream target genes (Batchelor et al., 2011, Purvis et al., 2012).

The reason why research on cellular heterogeneity has evolved recently is that our technical ability to examine large numbers of single cells has improved significantly over the past 10 years, as a result of advanced technologies and equipment. One of the most important techniques is fluorescent microscopy, which can be performed in two ways. The first division is using a fluorescent microscope to look at individual cells in the selected field at a specific moment, such as immunofluorescence and immunocytochemistry. Another division is time-lapse microscopy, which is able to record continuous behaviour of every single live cell in the selected field and produce a series of video clips at the end of recordings. Therefore, spatial and temporal order of gene expression, the dynamics of protein production, degradation, localisation, distribution and translocation at sub-cellular level, can be observed.

Moreover, time-lapse fluorescent microscopy makes more advanced fluorescent techniques feasible. For instance, detecting diffusion kinetics of protein molecules within a tiny sub-space (e.g. 1µm³) and a temporal window, ranging from seconds to
minutes, can be achieved by using fluorescence recovery after photobleaching (FRAP). The basic principal of FRAP is that the laser source of a fluorescent microscope system sheds a highly intensive and short light pulse to permanently bleach the fluorescent signal in the selected area; if the system detects the recovery of fluorescent signal, it means that fluorescence-tagged protein molecules outside of the selected area move into the bleached area; then the system measures the recovery rate of the fluorescent signal in the given range of time and area to acquire protein diffusion rate. Another similar advanced fluorescent microscope-based technique is fluorescence correlation spectroscopy (FCS), which aims to produce a series of physical parameters of fluctuations of fluorescence emitted by fluorescent particles or molecules, such as diffusion coefficients, average concentration and kinetics of chemical reactions, etc.

There are new non-microscope-based approaches for single-cell study as well. For instance, fluorescence-activated cell sorting (FACS), which is based on flow cytometry platform, is a very popular method for fast inspecting and screening cells one by one. Basically, fluorescence-labelled live cells (achieved by being transfected with fluorescence-tagged vectors or incubated with fluorescence-conjugated antibodies) are pipetted in the growth medium to form single-cell suspension and transferred to the flask connected with the system. The system then transforms the suspension into a high speed liquid beam and injects it to the detection area, where a laser source, a detector and a charger are mounted next to the bottom of the injector. As the suspension of individual cells passes into the laser beam, the fluorescent signal is detected and analysed; then the cell is charged accordingly, which is dependent on the fluorescence or other optical characteristics. Due to the existence of an electric field across the detection area, differentially charged or neutral cells are deflected and collected. So by repeating this process, a heterogeneous population of cells is analysed and sorted accordingly to specific criteria of properties. Other approaches, such as micro-bead and micro-fluidics technology, are also primarily based on this sorting philosophy. Although these techniques are widely used nowadays, they share a common limitation compared to time-lapse microscopy, which is that they are all end-point methods and thus merely able to investigate the single-cell state at a particular moment. Therefore, since our study aimed to look at p53 dynamics and how miRNAs modulate its behaviour and the
associated cell fate in response to DSBs at single-cell level, we had to use time-lapse imaging to meet our demands.

### 4.2 Objectives

In this project, the following experiments are considered as the first step of our detailed investigation of p53-MDM2 dynamics in response to DSBs in individual cells because they aim to verify the reliability and reproducibility of the cell model that Galit Lahav and her colleagues have used and published. We are also going to use Western Blotting assay to present DSB-induced p53-MDM2 dynamics at cell population level so as to make comparison with single cell studies. Moreover, we will use immunofluorescence and advanced statistical analysis on p53-MDM2 dynamics at the single-cell level to better show how heterogeneously every single [isogenic] cell in the same population responds to the same level of DNA damage.

### 4.3 The Cell Model

The cell line used in the following experiments was MCF7 that had been stably co-transfected with two plasmids: p53-CFP and MDM2-YFP (Figure 4.1), both of which were generous gifts from Galit Lahav. p53-CFP plasmid expresses a CFP-tagged p53 protein from a p53 mini-gene that spans 1.2kb compared to full-length p53 gene that is over 20kb. Most importantly, the two native p53 promoters were replaced by a Zn$^{2+}$ responsive metallothionein (MT) promoter, which means that this mini p53 is only driven by Zn$^{2+}$ but cannot be activated by stress signals, including DNA damage. The sequence of MDM2 gene in MDM2-YFP plasmid is also heavily truncated as the full size of the MDM2 gene is over 40kb. Expression of the DMD2-YFP protein is driven by the native MDM2 promoters (two promoters with two p53 REs included). Notably, although only 1.2kb of full-length p53 gene content is retained in this plasmid, its product is capable of recognising and binding to p53 REs, including two REs in MDM2 native promoter. Hence, an artificially manipulated p53-MDM2 negative feedback loop is stably present in single WT MCF7 cells and can be investigated by fluorescence-based time-lapse imaging microscope once Zn$^{2+}$ is added.
It is critical to understand that this ectopic reporter system will mimic the behaviour of the natural system one expression of the fluorescent proteins is induced. However, the system has additional versatility because the fluorescent p53 is switchable, using Zn addition, and can be activated either with or without induction of DNA damage. The fluorescent MDM2 protein expression, in contrast, is induced by the normal DNA damage response. Hence, if required, MDM2-YFP provides a reporter system in which MDM2-YFP dynamics in response to DSBs can be used to infer or represent the single-cell behaviour of the p53 system (Lahav et al., 2004). Notably, previous research has revealed that both p53 and MDM2 oscillations had similar periodic behaviour but were anti-phase, which is consistent with the fact that p53 and MDM2 form a direct negative feedback loop. However, this system has some limitations for the analysis of miRNA-based regulation on p53-MDM2 dynamics as the p53 mini-gene lack the 3’UTR on mRNA that is a major target for regulation of p53 regulation. In spite of this, the use of MDM2-YFP dynamics in response to induction of DDR is still informative and this cell model provides a useful tool to look at basic p53-MDM2 dynamics under normal or stressed conditions.

Figure 4.1: The Basic Structure of p53-CFP and MDM2-YFP Plasmids (Lahav et al., 2004). pMT-1 stands for Zn$^{2+}$-induced metallothionein promoter; pMDM2 represents native MDM2 promoter.

4.4 The Level of DNA Damage Varies in Individual Cells after NCS Treatment

Before the investigation of p53-MDM2 dynamics in response to DSBs at the single-cell level and analysis of its potential heterogeneity, it is necessary to understand the range of possible responses of NCS-induced DSBs within single cell. If the level of DNA damage
varies between individual cells, it would be of great help for us to further investigate the heterogeneity of p53-MDM2 dynamics. We set out to use immunofluorescence assays to look at the level of DNA damage by detecting the level of phosphorylated γ-H2A.X as this chromatin marker provides a very reliable reporter of DDR induction.

So specifically, every well of two 6-well plates was seeded with $1 \times 10^4$ Lahav's modified MCF7 cells (described in 4.3) and all cells were grown for 48 hours. In plate 1, cells were treated with 200, 300, 500, 800, 1000 and 1200ng/ml (final concentration) of NCS for 2 hours, respectively; in plate 2, cells were treated with 400ng/ml (final concentration) of NCS for 0.5, 1, 2, 4 and 6 hour(s) prior to the experiments, respectively. Non-treated cells were grown in plate 2 as a negative control. All cells were then fixed and permeabilised to make antibodies much more accessible to cellular antigens. Cells were then incubated with γ-H2A.X antibody, followed by RFP-conjugated secondary antibody and then DAPI. Eventually, cells on coverslips were observed and imaged under a fluorescent microscope.

As Figure 4.2 A and B show, with the increase of NCS concentration, both the number of cells with the expression of phosphorylated γ-H2A.X (pink) and the average level of DNA damage increased. Figure 4.2 C was generated by quantifying the RFP level in each cell using image quantification software CellTracker® and revealed more detail about the expression level of phosphorylated γ-H2A.X in individual cells. The values in this figure represent the relative fluorescent intensity of phosphorylated γ-H2A.X antibody in every single NCS-treated cell against the average fluorescent intensity of non-treated cells. From Figure 4.2 A and C two facts can be seen: 1) As NCS concentration increases more and more individual cells showed relatively higher level of phosphorylated γ-H2A.X expression (≥41); notably, the distribution of the positive (pink) cells was completely random in each field, ruling out the possibility of uneven NCS diffusion. 2) The variation in DNA damage level in single cells was high and potentially NCS dose-dependent, as it is more significant in cells treated with 800, 1000 and 1200ng/ml NCS than in cells treated with 200, 300 and 500ng/ml NCS (Figure 4.2).
A

From left to right: The expression level of phosphorylated γ-H2A.X in individual Lahav's modified MCF7 cells treated with 200, 500 and 1200ng/ml NCS (final concentration) for 2 hours, respectively. Merged with DAPI. Pink represents phosphorylated γ-H2A.X while blue represents DAPI.

B
Figure 4.2: The Alteration and Heterogeneity of Phosphorylated γ-H2A.X Expression in Response to NCS-induced DSBs in Individual Cells Treated with Different Concentration of NCS for 2 Hours, Respectively. A. The images (merged with DAPI, blue) obtained from immunofluorescence assays showed that the expression level of phosphorylated γ-H2AX (pink) varied in individual cells treated with 200, 500 and 1200ng/ml (final concentration) NCS for 2 hours, respectively. The images of cells treated with 300, 800 and 1000ng/ml (final concentration) NCS for 2 hours were not shown. B. The mean relative fluorescent intensity of phosphorylated γ-H2AX of each set of individual cells treated with 200, 300, 500, 800, 1000 and 1200ng/ml NCS for 2 hours, respectively. C. The distribution in terms of relative fluorescent intensity of phosphorylated γ-H2AX in individual cells treated with 200, 300, 500, 800, 1000 and 1200ng/ml NCS for 2 hours, respectively. All values of fluorescent intensity shown in this figure were relative and had been normalised against the negative control (non-treated, normal growing cells seeded in plate 2).

Similar to Figure 4.2, Figure 4.3 demonstrates the change of fluorescent intensity of phosphorylated γ-H2A.X antibody both at single-cell level (A and C) and cell population level (B); the difference is that this time all Lahav’s modified MCF7 cells were treated with 400ng/ml NCS (final concentration) but for different periods of time. As A and B indicate, the level of phosphorylated γ-H2A.X expression appeared to peak at 1 post NCS addition, followed by gradual decline. Single-cell result shown in Figure 4.3 C supports this conclusion because the highest percentage of cells with relatively high level of phosphorylated γ-H2A.X expression (≥41) was shown at 1 hour following NCS treatment. The variation in DNA damage levels in individual cells was seen again in
Figure 4.3 C but appeared to be less significant than that shown in Figure 4.2 C. Figure 4.3 C also seems to suggest that this variation has correlation with the length of NCS treatment period to some extent, as it looked more significant around the first hour after NCS addition but then gradually reduced.

**From left to right:** The expression level of phosphorylated γ-H2A.X in individual Lahav’s modified MCF7 cells treated with 200, 500 and 1200 ng/ml NCS (final concentration) for 2 hours, respectively. Merged with DAPI. Pink represents phosphorylated γ-H2A.X while blue represents DAPI.
Figure 4.3: The Alteration and Heterogeneity of Phosphorylated γ-H2A.X Expression in Response to NCS-induced DSBs in Individual Cells Treated with 400ng/ml (Final Concentration) NCS for Different Length of Periods, Respectively. A. The images (merged with DAPI, blue) obtained from immunofluorescence assays showed that the expression level of phosphorylated γ-H2A.X (pink) varied in individual cells treated with 400ng/ml (final concentration) NCS for 1, 2 and 4 hour(s), respectively. The images of cells treated with 400ng/ml (final concentration) NCS for 0.5 and 6 hour(s) were not shown. B. The mean relative fluorescent intensity of phosphorylated γ-H2A.X of each set of individual cells treated with 400ng/ml (final concentration) NCS for 0.5, 1, 2, 4 and 6 hour(s), respectively. C. The distribution in terms of relative fluorescent intensity of phosphorylated γ-H2A.X in individual cells treated with 400ng/ml (final concentration) NCS for 0.5, 1, 2, 4 and 6 hour(s), respectively. All values of fluorescent intensity shown in this figure were relative and had been normalised against the negative control (non-treated, normal growing cells seeded in plate 2).

4.5 The Investigation of p53-MDM2 Dynamics in Response to DSBs at Single-cell Level Based on Lahav’s Cell Model

4.5.1 The Classification of p53-MDM2 Dynamics in Response to DSBs at Single-cell Level and the Definition of Dynamical Parameters

From the results of the immunofluorescence assays, we understood that the level of NCS-induced DSBs varied greatly in single cells, even though a clear dose response was seen, with more responding cells and higher signals seen at greater doses. This fact,
together with previous results published by Lahav’s lab, made us hypothesise that p53-MDM2 dynamics in response to NCS-induced DSBs at single-cell level would be also heterogeneous. To test this hypothesis, we employed time-lapse fluorescent microscopy to look at p53-MDM2 dynamics at the single-cell level.

1×10^5 Lahav’s modified MCF7 cells were seeded in a glass-bottom dish and grown for 72 hours before imaging. 400ng/ml (final concentration) NCS applied 4 hours after the beginning of imaging to allow the microscope to record the baseline of the cell model. As mentioned above, the promoter of p53-CFP was only driven by Zn^{2+}, so apart from NCS, 200µM (final concentration) ZnCl2 was also used for switching on p53-CFP expression as required, which was added just at the beginning of imaging. Notably, because p53-CFP expression from the mini-gene cannot be activated by DNA damage no signal was expected in the absence of Zn. However, in such cases we had to use MDM2-YFP signal only as the reporter of p53-MDM2 dynamics in single cells to keep the format of our results consistent. Fortunately, due to the stable and straightforward negative feedback loop formed between p53 and MDM2, previous studies had demonstrated that in this set of assays the structure of MDM2 oscillations were reliable mimics or reporters of p53 dynamics. The output of the imaging assays was a series of video clips recording the real-time p53 and MDM2 behaviour in single live cells in each selected field. These video clips were input into an image quantification programme called CellTracker® to transform imaging results into numerical data, which was then analysed and plotted by Excel®. Some representatives of final results are shown below (Figure 4.4):
As shown in Figure 4.4, we classified NCS-induced p53-MDM2 dynamics at the single-cell level into three types, which was based on the number of peaks and their relative positions in time-lapse traces. Each classification is explained below:

Type 1: This signalling type shows no peaks of expression in response to DSBs, which represents a background expression profile where DDR is not induced. Note that classical peaks of expression are generally regular and fall on top of a stable baseline (see Type 3 pattern in Figure 4.4) whereas baseline modulations that show transient changes that are rapid, extended on shallow do not reflect true peaks of expression. Hence, minor changes in MDM2-YFP fluorescence that can be seen in some cells merely represents background fluctuations (Figure 4.4 Type 1 pattern).
Type 2: This signalling type includes diverse transition states between non-responsive (Type 1) and oscillatory behaviour (Type 3). Peaks of expression can be identified but these are often erratically spaced and do not reflect a robust limit cycle of periodic peaks of expression that defines Type 3 classification. The heterogeneity of Type 2 patterns indicates that p53-MDM2 oscillations in response to DSBs are disrupted and cell fate may vary as a consequence.

Type 3: These signalling traces show classical oscillatory behaviour. According to previous studies and our own data, we defined the oscillations seen in Type 3 traces as follows:

1. At least four consecutive peaks of MDM2 expression emerge during imaging.

2. The period, which means the distance between the highest value points of two neighbouring peaks (Figure 4.5), ranges from 4 to 7 hours.

3. As shown in Figure 4.5, the robust oscillations commonly display a series of similar peaks of MDM2 expression, as defined by the variability of peak width and amplitude limited to ±25%. In addition, in a stable limit cycle the minimum expression seen in troughs is also comparable from peak to peak; in other words, the baseline is stable with no significant drift.

**Figure 4.5: The Definition of Parameters of p53-MDM2 Dynamics.**
The classification of p53-MDM2 dynamics is very important as it will be involved in all of the single-cell data analysis in this thesis. Furthermore, on the basis of this classification, we will develop the connection between the profile of p53-MDM2 dynamics and subsequent cell fate determination.

4.5.2 The Analysis of p53-MDM2 Dynamics at Single-cell Level under Various Conditions in Lahav’s Modified MCF7 Cells

As the start of the investigation of p53-MDM2 dynamics at single-cell level, we treated Lahav’s modified MCF7 cells with 400ng/ml (final concentration) NCS, which was added 4 hours after the beginning of time-lapse imaging. The behaviour of MDM2 expression in the single cells randomly selected was tracked and then input into Excel® for analysis. Traces of MDM2 expression in 87 Lahav’s modified MCF7 cells were produced in this way. According to the criteria of classification listed in 4.5.1, the number of cells in each division was determined (Figure 4.6).

![Figure 4.6: The Number of NCS-treated Lahav's Modified MCF7 Cells in Each Classification.](image)

It can be seen from Figure 4.6 that more than half of the cells showed intermediate state of dynamics (Type 2) in response to DSBs, while the percentage of non-responsive cells and oscillatory cells was comparable. Moreover, the dynamics of MDM2 expression in single cells, even they were in the same classification, were heterogeneous. For example, among 46 cells in Type 2 division, 24 of them showed only one peak during the
experiment; another 17 cells showed two peaks and the rest showed three peaks. The single peaks of MDM2 expression in 14 cells of 24 cells occurred within the first 10 hours since the beginning of the experiment. The number of peaks, oscillatory periods, peak width and amplitude varied among Type 3 cells as well. Given these facts, we set another 2 variables, oscillatory period and the timing of the first peak, as the indicators of heterogeneity of p53-MDM2 dynamics. The definition of oscillatory periods has been shown in Figure 4.5. Regarding to the timing of the first peak, it was defined as the moment when the first p53 or MDM2 peak of a cell in response to NCS-induced DSBs reaches its highest point.

There were three reasons to choose these features: 1. By looking at the variability of oscillatory periods, we were able to know how stable the oscillations were and if the imaging traces were comparable to those described in previous studies published by Lahav and her colleagues; 2. The timing of the first peak is critical because it represents how a cell reacts to DSBs and how sensitive a cell to DSBs and its heterogeneity has not been investigated yet; 3. Previous studies demonstrated that baseline, peak width and amplitude were dose-independent variables, suggesting that they were not ideal indicators to describe p53-MDM2 dynamics.

So going through MDM2 traces in all 87 cells, we found the timing of the first peak of every single cell and put it into a corresponding division, which was set as follows: 0-10 hour(s), 10-20 hours, 20-30 hours and 30-40 hours. The result was shown below:

Figure 4.7: The Number of NCS-treated Lahav's Modified MCF7 Cells in Each Division of the Timing of the First Peaks. 400ng/ml (final concentration) NCS was added 4 hours after the beginning of time-lapse imaging. This experiment was performed twice and 64 of 87 cells were analysed, with 23 cells having no peak expression in the MDM2 traces.
Figure 4.7 shows 50% of cells showing at least one peak of MDM2 expression were significantly responsive to DSBs within 6 hours after NCS addition (performed at the fourth hour after the beginning of the experiment); and over 70% of treated cells reacted to DSBs within 16 hours. Combined with the results listed in 4.4, these facts suggest that while cells in the same population responds to NCS-induced DSBs in a heterogeneous way, the majority of cells tend to response to DNA damage within ~10 hours of inducing DNA damage. Note that while MDM2 expression is used as a reporter of DDR and indirectly p53 signalling, there will be an offset of ~3 hours between peaks of p53 expression and peaks of MDM2 expression, as these protein oscillate out of phase during the DNA damage response.

We further looked at the variability of oscillatory periods in those 18 cells performing robust MDM2 oscillations. Every value of period of MDM2 oscillations in every cell was measured to determine peak to peak heterogeneity and a range of variation from 4 to 7 hours seen, according to the definition of oscillations listed in 4.5.1. As a result, together 62 values were collected. Specifically, of all 62 values, 44 values ranged from 4 hours to 5.5 hours, which was a little bit more than 70%; 18 values were between 5.5 hours and 7 hours. However, if we want to learn whether the period of p53-MDM2 oscillation is fixed or context-dependent, we still have to do similar investigation under different conditions.

Hence, we looked at these three factors (peak numbers, the timing of the first peak and periods) in the same cell line under an additional two different conditions. The first was following treatments of Lahav’s modified MCF7 cells using a combination of NCS and ZnCl$_2$. Under this condition, MDM2-YFP dynamics is not only defined by the endogenous p53, but also by Zn$^{2+}$-dependent expression of p53-CFP. Thus we can establish if the addition expression of p53-CFP contributes to any altered behaviour in MDM2 dynamics. In this experiment, 200µM (final concentration) ZnCl$_2$ was added at the beginning of imaging and 400ng/ml (final concentration) NCS was added 4 hours after the beginning of imaging as usual. Again, we first looked at the number of cells in each classification defined in 4.5.1. Figure 4.8 shows that the percentage of each division is very similar to Figure 4.6, implying that Zn$^{2+}$ addition does not affect the overall profile of MDM2 dynamics.
Figure 4.8: The Number of NCS- and Zn\(^{2+}\)-treated Lahav’s Modified MCF7 Cells in Each Classification. 200µM (final concentration) ZnCl\(_2\) was added at the beginning of the experiment; 400ng/ml (final concentration) NCS was added 4 hours after the beginning of time-lapse imaging. This experiment was conducted twice and 92 cells were tracked and analysed.

We then determined the timing of the first peak from 71 Type 2 and 3 cells (Figure 4.9).

Figure 4.9: The Number of NCS- and Zn\(^{2+}\)-treated Lahav’s Modified MCF7 Cells in Each Division of the Timing of the First Peaks. 200µM (final concentration) ZnCl\(_2\) was added at the beginning of the experiment; 400ng/ml (final concentration) NCS was added 4 hours after the beginning of time-lapse imaging. This experiment was conducted twice and totally 71 cells were analysed as the rest of cells showed no peak in MDM2 traces.

It can be seen from Figure 4.9 that half of cells showing at least one peak in MDM2-YFP trajectories reacted to Zn\(^{2+}\) and/or DSBs within 10 hours, which was equivalent to the patterns of expression seen in the cells treated with 400ng/ml NCS only. When expression of the ectopic p53 reporter was induced slightly more cells (almost 80%) were responsive DDR induced by NCS treatment. This shows that Zn\(^{2+}\) addition and subsequent induction of expression of the ectopic p53-CFP reporter has no measureable
effect on the behaviour of the p53-MDM2 system at the level of cells with sustained oscillations or timing of the first peak.

Was the presence of Zn\(^{2+}\) able to change the periods of MDM2-YFP oscillations? We collected all values of oscillatory period from 19 cells where MDM2-YFP oscillations were seen. 74 values of period were collected in total, indicating that MDM2-YFP oscillations with more than four peaks appeared in a slightly higher proportion of Zn\(^{2+}\)-treated cells, which express p53-CFP. Specifically, of all 74 figures, 51 figures (nearly 70\%) were between 4 and 5.5 hours; 23 values ranged from 5.5 to 7 hours. So the percentage of values of period was almost identical to that in cells treated with NCS only (51/74 versus 44/62); we also found no significant difference (P>0.05) between two series of values of period by one-way analysis of variance (ANOVA) (Table 4.1). This fact suggests that the addition of Zn\(^{2+}\) does not affect the behaviour of p53-MDM2 oscillations in Lahav’s modified MCF7 cells, implying that the system maintains oscillations under conditions where small changes in the overall expression of p53 and MDM2 result from ectopic reporter gene expression.

<table>
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<th>F crit</th>
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Table 4.1: The Values of Oscillatory Period Measured in Lahav’s Modified MCF7 Cells Treated with NCS or NCS/ZnCl\(_2\) Respectively Were Analysed by One-way ANOVA.

Under the second alternative condition, Lahav’s modified MCF7 cells were treated with 200\(\mu\)M ZnCl\(_2\) and 400ng/ml NCS (both are final concentration). The difference from the first condition was that 400ng/ml NCS was first added at the 4\(^{th}\) hour following the beginning of the experiment and further added every 4 hours thereafter; which meant that 400ng/ml was added at the 4\(^{th}\), 8\(^{th}\), 12\(^{th}\), 16\(^{th}\) and so on. This experiment was conducted as we wanted to know if continuous induction of DSBs would alter the
behaviour of p53-MDM2 oscillations in single cells. As usual, we first looked at the number of peaks in each cell (Figure 4.10).

![Figure 4.10: The Number of NCS- (Pulsatile Addition) and Zn²⁺-treated Lahav’s Modified MCF7 Cells in Each Classification. 200µM (final concentration) ZnCl₂ was added at the beginning of the experiment; 400ng/ml (final concentration) NCS was added every 4 hours following the beginning of time-lapse imaging (4th, 8th, 12th, 16th and so on). This experiment was conducted only once and 36 cells were tracked and analysed.](image)

As Figure 4.10 shows, the percentage of Type 1, 2 and 3 cells is still very similar to that in cells treated with NCS only and with NCS and ZnCl₂, suggesting that pulsatile addition of NCS does not promote a dramatic increase in p53-MDM2 oscillation. In addition, regarding to the timing of the first peak, Figure 4.11 shows that of all 28 cells that have at least one peak, 15 cells (53.6%) showed their first peaks in the first ten hours; only 4 first peaks (14.3%) did not occur in the first 20 hours; no cells showed the first peak later than 23 hours after the beginning of the experiment. Again, although the number of samples was much smaller under this condition, the percentage of each division is comparable to two cases demonstrated above.
Finally, we investigated if pulsatile addition of 400ng/ml NCS was able to change the overall period of p53-MDM2 oscillations. 26 values of period were collected from 7 cells where MDM2-YFP expression oscillated. 19 of 26 values of period ranged from 4 hours to 5.5 hours, also a bit more than 70%; 7 values were between 5.5 hours to 7 hours. These figures were very similar to those results shown earlier. To consolidate this conclusion, we compared this data to that obtained in the cells treated with ZnCl₂ and NCS (non-pulsatile addition) by one-way ANOVA:

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<th>MS</th>
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<th>P-value</th>
<th>F crit</th>
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Table 4.2: The Values of Oscillatory Period Measured in Lahav’s Modified MCF7 Cells Treated with NCS/ZnCl₂ or NCS (Pulsatile Addition)/ZnCl₂ Respectively Were Analysed by One-way ANOVA.

Again, Table 4.2 shows that P>0.05, which means that with 95% confidence, the periods in the single cells treated with NCS (pulsatile addition)/ZnCl₂ have no significant difference from that in the individual cells treated with NCS (added just 4 hours after the beginning of the experiment)/ZnCl₂. Combined with the fact that there was no
significant difference between the periods in the single cells treated with NCS and/or ZnCl$_2$ at 95% confidence interval, we can conclude that under such conditions the periods of p53-MDM2 oscillations in response to DSBs are consistent at the level of the frequency of oscillating cells across the cell population and timing of induction of the p53 system. My data shows that the properties of the system are robust and change little when levels of p53 and MDM2 expression are altered through expression from ectopic reporter genes.

4.6 The Behaviour of p53 Signalling in Response to DSBs at Population Level

Following the single-cell analysis, next we were going to compare the single-cell results with the population-based data. Therefore, Western blotting assays were conducted in parallel with time-lapse microscopy. At first, Lahav’s modified MCF7 cells were treated with 400ng/ml NCS and/or 200µM ZnCl$_2$ (both are final concentration) for 4 hours prior to protein extraction; a non-treated sample was also prepared as the negative control (Figure 4.12).

<table>
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<tr>
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<th>Zn</th>
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Figure 4.12: The Expression of p53-CFP and Endogenous p53 in Response to 400ng/ml NCS and/or 200µM ZnCl$_2$ Treatment for 4 Hours in Lahav’s Modified MCF7 Cells. Non-treated cells were also prepared as the negative control. Both are final concentration. This experiment was repeated twice and this figure is a representative.
As expected, p53-CFP expression was activated in response to 4-hour Zn\(^{2+}\) treatment but was absent when Lahav’s modified MCF7 cells were only treated with 400ng/ml NCS. In contrast, endogenous p53 was only responsive to NCS but the sensitivity was less than that of p53-CFP to Zn\(^{2+}\). Then cells were treated with 400ng/ml (final concentration) NCS for 1 to 9 hour(s) to demonstrate p53 dynamics in response to DSBs in a population of Lahav’s modified MCF7 cells (Figure 4.13), which was used for the direct comparison with the single-cell data shown in Figure 4.6 and Figure 4.7.

**Figure 4.13:** The Expression of p53-CFP and Endogenous p53 in Response to 400ng/ml NCS Treatment for 1-9 hour(s). Non-treated cells were also prepared as the negative control. Both are in final concentration. This experiment was repeated twice and this figure is a representative.

The result was very similar to Figure 3.2 A, which was conducted in WT MCF7 cells. The profile of p53 expression looked like a single-peak and the highest level of p53 expression was found in the cells treated with 400ng/ml for 4 hours. It also appeared that p53 expression increased very quickly after NCS addition but declined gradually. To our surprise, p53-CFP was also activated at a much lower level. This implies that the Zn-based induction system appears to be slightly leaky under these conditions, and while no p53 expression in seen in untreated cells some traces can be seen at the peak of expression following NCS treatment (peak at the 4\(^{th}\) hour following NCS addition). Importantly, this very low level of the ectopic gene expression was almost identical to endogenous p53 dynamics.
4.7 Discussion

Almost all of studies on p53-MDM2 dynamics at the single-cell level published by Lahav and her colleagues were conducted in the artificial cell model introduced in 4.3, as well as its improved versions. Despite this fact, we still had to verify its behaviour at single-cell and cell population level in order to define the properties of the system under our experimental conditions. So in this chapter we investigated p53-MDM2 dynamics in response to NCS-induced DSBs in Lahav's cell model by performing both single-cell and cell population based experiments.

We first conducted immunofluorescence assays to reveal that the sensitivity of every single cell in a population to the same dosage of NCS massively varied, although a dose-dependent pattern was indeed shown both at single-cell and cell population levels and was consistent to population-based data demonstrated in section 3.3. Therefore, this result supports the fact that cellular heterogeneity is indeed masked by population-based assays; but it does not necessarily mean that the results obtained from respective level cannot draw a common conclusion. This result also inspired us with great interest in looking at p53 dynamics as its heterogeneity, which has been reported in Lahav's papers, might be the source.

Then by time-lapse microscopy, we illustrated the profile of p53-MDM2 dynamics in individual Lahav's modified MCF7 cells, followed by defining key parameters of p53-MDM2 dynamics for a better description of its properties. From all of the single-cell results demonstrated in 4.5.2, it can be seen that NCS-induced p53-MDM2 dynamics at single-cell level was highly heterogeneous in terms of following parameters: the number of peaks, the timing of the first peak, peak amplitude, periods of oscillations, duration of p53-MDM2 dynamics and dynamical baseline, etc. Based on previous studies, three parameters, which were the number of peaks, the timing of the first peak and periods of oscillations, were chosen as markers to represent the pattern of p53-MDM2 dynamics because their behaviour was more predictable than other parameters (Lahav et al., 2004, Loewer and Lahav, 2011, Geva-Zatorsky et al., 2006). Some parameters, such as peak amplitude and peak width, were reported as very variable dynamical factors from peak to peak and their variability almost had nothing to do with changes of context (Lahav et al., 2004); instead, they were comprehensively determined
by a series of intracellular events that were very complicated, stochastic, variable and
difficult to be quantified, including chromatin conformation, epigenetic modifications,
transcription bursting and the equilibrium of protein synthesis and degradation
(Loewer and Lahav, 2011).

Due to the great heterogeneity of p53-MDM2 dynamics in response to NCS and/or ZnCl₂
at single-cell level, we had to define three classifications of dynamics based on three
chosen markers for our analysis. Then p53-MDM2 dynamics obtained in every single
cell was put into the corresponding division according to the specific criteria of each
classification. From our results, we concluded the following characteristics of p53-
MDM2 dynamics in response to NCS and/or ZnCl₂ in single Lahav’s modified MCF7 cells:

1. At three different conditions, the numbers of cells performing Type 2 dynamics, an
intermediate state between non-responsive and oscillatory dynamics, were all over
50%; the numbers of cells showing Type 1 and 3 were comparable. More importantly,
according to Figure 4.6, Figure 4.8 and Figure 4.10, the percentage of cells in each
classification were consistent, respectively, suggesting that the number of peaks in p53-
MDM2 dynamics is not susceptible to NCS and/or ZnCl₂ treatment. Interestingly, this
conclusion was contrary to the conclusions of some previous studies; for example, it has
been reported that the number of peaks in p53-MDM2 dynamics in response to γ-
irradiation at single-cell level was dose-dependent (Lahav et al., 2004, Batchelor et al.,
2011). We speculated that the difference may result from a series of factors. The first
one is cell treatment. Although NCS is considered as a radiomimetic drug that leads to
DSBs as well, two methods are physically different in terms of operation, damage source
and dosage, etc (Shiloh et al., 1983, Lahav et al., 2004). The second one is microscope
system. Different instruments and analytical approaches might result in slightly
different interpretations of the data. The third one is sample size. We investigated over
200 cells at three different conditions, two times more than what Lahav and her
colleagues observed (Lahav et al., 2004). Regarding our own results, we suppose that
p53-MDM2 dynamics is subject to robust regulation by p53 upstream and downstream
components, such as ATM and WIP1 (Batchelor et al., 2008). Moreover, multi-negative
feedback loop architecture is a relatively stable biological system because it can achieve
sophisticated auto-regulation (Toettcher et al., 2010). However, we cannot totally
disagree with the previous conclusion because we did not actually observe p53-MDM2
dynamics in individual cells treated with higher NCS final concentration than 400ng/ml; instead, we just introduced a new source that can activate this dynamics (ZnCl$_2$) and increased the frequency of stimulation (pulsatile NCS addition), which might not be equivalent to higher NCS concentration.

And the results of immunofluorescence listed in 4.4 also show that DDR is dose-dependent. In fact, the dose-dependent manner is probably a more adaptive response to stress signal than fixed behaviour as it allows sophisticated native regulation that can improve the efficiency and coordination of cellular machineries. However, context-dependent systems generally lack autonomy, implying that the network may not remain stable under all conditions. So if the number of peaks in p53-MDM2 dynamics in individual cells is indeed proportional to the number of DSBs, it is highly likely that the fraction of cells in which MDM2-YFP oscillates will increase. However, since the number of peaks in p53-MDM2 dynamics is of great cell-to-cell variability, it is almost impossible to see the increase of the number of peaks in every single cell, which is also supported by the result demonstrated in Figure 4.2.

2. At three different conditions, the numbers of the values of oscillatory period ranged from 4 to 5.5 hours were all over 70%, whereas the rest of values were between 5.5 and 7 hours. Furthermore, we found no significant difference of the values of oscillatory period among three groups (see Table 4.1 and 4.2). Therefore, we are able to conclude that at 95% confidence interval, the periods of p53-MDM2 oscillations are relatively fixed under our conditions, which is consistent with previous reports (Lahav et al., 2004, Batchelor et al., 2011). In theory, the oscillatory period relates biologically to the interval between each p53-MDM2 activation event, which is largely equivalent to the gap of recurrent initiation of ATM for the detection of DNA integrity (Batchelor et al., 2008). Although the factors that actually shape this ATM dynamics are still under investigation, we believe that it is determined mainly by native elements because maintaining DNA integrity is so essential to cell survival that cells must guarantee the robustness of the regulatory network that controls DDR.

3. At three different conditions, the numbers of cells performing their first peaks within the first ten hours following the beginning of imaging (the first six hours in response to NCS addition) were over 50%; all values of percentage increased to 80% when the
range was extended another 10 hours (see Figure 4.7, 4.9 and 4.11). Given the fact that
this dataset is also relatively fixed under such conditions, we indicate that most cells are
primed to respond to stress as soon as possible following DNA damage. However, it
does not necessarily mean that the cells responding to NCS later than 10 or 20 hours
since the beginning of imaging die because every single cell is unique and its
susceptibility to DSBs is determined by many factors; so it is highly possible that the
potential negative effect that results from later response to NCS addition can be
compensated by other factors, preventing cells from suffering negative fates.

In 4.6, we demonstrated the Western Blotting results in Lahav's modified MCF7 cells
treated with NCS and/or ZnCl₂, which aimed to make a comparison with single-cell data
in parallel. These population-based results (see Figure 4.12 and 4.13) reveal ZnCl₂–
induced p53-CFP activation and reproduced the immunoblots data conducted in WT
MCF7 cells (see Figure 3.2). Nevertheless, due to the limited time range of NCS
treatment, we do not know whether there is any difference between population-based
results and single-cell results because both of them show at least a peak within 10 hours
following the beginning of imaging but p53 dynamics after 9 hours is unknown at cell
population level. Similar immunoblot assays were performed in previous studies, where
p53 dynamics after 10 hours were not shown as well. So we even cannot predict the
trend of p53 dynamics after 10 hours. However, in a couple of studies published by
Lahav and her colleagues, two damped p53 peaks were found within 10 hours following
the beginning of imaging, which was not reproduced by us (Lahav et al., 2004, Batchelor
et al., 2008, Batchelor et al., 2011). This fact has been discussed in 3.6. Another key
point that should be mentioned is the minor activation of p53-CFP in response to NCS,
which is not supposed to happen in that p53-CFP is driven by a Zn²⁺-responsive pMT
promoter instead of native promoters. A reasonable explanation is that due to a slightly
leaky promoter, the ectopic p53-CFP normally has a low level of synthesis, which is not
seen in the absence of DNA damage because of MDM2-mediated turnover; but this minor
expression becomes detectable when MDM2 is inhibited following DNA damage when
the turnover of the fluorescent p53 transgene is substantially reduced.

In conclusion, although we did not fully reproduce p53-MDM2 dynamics at single-cell
level based on the same cell model and techniques, we produced new findings and
conclusions. According to these results, we believe Lahav's cell model is still competent.
to carry out the investigation of p53-MDM2 dynamics at single-cell level because it is easy to manipulate, highly responsive and reliable. However, due to the lack of 3’-UTR in p53-CFP and MDM2-YFP plasmids, this cell model is not ideal for studying how miRNAs regulate p53-MDM2 dynamics in response to stresses, which is what we are going to show in the next chapter. Therefore, it will be necessary to modify this model system before continuing our project.
Chapter 5  The Retrofitting of the BAC Vector and the Analysis of NCS-induced p53-MDM2 Dynamics in Single Cells Transfected with BAC and/or miRNAs

5.1 Objectives

At the end of chapter 4, we mentioned that modification of Lahav's cell model was required for looking at how miRNAs regulate p53-MDM2 dynamics in response to DSBs at the single-cell level because the two plasmid vectors expressed in Lahav's cell model do not express the miRNA targets. This fact suggests the great importance of selecting an appropriate transgenic system on the basis of the objective of the study. As mentioned in 1.6, the ultimate goal of our project was to see if p53-related miRNAs can regulate NCS-induced p53-MDM2 dynamics in single cells and if changes could influence cell fate determination. Although DNA damage-induced p53-MDM2 dynamics has been well-studied both by computational and experimental approaches, and even the link between the profile of this dynamics and cell fates was partially revealed in Lahav's cell model (Purvis et al., 2012), there is very limited data on the role of miRNAs in this process. Furthermore, we are going to look at mechanisms of cell fate determination from the perspective of individual cells, which is a novel feature of our study. To testify our thoughts and hypothesis, we designed and conducted the following experiments, which include developing a versatile BAC-based vector, introducing the BAC vector into original Lahav's cell model and analysis of the signalling parameters using time-lapse microscopy.
5.2 Developing p53-DsRedXP-Lamin B1-eCFP BAC Vector

5.2.1 Why the BAC Construct Is Required

Generally speaking, due to capacity limitations, plasmid-based vectors are typically designed for a very specific purpose, which often involves removal of the vast majority of a gene that is not directly relevant to the purpose under study; such mini-genes with often exclude non-coding elements and remote regulatory sequences. Take Lahav’s cell model as an example. As previously described, Lahav’s cell model is plasmid-based, which is stably transfected with p53-CFP plasmid and MDM2-YFP plasmid (Figure 4.1). This p53-CFP plasmid merely retains the content of p53 gene responsible for trans-activating MDM2 expression and binding with MDM2; MDM2-YFP plasmid only has the components that express the domains to interact with p53 and inhibit its level subsequently. That is why these two plasmids are so short and simple, which makes Lahav’s cell model an ideal tool to observe the dynamical interaction between p53 and MDM2 in response to DSBs in single cells.

However, it is also because of this simplicity that the Lahav’s cell model is not suitable for more complicated studies. For instance, in our case it is not appropriate for performing miRNA-related studies as both mRNAs transcribed from the two plasmids do not express 3’-UTRs that contain the natural miRNAs target sequences. For our study, this is the most critical defect of Lahav’s cell model because it means that any miRNAs effects cannot be directly related to the change of fluorescent intensity that is further proportional to the expression level of p53 and MDM2. This will rule out the use of this model to test how miRNAs affect DSBs-induced p53-MDM2 dynamics in single cells. Another disadvantage of Lahav’s cell model is that p53-CFP plasmid is driven by the promoter of metallothionein instead of native p53 promoter (both P1 and P2). This promoter is only activated by some kinds of metallic ions such as Zn$^{2+}$ and Cu$^{2+}$ but not responsive to DNA damage. Hence, in the presence of NCS (but absence of metal ions added endogenously), p53-CFP will not be activated and thus not seen by fluorescent microscope; this is the reason why only MDM2-YFP traces were shown in chapter 4. Moreover, apart from the absence of 3’-UTR, p53-CFP and MDM2-YFP plasmids lack additional regulatory components that contribute to the genomic context of p53 and MDM2 gene, such as most parts of coding sequence and almost all non-coding sequence...
including introns, locus control region and enhancers. Thus natural p53-MDM2 expression and dynamics cannot be observed in Lahav’s modified MCF7 cells.

Therefore, to reproduce native p53-MDM2 dynamics and understand how miRNAs regulate p53-MDM2 dynamics behave in response to DSBs at single-cell level, it is necessary to develop a more suitable transgenic tool. To meet our needs, the vector must have large capacity of DNA content as full length of either p53 gene or MDM2 gene is over 20kb. In addition, a fluorescent protein’s gene that co-expresses with p53 gene, antibiotic-resistant markers and constitutive elements of vectors are required, which further increases the need for capacity. Therefore, most of vectors commonly used are not able to meet our requirements. Fortunately, our the M. White lab had previously generated a basic BAC construct, which contains a p53-DsRedXP cassette expressing a fusion protein consisting of a complete WT p53 protein and an advanced version of RFP called DsRedXP (Figure 5.1).

BACs provide a high-capacity circular double-strand DNA vector that can accommodate inserts over 100kb. These circular DNA elements can also be transformed and replicated in various species of bacteria such as E.coli because it is incorporated with a self-replication motif in prokaryotic organisms ("Vector" in Figure 5.1). Although these properties perfectly meet our need, BAC do have a some shortcomings due to their large size, typically 200kbp of DNA, means that they are prone to be mechanically damage while pipetting, mixing and centrifugation. The second disadvantage is that their transfection efficiency is very low (generally lower than 5%). Another consideration is the low copy number with which BACs are generally delivered into host cells, with only 2-3 copies per transfected cell on average; though providing the level of expression allows visualisation of the fluorescent report this can be an advantage as the expression of the native gene will not be overwhelmed. All of these facts lead to time-consuming preparation of BAC samples and search for BAC transfected cells by microscopy. Despite these experimental challenges, we were still determined to use BAC not only because of their high cloning capability, but also great expertise our lab has in BAC practice and its use in microscopy; however, to make the experiment simpler and appropriate for comparison with our earlier studies, in this chapter we have used the p53 BAC vectors transfected into Lahav’s cell model. As the first step toward establishing this technique,
further modification of the core p53-BAC was required at first, which will be addressed in 5.2.2.

**Figure 5.1: The Map of p53-DsRedXP BAC.** Vector is the component required for basic function such as replication and damage repair. p53 is native p53 gene, including native p53 promoter and all other regulatory elements for gene expression.

### 5.2.2 The Design of p53-DsRedXP BAC Retrofitting

p53-DsRedXP BAC produces a fluorescent fusion protein consisting of native p53 and DsRedXP. The expression of this cassette is driven by native p53 promoter (both P1 and P2 included); hence the DsRedXP gene is co-expresses with the native p53 gene and thus its fluorescent intensity directly correlates with p53 expression level. So by measuring the fluorescent intensity of DsRedXP in real time using time-lapse microscopy, we can subsequently acquire real-time p53 levels in single cells in the
presence of NCS. Activated by native p53, MDM2-YFP dynamics can also be obtained as normal, which has been demonstrated in the last chapter. Therefore, both p53 and MDM2 dynamics in a BAC transfected Lahav’s modified MCF7 cells can be illustrated in the same single cells by time-lapse microscopy.

Although the performance of p53-DsRedXP BAC listed above meets our basic need for this study, we found another defect of this vector after performing preliminary imaging, which was that it was almost impossible to identify transfected cells. This is because of two facts. The first one is that p53-DsRedXP is controlled by native p53 promoter, which merely works under stressed conditions; but according to our experience, even when NCS was added, p53-DsRedXP signal below the detection limits of our microscopes for at last 2 hours after NCS addition. This phenomenon will be analysed later in this chapter in terms of the timing of the first peak. The second one is the extremely low transfection efficiency (below 3% in our case). So in order to overcome these serious technical limitations it was considered necessary to retrofit the p53-DsRedXP BAC by inserting a constitutively expressed fluorescent cassette to make those BAC-transfected cells visible under non-stress conditions.

Because this fluorescent component is used as a transfection marker, constitutive, stable and context-independent expression of the report protein is required. In addition, for better observation by time-lapse imaging, it is required that the reporter is clearly localised and highly expressed in at least 48 hours. So the requirements mentioned above lead to very demanding criteria for selecting a proper fluorescent cassette and the promoter that drives its expression.

Regarding to the selection of fluorescent proteins, the key parameters that should be taken into account include half-life, fluorescent intensity, stability at different conditions (e.g. pH, temperature and ion intensity) and excitation/emission wavelength. Generally speaking, fluorescent proteins with short half-life are suitable for monitoring quick biological events and short half-life proteins because they are less likely to accumulate in cells, producing more accurate readout; in contrast, long half-life species are required for looking at target proteins with long half-life or events that last for a long period of time. If two or more fluorescent proteins are used at the same time, it is important to make sure that their spectrum does not interfere with each other, unless fluorescence
resonance energy transfer (FRET), the technique that requires much larger overlapped area of spectrum of two fluorophores to allow the transfer of excited fluorescent energy between them, is exploited to identify if any interaction occurs among proteins of interest.

Fortunately, our lab had already generated and used a modified pL451 plasmid in which a human Lamin B1-eCFP cassette driven by a constitutively and moderately active promoter derived from CMV was inserted. Lamin B1 protein is a highly conserved and constitutive component of nuclear lamina, which is located next to the inner nuclear membrane (Lin and Worman, 1995). eCFP is an improved version of normal CFP, which has longer half-life and higher fluorescent intensity. In addition, its spectrum (excitation peak wavelength 434 nm; emission peak wavelength 477 nm) is separated from DsRedXP spectrum (excitation peak wavelength 555 nm; emission peak wavelength 584 nm) so the mutual interference between them is minimal. Hence, the expression of pCMV-Lamin B1-eCFP cassette in single live cells sketches the contour of nuclei thanks to its clear localisation and constitutively high intra-cellular level; this helps to observe morphological changes of nuclei in response to DNA damage-induced p53 activation during imaging. Above all, it can be concluded that pCMV-Lamin B1-eCFP cassette in pL451 plasmid is an ideal indicator to mark transfected cells and their nuclear compartment.

### 5.2.3 The Result of p53-DsRedXP BAC Retrofitting

After clarifying why a BAC vector is required for our study and what components should be built in the BAC required, we next need to identify how to extract the lamin B cassette from pL451 and insert it into the desired location in the p53-DsRedXP BAC. By investigation of both sequence of pL451 plasmid and p53-DsRedXP BAC, we found that the sequence flanked on both ends of pCMV-Lamin B1-eCFP cassette (the two red boxes in Figure 2.1) in pL451 plasmid is homologous with the sequence near the vector cassette in p53-DsRedXP BAC (Figure 5.1), which offered an opportunity to transfer pCMV-Lamin B1-eCFP to p53-DsRedXP BAC by homologous recombination. The inserted location of pCMV-Lamin B1-eCFP in the BAC is excellent because it is far from
p53-DsRedXP cassette, which means that the mutual interference in terms of gene expression between two cassettes is unlikely to occur.

Since the strategy had been determined, the next step was isolating pCMV-Lamin B1-eCFP cassette from pL451 plasmid by restriction endonuclease digestion. Pvu I and Pci I were chosen because their digestion fully retained pCMV-Lamin B1-eCFP cassette and its 5'/3' homologous ends. It also keeps the entire insert as short as possible (Figure 2.1), which makes the following homologous recombination easier. Another advantage is that the size of pCMV-Lamin B1-eCFP fragment, which is 6219bp according to the map of pL451 plasmid, is much larger than the other two fragments, thus simplifying isolation of the DNA fragment by gel electrophoresis (Figure 2.1).

Following Pvu I- and Pci I-catalysed restricted digestion described in 2.5.4, the digestion products were separated by gel electrophoresis. By comparing with the DNA ladder, intact pL451 plasmid and the products generated by either Pvu I- or Pci I-mediated restrictive digestion, we confirmed the desired pCMV-Lamin B1-eCFP band on the gel, which was then extracted from the gel and purified (data not shown). As described in 2.5.5, the purified pCMV-Lamin B1-eCFP fragment was incorporated into the designated site of p53-DsRedXP BAC by electroporation-induced homologous recombination in bacteria, based on the fact that both 5' and 3' ends of pCMV-Lamin B1-eCFP fragment are homologous with the sequence near the vector cassette of p53-DsRedXP BAC. Notably, the sequence in this region of p53-DsRedXP BAC contain a chloramphenicol-resistant gene, which was supposed to be disruptive by the homologous recombination; however, the retrofitted BAC acquired kanamycin- and neomycin-resistant gene as this was contained within the chosen pCMV-Lamin B1-eCFP fragment/cassette, which were carefully designed for screening. The promoter of kanamycin-resistant gene only works in prokaryotic cells so this gene can be used for identifying potential retrofitted BAC-transformed *E.coli*; in contrast, neomycin-resistance is seen when the gene is expressed in eukaryotes, which is required for G418 screening following BAC stable transfection.

According to the difference in antibiotic resistance between original and retrofitted BAC, the colonies that can grow on LB-agar plates with kanamycin but not chloramphenicol were considered as potential retrofitted BAC-transformed *E.coli*. However, we were still not able to guarantee that BACs in these bacteria were the exact ones we need, as it was
possible that only a part of pCMV-Lamin B1-eCFP fragment with the kanamycin-resistance gene was inserted. Therefore, we picked 9 Kan+/Chl-colonies and extracted BAC DNA from them, which were then used for further characterisation by means of endonuclease digestion. The principle of this method is to select restriction endonucleases that have at least one restricted site in the sequence between 5’ and 3’ homologous arms in both original and retrofitted BAC. Hence, the difference in size between original and retrofitted BAC can be seen from the result of gel electrophoresis in terms of the position and/or the number of bands generated. Moreover, the significance of difference matters because the resolution of gel electrophoresis is limited. Thus, based on the sequence of the original and retrofitted BAC, the more significant the altered size of the DNA fragments the higher the chance that the precise retrofitting will be confirmed. Mlu I and Sal I were selected according to this rule. Then purified BAC samples extracted from 9 colonies respectively were subject to Mlu I and Sal I digestion, respectively. Of these samples, only sample 3 and 9 were positive for the desired homologous recombination event. The structure of retrofitted BAC from these positive BAC clones were then compared with the parental p53DsRedXP BAC (P53), as shown in Figure 5.2.
Figure 5.2: The Characterisation of Retrofitted p53-DsRedXP BAC by Sal I- and Mlu I-induced Restriction Digestion. From left to right: DNA marker, blank, Mlu I-induced digestion products of the original p53-DsRedXP BAC sample, Mlu I-induced digestion products of the retrofitted p53-DsRedXP BAC sample 9, Mlu I-induced digestion products of the retrofitted p53-DsRedXP BAC sample 3, Sal I-induced digestion products of the original p53-DsRedXP BAC sample, Sal I-induced digestion products of the retrofitted p53-DsRedXP BAC sample 9 and Sal I-induced digestion products of the retrofitted p53-DsRedXP BAC sample 3.

The two red circles in Figure 5.2 mark the difference between original and retrofitted p53-DsRedXP BAC in size and also show that sample 3 and 9 appear to be identical. The sizes shown are consistent with these two BAC clones being the p53-DsRedXP BAC that has been retrofitted with pCMV-Lamin B1-eCFP cassette, thus generating p53-DsRedXP-Lamin B1-eCFP. Both of these clones were then used in transfection trials to monitor p53-DsRedXP and Lamin B1-eCFP expression in Lahav’s modified MCF7 cells.
5.3 p53-DsRedXP-Lamin B1-eCFP BAC Transfection into Lahav’s Modified MCF7 Cells

5.3.1 Stable Transfection

Following p53-DsRedXP BAC retrofitting, then next step was to introduce the new BAC into Lahav’s modified MCF7 cells and perform time-lapse microscopy. The ideal way of monitoring expression by time-lapse microscopy would be generate stable clones that express a known number of copies of the BAC DNA. So called stable transfection, in this scenario the exogenous gene is integrated into the genome and expressed as the endogenous genes. This fact also indicates another advantage of stable transfection, which is as cell clones are generated the copy number of the ectopic gene of interest should have the same of similar patterns of expression in all cell of the population. Therefore, performing p53-DsRedXP-Lamin B1-eCFP BAC stable transfection in Lahav’s modified MCF7 delivers p53-DsRedXP expression from its native genomic context (BAC), Mdm2-YFP expression from a normally regulated mini gene (host MCF7) and the Lamin B1-eCFP nuclear reporter expressed from the CMV mini gene (BAC). However, in order to obtain stable cells, screening-by-antibiotic must be required, which may take from weeks to months. And given extremely low efficiency of BAC transfection and homologous recombination, collecting sufficient number of transfected samples is a challenge.

In contrast, transient transfection does not have such shortcomings because exogenous genes are not incorporated with genome; as expected, the advantages of stable transfection become the disadvantages of transient transfection, which suggests that variable copy number might be an issue and that copy number might not be stable over long period of time. Based on those facts, we decided to do BAC stable transfection first because we believed that more consistent, stable and regulated gene expression would make results more comparable among single cells and biological repeats. This is very important for single-cell studies, which has more variables than population-based research. In addition, although stable transfection is very complicated and time-consuming, it does not require any other effort to transflect cells and identify transfected cells before imaging. Instead, transient transfection needs to be done every time before
time-lapse imaging and additional time has to be spent in searching for BAC transfected cells by microscopy, both of which further increase variability of single-cell data. If BAC stable transfection is not able to be achieved due to the difficulties listed above, then transient transfection will be conducted as an alternative.

The most significant difference in terms of experimental protocol between stable and transient transfection is that antibiotic selection is required for developing a stable cell line. As previously described, p53-DsRedXP-Lamin B1-eCFP BAC contains a neomycin-resistance gene constitutively expresses in eukaryotes, which indicates that stable cells can survive in the presence of geneticin and thus be isolated by long-term geneticin screening.

Prior to stable transfection, the proper concentration of geneticin for screening needed to be determined. Although previous data generated in MCF7 cells can be directly used, this step was considered to be essential because it is highly context-dependent. Theoretically, the proper concentration of geneticin for cell screening is the lowest that leads to death of all cells in a single well in 240-336 hours following the addition of geneticin. In our project, due to the low tolerance of geneticin of Lahav’s modified MCF7 cells, we found that only 0.6 mg/ml (final concentration) geneticin could kill all of 5000 cells in a well of a 24-well plate in 240 hours. As mentioned in 2.6.2.1, the initial concentration for cell screening should be a bit higher than the lethal concentration so in fact 0.8 mg/ml (final concentration) geneticin was used.

The generation of stable BAC cell lines started with BAC transient transfection using ExGen500 or PEI40 and Lahav’s MCF7 cells. Antibiotic screening was then performed 48 hours after the BAC transfection by adding geneticin into growth medium to the final concentration of 0.8 mg/ml. Then every 48 hours growth medium was refreshed and fresh geneticin was added (0.8 mg/ml, final concentration) to maintain antibiotic stress for screening. We kept repeating this process for 576 hours and 9 single colonies were obtained. Screening was stopped at the 576th hour because the latest colony to develop had been obvious for more than 100 hours and no further colonies had appeared since. These 9 colonies were treated as cells clones in which p53-DsRedXP and Lamin B1-eCFP should be co-expressed. However, when these was checked using fluorescent microscopy the imaging result indicated that no eCFP signal was shown in any single
colony at normal condition in a fluorescent microscope. Furthermore, while single colonies were treated with 400ng/ml NCS, no p53-DsRedXP signal was detected during 24-hour time-lapse imaging. Images shown in Figure 5.3 were representatives of time-lapse imaging results of 5 out of 9 single colonies. It can be seen that only auto-fluorescence signal (small pink dots in each picture) was shown. The rest of 4 colonies had no fluorescence detected so their images were not shown. The reason why these signals were defined as auto-fluorescent signal lies in two facts. The first one was that they were all point-focused seen from a fluorescent microscope, which meant that it was not able to indicate the shape of the sub-cellular components in which it located. All of fluorescent signals in Figure 5.3 met this characteristic. The second reason was that their intensity remained almost unchanged during time-lapse microscopy (data not shown but the intensity can be seen from Figure 5.3, which was high), which was against the basic property of native p53 gene expression in response to DSBs. Based on these reasons, we believed that these signals were auto-fluorescence and that BAC stable transfection did not succeed.
Figure 5.3: Only Auto-fluorescence Was Show in 5 out of 9 Single Colonies Obtained from Long-term Geneticin Screening Following p53-DsRedXP-Lamin B1-eCFP BAC Transfection. The small pink dots are auto-fluorescent signals, indicating that BAC stable transfection did not succeed. The remaining 4 single colonies had no fluorescent signal detected.

To double-check the imaging results, Western Blotting was also conducted in 9 single clones of Lahav’s modified MCF7 cells following 4-hour NCS treatment. Figure 5.4 indicates that only endogenous p53 expression was detected in response to NCS treatment but there was no p53-DsRedXP (140kDa) shown, further reinforcing the imaging result.

Our attempt to generate p53-DsRedXP expressing single cell clones was attempted a further time in MCF7 cells and in parallel in HCT 116 cells. In both case, while clones were developed none of these clones were positive for expression of the fluorescent transgenes contained on the tranfected BAC (data not shown). These observations imply that while the BAC fragment containing the antibiotic resistance gene is retained
in the cell clones parts of the BAC are deleted during cloning. This implies that the BAC-dependent expression of ectopic p53 might induce cell apoptosis in transfected clones during selection. Given the timeline of the entire project, no further time was spent of attempting to establish stable transfection in Lahav’s modified MCF7 cells and transient transfection of the cell lines was used instead.

<table>
<thead>
<tr>
<th>Clone</th>
<th>NCS</th>
<th>p53-DsRedXP</th>
<th>p53</th>
<th>β-actin</th>
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<td>1</td>
<td>+</td>
<td>140kDa</td>
<td>53kDa</td>
<td>42kDa</td>
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<td>9</td>
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**Figure 5.4: p53-DsRedXP Expression Was Not Found after NCS Treatment in All of 9 Single Colonies Obtained from Long-term Genetin Screening Following BAC Transfection.** Cells were treated with 400ng/ml (final concentration) NCS for 4 hours prior to protein extraction and western blots performed.

### 5.3.2 Transient Transfection

Similar to BAC stable transfection assays, Lahav’s modified MCF7 cells were transfected with p53-DsRedXP-Lamin B1-eCFP BAC using either ExGen500 or PEI40. Then 48 hours later, cells were used directly for time-lapse microscopy. Seen using a fluorescent microscope, MDM2-YFP was persistently presented in most cells as expected under normal, non-stressed, growth condition. In the early experiments, p53-CFP present in the original Lahav’s cell line was not activated (Zn$^{2+}$ was not added). As a transfection marker, the presence of pCMV-Lamin B1-eCFP in individual cells showed the cells that had been transfected with the retrofitted BAC. Due to the clear localisation of Lamin B1, eCFP generally rims a nucleus, which makes a potentially BAC-transfected cell
highlighted. Nevertheless, it did not mean that looking for eCFP-marked cells was easy. As mentioned above, transfection efficiency of BAC DNA is generally low—typically in the range 1-5% of cells contain the BAC—as the BAC DNA (at 200kbp DNA) is very much longer than plasmids (10kbp DNA), which are normally used for ectopic gene expression. So in this experiment collecting sufficient eCFP-marked cells was very time-consuming. Moreover, p53-DsRedXP cassette did not necessarily express in eCFP-marked cells and was only expressed at detectable levels once the expression of the fluorescent p53 transgene has been stabilised by NDA damage. Therefore, the difficulty of identifying BAC transfected cells prior to or immediately following DNA damage was further increased. However, since we failed to make the BAC stable transfection happen, then we had to try hard to overcome those difficulties in BAC transient transfection as it was still the preferred way to achieve the goals we set in 5.1.

miRNA mimics were introduced to Lahav's modified MCF7 cells by transient transfection as well. As shown in Figure 3.3 B, the transfection efficiency of miRNAs was very high. So miRNAs transfection was conducted 24 hours before imaging, which was 24 hours later than BAC transfection. The reagent used for miRNAs transfection was Invitrogen's RNAiMAX as it was particularly designed for nucleotides of small size.

5.4 p53-MDM2 Dynamics in Response to DSBs in Single Cells Is Regulated by miRNAs

In a 35mm glass-bottom dish with four compartments, 5×10^4 Lahav’s modified MCF7 cells were seeded in each compartment. 0.5μg p53-DsRedXP-Lamin B1-eCFP BAC was transfected 48 hours after seeding and 48 hours before imaging; 5pmol miR-34a or 5pmol miR-125b was transfected 24 hours before imaging, respectively. pCMV-Lamin B1-eCFP, MDM2-YFP and p53-DsRedXP were all recorded by time-lapse microscopy simultaneously. Some representatives of the results were shown in Figure 5.5.
Figure 5.5: The Typical Examples of p53-MDM2 Dynamics in Response to NCS-induced DSBs at Single-cell Level in the Retrofitted BAC-transfected Lahav’s Modified MCF7 cells. The traces of p53-DsRed, MDM2-YFP and Lamin B1-eCFP were all shown in the upper panels of A and B. The series of pictures shown in the lower panels of A and B demonstrated the fluorescent intensity of each transgenic cassette at the specific moments on each corresponding trace. NCS was added four hours after the beginning of the experiments. The vertical axis at the 4th hour following the beginning of imaging marks the moment of NCS addition.

In sum, 111 BAC-transfected Lahav’s modified cells, like the ones shown in Figure 5.5, were found and recorded. Due to the limited number of samples, the reliability of the conclusions drawn from the results of these experiments may be impaired at some extent. However, from the results we can still clearly see basic dynamical properties of p53-DsRedXP, MDM2-YFP and Lamin B1-eCFP in every single cell that displayed dynamics of p53 expression. As in other experiments, these examples showed great heterogeneity but shared the expected common characteristics described before. The
data was also able to reveal how the over-expression of p53-related miRNAs affected p53 and MDM2 dynamics. Moreover, the link between cell fate and p53-MDM2 dynamics was indicated and thus the two factors can be analysed together in an interactive way.

Regarding the general behaviour of each fluorescent cassette at single-cell level, let us start to demonstrate from Lamin B1-eCFP. The level of Lamin B1-eCFP fluorescence in most cells was quite stable and not affected by NCS treatment or miRNAs transfection. Hence, as expected the trace of Lamin B1-eCFP expression shown in Figure 5.5 A was not mostly stable across the time course recorded, though expression seems to be delayed during the early stages of imaging. The expression of p53 and MDM2 was not expected, with oscillations of both proteins seen and the period of p53 oscillations preceding those of MDM2, as would be expected from the fact that MDM2 is a target gene for p53. The dynamics of p53-DsRedXP and MDM2-YFP in response to DSBs and miRNAs transfection in individual cells still matched the model demonstrated in 4.5.1. In that model, p53-MDM2 dynamics was categorised as three types: non-responsive/no peaks (Type 1, see Figure 5.5 B), intermediate or heterogeneous (Type 2) and oscillatory (Type 3, see Figure 5.5 A). The definition of these types and parameters involved were identical to those described before, confirming that introduction of the BAC to Lahav's cells does not alter the natural properties of p53 signalling (see details in 4.5.1). Hence, we continued to use this model to analyse the heterogeneity of p53-MDM2 dynamics in response to DSBs and miRNAs transfection in these 111 cells. The following features were described: the number of peaks, the timing of the first peaks and the periods of oscillations. In addition, we focused on the fate of these 111 cells at the end of imaging and tried to link cell fate determination and p53-MDM2 dynamics, which has been described previously but to date has not been characterised in the unperturbed system. For example, Figure 5.5 A is a representative of an oscillatory cell that finally survived in the presence of NCS; Figure 5.5 B shows a Type 1 cell that did not activate oscillations in p53-MDM2 dynamics. However, due to the limited number of cells collected from the experiments and the need for simplifying the research model, we merged Type 1 and Type 2 dynamics and called the new category non-oscillatory dynamics; correspondingly, Type 3 dynamics was named oscillatory dynamics. Notably, if either p53 or MDM2 oscillations was seen in a cell, that cell was defined as an
oscillatory cell; this is appropriate as the p53 and MDM2 oscillations are coupled and any failure to visualise oscillations in one imaging channel only is a technical issue, which almost always relates to limited signal intensity. Thus, combined with another factor cell fate, four biological outcomes were present in the experiments, which were oscillation-death, oscillation-survival, non-oscillation-death and non-oscillation-survival.

Another exciting point that was demonstrated in the experiments was the effect of miRNAs transfection on p53-MDM2 dynamics. Two p53-related miRNAs, miR-34a and miR-125b, were selected because of their different modes of interaction with p53. miR-125b targets to 3'-UTRs of p53 mRNAs and leads to the inhibition of p53 protein synthesis. As a p53 downstream component, miR-34a expression is up-regulated by p53 and then followed by miR-34a-induced suppression of a deacetylase SIRT1 that can down-regulate p53 trans-activity. The mechanism of SIRT1 suppression lead by miR-34a is the same as that of p53 repression induced by miR-125b. Therefore, a positive feedback loop is formed among p53, miR-34a and SIRT1; while the relationship between miR-125b and p53 is a simple negative regulation. But why did we choose two miRNAs that have two different modes of interaction with p53? This is based on the fact that the interaction between signalling pathways with different dynamical properties can generate various network architectures, which might differ from the native structure in a way that is context specific. So it means that the dynamical outcomes produced by p53-MDM2 negative feedback loop and p53-miR-34a-SIRT1 positive feedback loop are not only different from any of original feedback loop, but also from the outcomes generated by p53-MDM2 negative feedback loop and miR-125b-p53 one-way inhibition. Hence, together with the previous conclusion that different patterns of p53-MDM2 dynamics give rise to different cell fates, we hypothesise that the over-expression of two miRNAs respectively, which potentially leads to different p53-MDM2 dynamics, will effect cell fate determination.

So combined with the three factors mentioned above, the overview of the core experimental result is as follows:
From Table 5.1 it can be seen that compared to the control mimics, miR-34a over-expression greatly increased the percentage of cells in which p53-MDM2 oscillations were seen (25.7% VS. 42.5%). In contrast, the fraction of miR-125b-transfected cells in which p53-MDM2 oscillations occurred slightly decreased by 9% in comparison with the control group (16.7% VS. 25.7%). Of course, the difference between miR-34a-transfected and miR-125b-transfected cells in terms of the percentage of cells showing p53-MDM2 oscillations was more significant (42.5% VS. 16.7%).

The correlation between the types of p53-MDM2 dynamics and the cell fates was demonstrated very clearly in Table 5.1 as well. p53-MDM2 oscillations dramatically facilitated cell survival in the presence of NCS because 30 of 32 cells kept alive at the end of imaging in total. In comparison, only 37 of 79 cells with non-oscillatory behaviour were alive at the end of imaging. That is to say, of all 67 survived cells, nearly half of them were the cells with p53 and/or MDM2 oscillations, although the total
percentage of the cells with p53 and/or MDM2 oscillations was only less than 30%. Regarding to non-oscillatory p53-MDM2 dynamics, in drawing a convincing conclusion we must be very cautious. On one hand, as the numbers of dead and survived cells with non-oscillatory p53-MDM2 dynamics were approximately equal in miR-34a-, miR-125b- and scrambled miRNA-transfected sets, respectively, it could be concluded that non-oscillatory p53-MDM2 dynamics did not make a significant impact on cell fate determination. On the other hand, there were two facts that made people believe the causal relationship between non-oscillatory p53-MDM2 dynamics and following cell death. The first one was that 42 of 79 cells showing non-oscillatory p53-MDM2 dynamics were found dead at the end of the experiments, compared to the fact that only two cells out of 32 cells with p53 and/or MDM2 oscillations were dead at the end of imaging. The second one was that, of all 44 dead cells, 42 of them were the cells with non-oscillatory p53-MDM2 dynamics. But no matter which one was correct or at least more reasonable, one thing was guaranteed: non-oscillatory p53-MDM2 dynamics did not play a positive role in promoting cell survival, which was against to that of p53-MDM2 oscillations.

Hence, combined with the analysis in last two paragraphs, we concluded that miRNAs transfection was able to manipulate p53 and/or MDM2 dynamical profiles, which further led to having different impact on cell fate determination, with persistent and stable oscillation limiting cell death.

To develop this further, we will look next at p53-MDM2 dynamics in these 111 cells by analysing another two parameters previously described in 4.5.2: the timing of the first peaks and the periods of p53-MDM2 oscillations.

### 5.4.1 The Timing of the First Peaks

Of all 111 qualified BAC-transfected cells, 84 cells showed peaks in p53 and/or MDM2 dynamics in response to NCS and miRNAs transfection. Particularly, 46 cells showed both p53 and MDM2 peaks; 22 cells had MDM2 peaks only and 16 cells had p53 peaks only. The timing of the first p53 and MDM2 peaks is present in Figure 5.6 A and B, respectively.
Previously described in 4.5, the majority of Lahav’s modified MCF7 cells responded to NCS-induced DSBs with a peak of p53 expression within 1-3 hour(s) of damage induction using NCS. As NCS was added at 4h, most of the first p53 and/or MDM2 peaks appeared within 5-7 hours after the beginning of imaging and took another 1-2 hour(s)
to reach their maximum expression. The moment of the highest point on the first peak was defined as the timing of the first peak because it was the easiest way to precisely localise a peak in terms of time course. Although p53-MDM2 dynamics at single-cell level were very distinct from one cell to another, over half of cells conformed to this pattern of timing, independently of whether BACs and/or miRNAs were transfected into cells (Figure 4.7, 4.9, 4.11 and 5.6).

It also can be seen from Figure 5.6 that in miR-125b-transfected cells, the first p53 and MDM2 peaks appeared within the first 6 hours following NCS addition was roughly 8%-20% earlier than its counterparts in the other two groups of cells. Moreover, in every single division of miR-125b-transfected cells, most values of the timing of the first p53 and MDM2 peaks, which were respectively designated to the same number of single miR-125b-transfected cells, were larger than those in their corresponding divisions of the other two sets. On the contrary, the higher percentage (10% more) of cells where the first MDM2 peaks were activated in response to NCS in the first 6 hours after treatment was produced in miR-34a transfection group than in the other two groups of cells (Figure 5.6 B). However, under the same condition, the fraction of those miR-34a transfected cells where the first p53 peaks were produced in the first 10 hours following the beginning of imaging was almost identical to the control (Figure 5.6 A).

Apart from these discrepancies among three groups, there were a couple of common facts in Figure 5.6. The first one was that it was very rare to see the first p53 and/or MDM2 peaks appeared after 15 hours, as only 2 and 3 values were larger than in all 10-20 hours divisions in Figure 5.6 A and B, respectively. The second one was that among all of 46 cells with both p53 peak(s) and MDM2 peak(s) appeared, the number of cells (28) where the first p53 peaks appeared earlier than the first MDM2 peaks was more than that of cells (18) in which the first MDM2 peaks appeared earlier than the first p53 peaks, no matter which miRNAs were transfected into them. And last but not the least, there were a small number of cells, regardless of which miRNAs being over-expressed, showing the first p53 and/or MDM2 peaks before NCS addition. These peaks were defined as “natural peaks” in our project, and intermittent activation of p53 expression has been described before by Lahav and colleagues. For example, natural p53 peaks were seen in 3 cells with miR-34a transfected, 2 cells with miR-125b transfected and another 2 cells with scrambled miRNA transfected, respectively. Similarly, a couple of
cells with miR-34a, miR-125b or control miRNA mimics transfected also showed natural MDM2 peaks, respectively.

From the above, it can be concluded that although the timing of the first p53 and MDM2 peaks was heterogeneous, it was able to be manipulated by miRNAs over-expression in a regular pattern. Particularly, miR-34a and miR-125b played opposite roles in fine-tuning the sensitivity of cells in response to NCS-induced DSBs by advancing or delaying the first p53 and/or MDM2 peaks, respectively. In addition, the timing of the first peaks demonstrates some traits that are independent of miRNAs transfection, suggesting that it is shaped by some intrinsic factors of the pp53 signalling network architecture.

5.4.2 The Periods of Oscillations

Another parameter of p53-MDM2 dynamics we set to investigate was the periods of p53 and MDM2 oscillations. Similar to 4.5.2, the values of periods of p53 and MDM2 oscillations were collected from BAC- and miRNAs-transfected cells that showed p53 and/or MDM2 oscillations. All of those values were according to Table 5.1, 32 cells showed p53 and/or MDM2 oscillations, which were listed in Table 5.2:
The periods of p53 and MDM2 oscillations were analysed separately. In order to look if the miRNAs over-expression had any impact on the periods of p53 and MDM2 oscillations at single-cell level, the cells transfected with different miRNA mimics were investigated separately. Similar to Table 5.2 where the numbers of cells performing p53 and/or MDM2 oscillations in each miRNA transfection group were demonstrated, the numbers of the values of oscillatory periods corresponding to those of p53 and/or MDM2 oscillations, together with the total numbers of cells showing p53 or MDM2 oscillations, were listed in Table 5.3:

<table>
<thead>
<tr>
<th></th>
<th>p53 Oscillations Only</th>
<th>MDM2 Oscillations Only</th>
<th>p53 and MDM2 Oscillations</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-34a</td>
<td>5</td>
<td>8</td>
<td>4</td>
<td>17</td>
</tr>
<tr>
<td>miR-125b</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>Control</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
<td>15</td>
<td>7</td>
<td>32</td>
</tr>
</tbody>
</table>

Table 5.2: The Numbers of BAC- and Different miRNAs-transfected Cells Showing p53 and/or MDM2 Oscillations
Table 5.3: The Overview of the Numbers of Cells Showing p53 or MDM2 Oscillations and the Numbers of Values of p53 or MDM2 Oscillatory Periods in Each Group of Cells Transfected with Different miRNAs

Then we made three sets of comparison of the periods of p53 and MDM2 oscillations, respectively, which were as follows:

Table 5.4: How the Periods of p53 and MDM2 Oscillations Were Analysed between Any Two Sets of Cells with Different miRNAs Transfected.
According to Table 5.4, the results of the comparison were tested for significance using one-way ANOVA statistics:

<table>
<thead>
<tr>
<th>$SS$</th>
<th>df</th>
<th>$MS$</th>
<th>$F$</th>
<th>$P$-value</th>
<th>$F$ crit</th>
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</thead>
<tbody>
<tr>
<td>0.031281</td>
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<tr>
<td>13.3914</td>
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<tr>
<td>13.42268</td>
<td>46</td>
<td></td>
<td></td>
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**p53 Oscillations: miR-34a VS. Control**

<table>
<thead>
<tr>
<th>$SS$</th>
<th>df</th>
<th>$MS$</th>
<th>$F$</th>
<th>$P$-value</th>
<th>$F$ crit</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.739797</td>
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<td>0.739797</td>
<td>3.030272</td>
<td>0.094532</td>
<td>4.259677</td>
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<tr>
<td>5.859248</td>
<td>24</td>
<td>0.244135</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.599044</td>
<td>25</td>
<td></td>
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**p53 Oscillations: miR-125b VS. Control**

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<th>$F$</th>
<th>$P$-value</th>
<th>$F$ crit</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.626716</td>
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<td>0.162475</td>
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<td>11.41584</td>
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<tr>
<td>12.04256</td>
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<td></td>
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</tr>
</tbody>
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**p53 Oscillations: miR-34a VS. miR-125b**
<table>
<thead>
<tr>
<th>SS</th>
<th>Df</th>
<th>MS</th>
<th>F</th>
<th>P-value</th>
<th>F crit</th>
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</thead>
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<tr>
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<td>55</td>
<td>0.14856</td>
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</tr>
<tr>
<td>8.55017</td>
<td>56</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

**MDM2 Oscillations: miR-34a VS. Control**

<table>
<thead>
<tr>
<th>SS</th>
<th>Df</th>
<th>MS</th>
<th>F</th>
<th>P-value</th>
<th>F crit</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.418466</td>
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<td>0.418466</td>
<td>3.811185</td>
<td>0.059707</td>
<td>4.149097</td>
</tr>
<tr>
<td>3.513579</td>
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<td>0.109799</td>
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<tr>
<td>3.932044</td>
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</table>

**MDM2 Oscillations: miR-125b VS. Control**

<table>
<thead>
<tr>
<th>SS</th>
<th>Df</th>
<th>MS</th>
<th>F</th>
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<th>F crit</th>
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<td>7.138668</td>
<td>50</td>
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</tbody>
</table>

**MDM2 Oscillations: miR-34a VS. miR-125b**

Table 5.5: The Comparison of the Periods of p53 and MDM2 Oscillations by One-way ANOVA (95% Confidence Interval) between Any Two Sets of Cells Treated with NCS and Transfected with miR-34a, miR-125b or Scrambled miRNAs, Respectively.

As shown in Table 5.5, at 95% confidence interval, miRNAs transfection was not able to produce any influence on the periods of both p53 and MDM2 oscillations in single cells, as there was no significant difference in every single set of comparison (all P values were over 0.05). In addition, we found that the range of variation in the period of MDM2 oscillations was reduced compared to that shown in 4.5.2. According to Table 5.3, 71 values of periods were collected from MDM2 oscillations, with 61 values were between
4 hours and 5.5 hours; no value was over 6 hours. As a comparison, in 4.5.2 the result indicated that 18 of 62 values were between 5.5 and 7 hours, with 13 of them were over 6 hours; and only 44 values were between 4 and 5.5 hours. Interestingly, in these experiments the range of variation of oscillatory periods between p53 and MDM2 oscillations were very different, as 30 of 56 values of p53 oscillations’ periods were in the interval of 4-5.5 hours and the rest of them ranged from 5.5 to 7 hours, with the maximum value near to 6.5 hours. Hence, the range of variation of p53 oscillations’ periods was wider than that of MDM2 oscillations’ periods, partially suggesting the greater heterogeneity of p53 oscillations than MDM2 oscillations.

5.5 Discussion

As detailed in 5.1 and 5.2.1, the ultimate objective of employing BAC transgenic system to investigate p53-MDM2 dynamics is not only to demonstrate DSBs-induced p53 and MDM2 dynamics in single cells, but also to link p53-MDM2 dynamics and cell fate determination, which is very challenging and has not been well studied yet. Although there have been several related pieces of work conducted by computational simulation, the biological analysis of p53 signalling in single cells is very specialised and so limited to a small number of labs with the relevant expertise. To date, perhaps the most well-known study was performed by Purvis et al, which was published on Science in 2012. Their work was based on the research published by Batchelor et al in 2011, which indicated that different types of DNA damage, such as SSBs and DSBs, resulted in different patterns of p53-MDM2 dynamics at single-cell level. Purvis et al went further to find that the discrepancies in the pattern of p53-MDM2 dynamics at single-cell level, which were attributed to different types of DNA damage, led to different cell fate from one cell to another. Interestingly, cell fate difference was present as the difference in transcription profiles of the typical p53 downstream genes responsible for cell apoptosis, cell cycle arrest, cell senescence and DNA repair. However, this study still had some limitation, which include: the cell model used was still based on plasmids with mini-genes lack some features of p53 regulation; different concentration of the inhibitor of p53 degradation, Nutlin-3, were used to artificially minic increased p53 expression; the amount of cells probed by time-lapse microscopy and the length of imaging were
both insufficient; the expression profiles of p53 downstream genes did not necessarily mean that the corresponding cell fates they lead to actually occurred; and most importantly, the authors did not demonstrate p53-MDM2 dynamics of single cells observed (microscopic images or dynamical traces) and their respective fates (microscopic images) together to show the causal relationships.

Luckily, our experiments overcame most of these disadvantages. We used bespoke BACs to introduce fluorescent p53 and the transfection marker cassettes and to create a completely native genomic environment for them to express naturally and stably in transfected cells. In our experiments, cells were transfected with p53-related miRNAs to give biologically relevant regulation of p53 expression. In contrast, Purvis et al used artificial (using Nutlin-3, a powerful MDM2 chemical inhibitor) treatment to alter p53-MDM2 dynamical patterns. In addition, we focused on DSBs-induced p53-MDM2 dynamics only so the switch between different types of DNA damage was not required. We also extended imaging to acquire more information about p53-MDM2 dynamics and cell fates. Finally, our data, and the approaches we took to obtain the data, revealed the direct link between p53-MDM2 dynamics in individual cells and their respective cell fate by presenting both results together (take Figure 5.5 and Table 5.1 as examples).

Consequently, we were able to draw clear conclusion on how miRNAs transfection affects p53-MDM2 dynamics at the single-cell level and how these changes produce an impact on cell fate determination. Basically, we found that miR-34a over-expression facilitated the formation of p53-MDM2 oscillations in single cells compared to the control group while the fraction of miR-125b-transfected cells undergoing p53-MDM2 oscillations was reduced relative to the control group. We also revealed that p53-MDM2 oscillations correlate with cell survival but p53-MDM2 non-oscillatory behaviour either played a neutral role in cell fate determination or promoted cell death. In addition, by looking at other parameters of p53-MDM2 dynamics, we concluded that miRNAs transfection can make more impact. For example, most cells in which miR-34a was over-expressed performed their first p53 and/or MDM2 peaks in response to NCS earlier than most cells transfected with scrambled miRNA mimics (control); in contrast, compared with the control group, miR-125b over-expression tended to delay the time at which the first p53 and/or MDM2 peaks appeared following NCS treatment. This result indicated that miR-34a and miR-125b over-expression were able to fine-tune the
sensitivity of individual transfected cells to DSBs. However, the over-expression cannot alter every aspect of p53-MDM2 dynamics at single-cell level, such as the periods of oscillations. This is perhaps not surprising as the period of signalling oscillations will be hard-wired by the key relevant parameters such as rates of expression and turnover of the relevant mRNA and protein species.

Besides these conclusions, there were some interesting points hiding in our results, which were worth analysing as well. The first one was that why stable transfection was not successful. As one of the approaches that aim to introduce an exogenous gene into cells, stable transfection is one of the most effective methods to harness the copy number and expression profile of the gene of interest. Despite of this fact, it might be extremely difficult for cells to remodel the p53 signalling network in a way that accommodates the altered expression of p53, in cells with ectopic p53 expression from the BAC. As a result, some cells, in which p53-DsRed and Lamin B1-eCFP cassettes may be integrated into their genomes successfully, died during selection because of higher p53 expression. As we observed, those cells that eventually survived during selection and stable transfection had at some point in the process deleted all or part of the p53 gene. This implies that over expression of p53 results in cell death in the MCF7 cell line. Then people will ask: why can cells survive following BAC transient transfection, since p53 was over-expressed as well? This is because exogenous gene integration in a genome is not involved in transient transfection so the vectors carrying an exogenous gene is very easy to be cleared from a transfected cell if it is not able to tolerate the over-expression of that gene. In addition, an exogenous vector generally can stay in a cell for no more than 100 hours following transient transfection, during which is not long enough to make lethal impact on most cells before being degraded.

So since BAC stable transfection was not successful, there were still three options that can potentially achieve the same goal. The first one was to stably transfect cells with the similar fluorescent MDM2 BACs before retrofitted p53 BAC stable transfection, which may help to recover the balance between p53 and MDM2. The second one was to precisely knock in a fluorescent protein gene at the downstream flank of p53 gene by clustered regularly interspaced short palindromic Repeats (CRISPR)/Cas9 genomic editing technique. In this scenario, p53 protein produced by genomic p53 gene is labelled and measured so exogenous vectors containing a fusion of p53 and fluorescent
genes, such as the retrofitted p53 BAC used in these experiments, are no longer required, which is definitely the best way to investigate endogenous protein dynamics at single-cell level in native context.

To take the first option, first we will need to copy the constitution of p53-DsRedXP-Lamin B1-eCFP BAC to build a similar MDM2 BAC from scratch; then we will still try hard to overcome low transfection efficiency; and we also have to worry if cells can tolerate two BACs at the same time and if sufficient amount of samples can be collected eventually. The second plan was not available during the early stages of our work, but since my project has been completed has become a routine approach for generating fluorescent fusion proteins that are expressed from the endogenous loci in mammalian cells and animal models.

Hence, we finally went for the third option, BAC transient transfection, which was the simplest solution but the most compromised one as well. The disadvantages of BAC transient transfection have been listed in 5.3.1. Apart from them, it is also noteworthy to be aware of the cellular toxicity of transfection reagents. In our project, ExGen500 or PEI40 used was both relatively less toxic. However, PEI40 had to be washed off 4-6 hours after BAC transfection to prevent cells from being damaged. ExGen500 was not necessary to be removed from growth medium after BAC transfection but still gave rise to cell death at some extent. The damage caused by transfection reagents was able to affect p53-MDM2 dynamics, which was one of the reasons why time-lapse microscopy has to be conducted at least 24 hours after transfection. In fact, we performed imaging 48 hours after BAC transfection, leaving sufficient time for the exogenous genes expression and miRNAs transfection.

Next, we need to clarify which cells were not tracked although they had been transfected with BACs. The first category of cells is those which survived less than 8 hours following the beginning of imaging in that their survival period was less than a period of p53-MDM2 oscillations since NCS addition, which was done at the 4th hour from the start; those which slipped out the field within 8 hours also belonged to this category. The second category of cells is those which did not express all of three fluorescence-labelled proteins. This phenomenon is highly likely to result from
accidental BAC truncation, shear or BAC/plasmid expression faults during manipulation and transfection, leading to incomplete and unreliable data.

The third point to be discussed is p53-MDM2 dynamical traces and images, in which several phenomena were found interesting. The first one is Lamin B1-eCFP's localisation. Chosen to be the transfection marker for our imaging assays due to its constantly stable expression and clear localisation at the inner rim of a nucleus, we surprisingly found that Lamin B1-eCFP's localisation was not always stable. In fact, in some normal BAC-transfected cells eCFP signal evenly or unevenly spread all over nuclei or kept changing against time, potentially suggesting that morphological change occurred in nuclei. In addition, in a minority of cells eCFP signal did not appear until several hours after the beginning of the experiments, which may result from the lag of BAC replication or expression often occurs in newly divided cells. This speculation, together with the variability of Lamin B1 localisation seen in some cells during time-lapse imaging, were consistent with the facts that eCFP signal generally disappeared or changed dramatically during cell division and only stabilised at the nuclear rim 2-3h later. Apart from dividing cells, the abnormal eCFP localisation can indicate dying cells because cell death is accompanied by Caspase-dependent degradation of the nuclear lamina general nuclear collapse. The second one is the occasional changes of eCFP signal seen in cells (Figure 5.5 A), which could be attribute to technical issues such as altered focus, heterogeneity of copy numbers during transfection and expression efficiency of p53-DsRedXP-Lamin B1-eCFP BAC in individual transfected cells. The third one is the behaviour of p53-DsRedXP.

According to the map of p53-DsRedXP-Lamin B1-eCFP BAC, p53-DsRedXP is driven by native p53 promoter, which means that p53-DsRedXP signal does not emerge until several hours following NCS treatment. But in a minority of cells p53-DsRedXP signal did appear at the beginning of imaging, implying that intrinsic stress existed in those cells. The localisation of p53-DsRedXP signal was variable as well. Because p53 is a nuclear protein, nuclei in most BAC-transfected cells were evenly filled with DsRedXP's fluorescence (Figure 5.5 A). However, in some cells, especially dying cells, DsRedXP's fluorescent signal often became condensed, accompanied with greatly boosted intensity. With the help of this characteristic and bright field mode, it was easy to distinguish dying cells. Moreover, DsRedXP’s fluorescence was seen in both nuclei and cytoplasm.
in a couple of cells, indicating that p53 translocation took place. Generally speaking, if seen in the cytoplasm, it means that p53 is about to be degraded in proteosomes. But why did we observe this normal process in only a couple of cells? One explanation is that at stressed condition, due to the dissociation with its inhibitor MDM2, p53 degradation is so slow this makes the translocation and signal in cytoplasm invisible in most cells.

Then we come to Table 5.1 and find another interesting question: why were p53-MDM2 oscillations enhancing cell survival? A previous study demonstrated that p53-MDM2 oscillations resulted from the recurrent initiation of p53 upstream components responsible for DNA damage checking, such as ATM/ATR and CHK1/2 (Batchelor et al., 2008). So why do these components rather perform more complex oscillatory behaviour? It is speculated that this behaviour helps to achieve regular inspection of DNA integrity, which is an energy-efficient and sensitive way to protect DNA from damage (Batchelor et al., 2008). So due to robust synchronisation and coordination among upstream and downstream components during p53 signalling, it was reasonable to expect that p53-MDM2 oscillations increase survival rate of BAC-transfected cells. Naturally, the fact that non-oscillatory p53-MDM2 dynamics did not lead to cell survival was not surprising. However, unlike oscillatory dynamics, we did not find a definite conclusion of how non-oscillatory p53-MDM2 dynamics can impact on cell fate determination. Perhaps it was because 400ng/ml NCS did not sustain sufficient damage to be lethal to most cells.

In addition, we also need to further look at why miR-34a over-expression facilitated p53-MDM2 oscillations. As mentioned previously, a positive feedback loop is formed between miR-34a and p53 via SIRT1. So miR-34a over-expression up-regulates p53, which has been shown in Figure 3.5 at the population level. But at the single cell level, p53 up-regulation does not necessarily mean that the increase of p53 level is due to highly heterogeneous properties of each cell. And as previous studies demonstrated that p53 behaviour was supposed to be oscillatory in response to DSBs at single-cell level, it was reasonable to see that p53 up-regulation was embodied by more widespread p53-MDM2 oscillations in single cells. This explanation is applicable to the effect of miR-125b over-expression as well.
Next, we note that it is worth going over the data in 5.4.1 and 5.4.2, to explore how the over-expression of miR-34a and miR-125b may fine-tune the two important parameters of p53-MDM2 dynamics, the timing of the first p53 and MDM2 peaks and the periods of p53-MDM2 oscillations. The timing of the first p53 and MDM2 peaks was considered as the sensitivity of each cell to stress and the data in 5.4.1 showed that both miR-34a and miR-125b transfection made an impact on this parameter. As we described in the last paragraph, at the single-cell level p53 up-regulation can be embodied in several ways. In these experiments, we demonstrated that miR-34a over-expression enhanced p53 in two ways: expanding the fraction of cells in which p53 and MDM2 oscillate to increase cell survival in the presence of NCS and advancing the timing of the first p53 and MDM2 peaks to improve the sensitivity of cells to NCS-induced DSBs. It is also noteworthy that the first p53 peaks were more likely to emerge earlier than the first MDM2 peaks in response to NCS, which was shown in all three groups. This fact, together with Figure 3.2 A, suggests the temporal order and the direction of signal flow in p53 signalling. In addition, Figure 5.6 implies that most cells tend to respond to stress as soon as possible following damage. Regretfully, we did not investigate the correlation between the timing of the first peaks and cell fates so we cannot assert that the earlier the first p53 and/or MDM2 peak emerge(s), the more likely it is that survival will be enhanced. In addition, we were unable to compare the importance of p53-MDM2 behaviour and the timing of the first p53/MDM2 peaks to cell fate determination. Furthermore, from the results in 5.4.1 and 5.4.2 we are aware that there are some aspects of p53-MDM2 dynamics that cannot be changed simply by miRNAs transfection, such as the periods of p53-MDM2 oscillations and the probability of “natural peaks” appearance, which suggests that p53-MDM2 dynamics can be partially dependent on intrinsic factors.

Finally, we have to bear in mind that all data shown in 5.4 were obtained from only 111 cells and all the conclusions were drawn on the basis of this relatively small dataset. This limitation originated from extremely low transfection efficiency of BACs. Another potential weakness is that BAC transfection is transient, which is considered less reliable than stable transfection. Therefore, to overcome these disadvantages, we will go back to the original Lahav’s cell model to further study how the over-expression of miR-34a and miR-125b affects p53-MDM2 dynamics at single-cell level in the next chapter. Although the deficiencies of the original Lahav’s cell model have been discussed in
Chapter 4, we believe that we can partially compensate for them using carefully controlled experimental designs.
Chapter 6  miRNAs Regulate NCS-induced p53-MDM2 Dynamics at Single-cell Level in the Plasmid-based Cell Model

6.1  Objectives

Following the last chapter, we found that although the BAC-based cell model had many advantages compared to original Lahav’s model, we cannot obtain BAC stably transfected cells and also cannot collect sufficient samples due to extremely low transfection efficiency of BACs, which potentially impair data reliability. Therefore, in this chapter we are going to get rid of BACs and use original Lahav’s cell model to look at how miR-34a and miR-125b over-expression affects p53-MDM2 dynamics at single-cell level. The reason to use original Lahav’s cell model again is that it is just able to overcome the two deficiencies of the BAC-based cell model, as it is a dual-plasmid stably transfected cell model and high transfection efficiency of miRNA mimics does not limit sample collection. Moreover, by comparing the data obtained in original Lahav’s cell model with the one acquired from the BAC-based cell model, we are able to compensate the shortcomings of each model at some extent to draw more convinced conclusions.

6.2  The Over-expression of miR-34a and miR-125b Fine-tunes DSBs-induced p53-MDM2 Dynamics in Individual Lahav’s Modified MCF7 Cells

We used to look at p53-MDM2 dynamics at single-cell level successfully in Lahav’s modified MCF7 cells in 4.5. So this time we still stuck to this cell model (including how p53-MDM2 dynamics were categorised and how each category was defined; please see 4.5.1 for details) and kept most of the cell treatment and experimental conditions the same (see 2.7 and 4.5 for details) in addition to the introduction of miR-34a and miR-125b. Hence, how the experiments were organised was listed in below:
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<th></th>
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<th>Control</th>
</tr>
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<tbody>
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<td>Completed</td>
<td>Completed</td>
<td>Completed</td>
<td>Completed</td>
</tr>
<tr>
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<td>Non-applicable</td>
<td>Completed</td>
</tr>
<tr>
<td>NCS+ZnCl₂</td>
<td>Completed</td>
<td>Completed</td>
<td>Non-applicable</td>
<td>Completed</td>
</tr>
</tbody>
</table>

**Table 6.1: How Cells Were Treated in the Following Experiments.** Control includes cells transfected with scrambled miRNA mimics (NC in Figure 6.1) and non-transfected cells (NT in Figure 6.1).

Again, we were going to analyse p53-MDM2 dynamics at single-cell level by three parameters: the number of peaks, the timing of the first peaks and the periods of oscillations. Note that still only MDM2-YFP traces were recorded as p53-CFP signal was not present without ZnCl₂; and the link between p53-MDM2 dynamics and cell fate determination was not involved in the following experiments as the interaction among miRNAs, p53-CFP/MDM2-YFP and cell fates was not direct in original Lahav's cell model. However, as mentioned above, we were going to manage to overcome its weakness by making a comparison with the data obtained in BAC-based cell model.

### 6.2.1 The Number of Peaks

In a 35mm four-compartment glass-bottom dish, 2.5×10⁴ Lahav's modified MCF7 cells were seeded per compartment 72 hours before imaging. Cells in each compartment were transfected with 5pmol miR-34a, 5pmol miR-125b and 5pmol scrambled miRNA mimics 24 hours before imaging, respectively. The cells in the rest of the compartment were left non-transfected in some experiments or transfected with 15pmol miR-125b in other experiments. ZnCl₂ was added just at the beginning of the experiments to the final concentration of 200µM; NCS was added at the 4th hour from the start, with the final concentration of 400ng/ml; pulsatile NCS addition means that after the first addition of
NCS to the final concentration of 400ng/ml at the 4th hour from the start, the same amount of NCS was repeatedly added every 4 hours.

miRNAs transfection and cell treatment were followed by time-lapse microscopy, producing MDM2 dynamics in individual cells in combination with cell tracking software. The latter processed acquired imaging data and transformed it into numerical raw data, from which three parameters we intended to study were extracted.

In order to analyse the number of peaks in MDM2 dynamics at single-cell level, we counted the number of MDM2 peaks in every single cell observed. Then according to the classification of p53-MDM2 dynamical profiles at single-cell level on the basis of the number of p53 and/or MDM2 peaks (see 4.5.1 for details), we presented the results of the number of MDM2 peaks by the percentage of each type of p53-MDM2 dynamics in single cells with different miRNAs transfected and NCS/ZnCl₂ treated (Figure 6.1):

![Graph A](image1)

![Graph B](image2)
Figure 6.1: The Fraction of Each Type of p53-MDM2 Dynamics in Individual Lahav’s Modified MCF7 Cells with Different miRNAs Transfected and NCS/ZnCl₂ Treated. The values on the bars in different colours were the numbers of cells performing the corresponding type of MDM2 dynamics. Every set of the experiment in every graph was done at least two times under the same condition. NC: negative control, means the group of cells transfected with scrambled miRNAs; NT: no transfection, means the group of non-transfected cells.

MDM2 dynamical traces at single-cell level were captured and recorded in 1044 cells in total, with which different drugs treated and/or miRNAs transfected. From Figure 6.1 we can see that even though in individual cells the types of MDM2 dynamics and the
number of MDM2 peaks were both heterogeneous, there were two common facts that were very clear:

1. Because non-transfected cells and cells transfected with scrambled miRNAs had no significant difference in terms of all dynamical features (including the periods of oscillations and the timing of the first MDM2 peaks shown later in this chapter), we merged them into one group named “control” (NC&NT in these experiments). In every control group, regardless of transfection and treatment, more than 50% of cells performed induced Type 2 MDM2 dynamics, suggesting the steady state of most cells in the presence of moderate stress. However, it does not necessarily mean that the heterogeneity of MDM2 dynamics in individual cells becomes less; in fact, because the categorisation was only based on the number of peaks, traces belong to the same type vary a lot in terms of many parameters, such as timing of the first peaks, periods of limit cycles, amplitude and width of peaks, range of baseline drift and so on. That is why we also studied some of the parameters listed other than the number of peaks in the last chapter and this chapter.

2. Compared to respective control groups, miR-34a over-expression increased the fraction of NCS-induced Type 3 MDM2 behaviour in individual Lahav’s modified MCF7 cells. In contrast, miR-125b-transfected cells showed higher proportion of Type 1 MDM2 dynamics but lower the percentage of Type 3 MDM2 dynamics at single-cell level. This phenomenon was seen in all sets of experiments, indicating that the over-expression of miR-34a and miR-125b can also modulate the number of peaks in NCS-induced p53-MDM2 dynamics at single-cell level in plasmid-based original Lahav’s cell model, even though there was no miRNA-targeted 3’-UTR in both MDM2-YFP and p53-CFP plasmids.

In the meantime, every different combination of transfection and treatment had its own traits, which were all listed below:

1. Although the cells in control groups and miR-125b-transfected groups were treated completely the same in Figure 6.1 A and C respectively, and their dynamical profiles also conformed to the common fact #2 listed just above, the number of MDM2 peaks and the percentage of each type of MDM2 dynamics in single cells greatly varied
between the respective set in each figure (e.g. miR-125b NCS in Figure 6.1 A VS. miR-125b NCS in Figure 6.1 C), especially the fraction of Type 3 of response. This fact reminds us of the huge heterogeneity of the data at single-cell level again.

2. According to Figure 6.1 C, with 3 times amount of miR-125b transfected, even higher fraction of Type 1 MDM2 dynamics in response to NCS was shown in single cells than that with normal amount of miR-125b transfected, indicating that the modulation of NCS-induced p53-MDM2 dynamics at single-cell level by miRNAs over-expression, which was in terms of the number of MDM2 peaks, was dose-dependent at some extent.

3. Compared to Figure 6.1 A, Figure 6.1 B reveals that pulsatile NCS addition vastly promoted the formation of MDM2 oscillations in single cells indeed, as the percentage of induced Type 3 MDM2 dynamics was expanded in every set of cells. However, if we compare Figure 6.1 B with Figure 6.1 C, we will find that pulsatile NCS addition does not actually alter the relative proportion of each cell division in control sets and miR-125b transfection sets. For miR-34a transfection set, as there is no other comparison, we tend to believe that miR-34a over-expression helps to increase the fraction of cells performing MDM2 oscillations. This result partially suggests that the existence of a sustained but not lethal source of stress facilitates p53-MDM2 oscillations, which matches with the previous conclusion that a robust p53-MDM2 limit cycle originated from the oscillatory behaviour of DNA damage sensors such as ATM/ATR and γ-H2A.X.

4. Similar to the effect of pulsatile NCS addition, Figure 6.1 D shows that ZnCl$_2$ treatment at the beginning of imaging can also help more cells perform p53-MDM2 oscillations in comparison to Figure 6.1 A, no matter which miRNA was transfected into cells. Again, the comparison with Figure 6.1 C erases the differences between the two control sets and between the two miR-125b transfection sets. We still do not know whether ZnCl$_2$ treatment actually works in the miR-34a transfection set due to lack of comparison as well. But the conclusion that ZnCl$_2$ treatment facilitates p53-MDM2 limit cycles in single BAC- and miR-34a-transfected cells could be drawn based on two facts: 1) The proportion of each cell division in miR-34a transfection set in Figure 6.1 D is very similar to its counterpart in Figure 6.1 B; 2) Given that the promoter of p53-CFP is Zn$^{2+}$-responsive and ZnCl$_2$ does not degrade automatically in growth medium, Zn$^{2+}$ is able to activate p53-CFP and then MDM2-YFP (driven by
MDM2 native promoter containing p53 RE) continuously, which is equivalent to pulsatile NCS addition that also aims to create a sustained activator of p53-MDM2 limit cycles.

6.2.2 The Timing of the First Peaks

After the investigation of the number of peaks in p53-MDM2 dynamics, then we followed the analytical pattern used in the last chapter to look at the timing of the first peaks and the periods of oscillations, aiming to better understand the heterogeneity of p53-MDM2 dynamics at single-cell level. As previously described, the timing of the first p53/MDM2 peaks in p53-MDM2 dynamics represents the sensitivity of cells to stress signal, thus it is one of the most important parameters of p53-MDM2 dynamics. As the first response of cells to DSBs, 689 values of the timing of the first MDM2 peaks we obtained from every single cell that showed either Type 2 or 3 dynamics were very heterogeneous and variable, ranging from less than 1 hour to over 20 hours. However, 366 of 689 values were between 5 hours and 10 hours, which accounted for the highest percentage of all values and were much higher than the second (167 values in the interval of 0-5 hour(s)) and the third place (85 values in the interval of 10-15 hours). The lower fraction compared to its counterpart obtained in the BAC-based model was because the addition of ZnCl$_2$ at the beginning of imaging vastly increased the number of cells performing their first MDM2 peaks earlier than the moment of NCS addition, which was supposed to be considered as rare “natural peaks” in the last chapter. If 256 values collected from the cells treated with ZnCl$_2$ from the start are ruled out, the rank of the top 3 will become: 283 of 433 values in 5-10 hours, 63 of 433 values in 10-15 hours and 39 of 433 values in 15-20 hours. Although the result was a bit more heterogeneous than that of BAC-based cell model, the timing of the first response to NCS-induced DSBs between 5 hours and 10 hours was the main pattern, which was the same as that can be seen in most individual BAC-transfected cells.

Specifically, the analysis of the impact of miRNA over-expression on the timing of the first MDM2 peaks was organised and conducted based on Figure 6.1. Note that the cells with Type 1 dynamics in every set of transfection were excluded as they did not show any peaks in MDM2 traces. For Figure 6.1 A, three sets of comparison were performed
by one-way ANOVA to distinguish if different miRNAs transfection can affect the timing of the first MDM2 peaks in single cells treated with NCS only. The results were shown below:

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The Timing of the First MDM2 Peaks: miR-34a VS. Control

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The Timing of the First MDM2 Peaks: miR-125b VS. Control

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The Timing of the First MDM2 Peaks: miR-34a VS. miR-125b

Table 6.2: The Comparison of the Timing of the First MDM2 Peaks by One-way ANOVA (95% Confidence) between Any Two Sets of Lahav’s Modified MCF7 Cells Treated with NCS and Transfected with miR-34a/miR-125b/Scrambled miRNA Mimics, Respectively.
Table 6.2 demonstrated that miRNA transfection was not able to produce an impact on the timing of the first MDM2 peaks, as all P values were above 0.05. Similar to the analysis above, the rest sets of comparison based on Figure 6.1 B, C and D were made and the results were listed below:

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The Timing of the First MDM2 Peaks: miR-34a VS. Control

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The Timing of the First MDM2 Peaks: miR-125b VS. Control

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The Timing of the First MDM2 Peaks: miR-34a VS. miR-125b

Table 6.3: The Comparison of the Timing of the First MDM2 Peaks by One-way ANOVA (95% Confidence Interval) between Any Two Sets of Lahav's Modified MCF7 Cells Treated with NCS (Pulsatile Addition) and Transfected with miR-34a/miR-125b/Scrambled miRNAs, Respectively.
Table 6.4: The Comparison of the Timing of the First MDM2 Peaks by One-way ANOVA (95% Confidence Interval) between Any Two Sets of Lahav’s Modified MCF7 Cells Treated with NCS and Transfected with 3×miR-125b/miR-125b/Scrambled miRNA Mimics, Respectively.
Table 6.5: The Comparison of the Timing of the First MDM2 Peaks by One-way ANOVA (95% Confidence Interval) between Any Two Sets of Lahav’s Modified MCF7 Cells Treated with NCS and ZnCl₂ and Transfected with miR-34a/miR-125b/Scrambled miRNA Mimics, Respectively.

All P values in the three tables above were over 0.05, which indicated two facts: 1. miR-34a and miR-125b were not able to affect the timing of the first MDM2 peaks in single cells treated with either NCS pulse or the combination of NCS and ZnCl₂; 2. Even three-fold amount of miR-125b was introduced and expressed, the timing of the first MDM2 peaks cannot be changed. These results contradicted the data and conclusion obtained
in BAC-based cell model that the timing of the first p53 and MDM2 peaks was able to be fine-tuned by miRNAs over-expression in the presence of NCS. The potential explanation will be addressed in the next chapter.

6.2.3 The Periods of Oscillations

To investigate the periods of MDM2 oscillations, every single time point corresponds with a peak value on MDM2 trajectories was collected from every single cell performing MDM2 oscillations (Type 3 dynamics). In sum, 863 values of periods of MDM2 oscillations were obtained from 228 qualified cells. According to the definition of oscillations listed in 4.5.1, the periods of p53-MDM2 oscillations must be between 4 hours and 7 hours. If we divide the defined interval of the periods of MDM2 oscillations into two halves, 486 values will be in 4-5.5 hours and 377 will be in 5.5-7 hours, which is consistent with the result in the BAC-based cell model that the values between 4 hours and 5.5 hours are more than that between 5.5 hours and 7 hours.

Similarly, we evaluated the effect of miRNAs transfection on the periods of MDM2 oscillations based on the divisions shown in Figure 6.1. Again, one-way ANOVA was used for making the comparison between any two sets of cells with different miRNAs transfected in every group of cell treatment. The results were shown below:

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**The Periods of MDM2 Oscillations: miR-34a VS. Control**
The Periods of MDM2 Oscillations: miR-125b VS. Control

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The Periods of MDM2 Oscillations: miR-34a VS. miR-125b

Table 6.6: The Comparison of the Periods of MDM2 Oscillations by One-way ANOVA (95% Confidence Interval) between Any Two Sets of Lahav's Modified MCF7 Cells Treated with NCS and Transfected with miR-34a/miR-125b/Scrambled miRNA Mimics, Respectively.

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The Periods of MDM2 Oscillations: miR-34a VS. Control

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The Periods of MDM2 Oscillations: miR-125b VS. Control

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The Periods of MDM2 Oscillations: miR-34a VS. miR-125b

Table 6.7: The Comparison of the Periods of MDM2 Oscillations by One-way ANOVA (95% Confidence Interval) between Any Two Sets of Lahav’s Modified MCF7 Cells Treated with NCS (Pulsatile Addition) and Transfected with miR-34a/miR-125b/Scrambled miRNAs, Respectively.

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82.195  152

The Periods of MDM2 Oscillations: miR-125b VS. Control

Table 6.7: The Comparison of the Periods of MDM2 Oscillations by One-way ANOVA (95% Confidence Interval) between Any Two Sets of Lahav’s Modified MCF7 Cells Treated with NCS (Pulsatile Addition) and Transfected with miR-34a/miR-125b/Scrambled miRNAs, Respectively.

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40.93082  117

The Periods of MDM2 Oscillations: 3×miR-125b VS. Control

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62.61982  153

The Periods of MDM2 Oscillations: miR-125b VS. Control
The Periods of MDM2 Oscillations: $3\times$miR-125b VS. miR-125b

Table 6.8: The Comparison of the Periods of MDM2 Oscillations by One-way ANOVA (95% Confidence Interval) between Any Two Sets of Lahav’s Modified MCF7 Cells Treated with NCS and Transfected with $3\times$miR-125b/miR-125b/Scrambled miRNA Mimics, Respectively.

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The Periods of MDM2 Oscillations: miR-34a VS. Control

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The Periods of MDM2 Oscillations: miR-125b VS. Control
### Table 6.9: The Comparison of the Periods of MDM2 Oscillations by One-way ANOVA (95% Confidence Interval) between Any Two Sets of Lahav’s Modified MCF7 Cells Treated with NCS and ZnCl₂ and Transfected with miR-34a/miR-125b/Scrambled miRNA Mimics, Respectively.

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<td>239</td>
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The Periods of MDM2 Oscillations: miR-34a VS. Control

Table 6.6-6.9 demonstrated that the periods of MDM2 oscillations showed no statistical significance with 95% confidence between any two sets of cells transfected with different miRNAs in the same group of cell treatment, indicating that miRNAs over-expression was not able to influence the periods of MDM2 oscillations at these given conditions. This result is consistent with the conclusion drawn in the BAC-based cell model, further consolidating the fact that the range of periods of p53-MDM2 oscillations is highly dependent on cellular intrinsic factors. It is also noteworthy that the comparison between the sets of cells with the same miRNA transfected but with different cell treatment was not conducted as we did not look at the impact of different cell treatment on p53-MDM2 dynamics at single-cell level.

### 6.3 Discussion

The content of discussion for this chapter was integrated into the next chapter, where the general conclusions of our project, the further data comparison and analysis among Chapter 3, 4, 5 and 6, as well as the future plan of our work, were addressed in detail. This arrangement aimed to reduce repeated content.
Chapter 7  General Conclusions and Discussion

With countless studies conducted during the past three decades since discovered, p53 has been well recognised as a central mediator in the network of stress response, indicating its essential role in cell fate determination and tumour suppression (Vousden and Prives, 2009). Basically, p53 becomes stabilised and activated once receiving stress signals from its upstream components, such as ATM, ATR, CHK1/2 and PARPs. The process of p53 activation is achieved by the alteration of PTM status and then the dissociation with the inhibitor MDM2. Activated p53 acts as a powerful transcription factor to induce the expression of various downstream target genes, leading to different biological outcomes such as DNA repair, cell apoptosis and temporary or permanent cell cycle arrest (Brady and Attardi, 2010). Therefore, by analysing the intensity of stress signals and then initiating downstream pathways accordingly, p53 is able to manipulate cellular processes and subsequent cell fate, which is the fundamental reason why p53 has been crowned as “the guardian of the genome” and “cellular gatekeeper” (Lane, 1992, Levine, 1997).

While earlier research mainly focused on p53’s structure (both gene and protein), biological function and the architecture of p53-mediated network of stress response, recent studies have placed more emphasis on the molecular mechanisms of p53 regulation. Generally speaking, the fact that p53 regulation is governed by a very complicated network has been widely accepted, which means that it takes place at various levels. For example, at genetic level, various mutations and epigenetic modifications are frequently found in p53 gene in more than half of human cancer cell lines (Vousden and Prives, 2009); at transcription level, the expression of p53 gene is regulated by a complex network containing many transcription factors and other co-regulators (Reisman et al., 2012); during translation process, some miRNAs are able to perturb mRNAs stability and efficiency of translational machinery (please see Figure 1.9 and 1.10) (Hermeking, 2012); and at post-translation level, p53 properties, such as stability and trans-activity, can be modulated as well, which is achieved by PTMs and protein-protein interactions (Kruse and Gu, 2009).
Although p53 regulation has been widely and deeply investigated, a lot of essential questions cannot be properly answered at current stage, some of which include: 1) Is there any uniform mechanism that can co-regulate p53 stability and trans-activity \textit{in vivo}? 2) How does p53 regulation at different levels cooperate to achieve a common biological outcome \textit{in vivo}? 3) How does p53 coordinate its roles both as a transcription activator and a repressor in cell fate determination? 4) What is the primary molecular mechanism of the fact that the importance of various modes of p53 regulation varies in different cell lines, tissues and species? (Kruse and Gu, 2009, Fischer et al., 2014)

Now that many broad topics regarding to p53 regulation need to be further investigated, let alone much more specific questions and controversies came from thousands of published studies. For instance: 1) Are Ser15 and Ser20 phosphorylation indispensable to p53 activation, as the conclusions drawn from \textit{in vitro} studies contradicted those acquired from \textit{in vivo} studies (Shieh et al., 1997, Garcia and Attardi, 2014)? 2) What are the major determinants that lead to the fact that the two almost identical TADs of p53 gene play completely different roles in different cellular processes, such as in tumour suppression and DDR (Garcia and Attardi, 2014)? 3) Is the activation of HATs like CBP/p300 and TIP60/MOF the consequence or the cause of p53 activation at post-translation level (Kruse and Gu, 2009)?

The examples listed above are only a very tiny part of unknown, unclarified or unexplored area in the field of p53 research, revealing the complexity of p53 and its regulatory network at some extent. Adding to this, a series of latest studies has been employing a combination of advanced single-cell and computational modelling approaches to mimic, investigate and predict the dynamical behaviour of p53 and its regulators in response to stress in single live cells, which emphasises the importance of cell heterogeneity, spatial and temporal order in p53 signalling and p53 regulatory network; those studies also unveiled a level of information that is usually lost in population-based analysis (Batchelor et al., 2009). All of these studies were initially based on a common hypothesis that the spatial and temporal behaviour of p53 in response to stress in single cells is different from the results previously obtained in a population of cells. As the field of single-cell research has been gradually developing, the objectives have been expanded to unveil the following facts: 1) Cell heterogeneity is permanently present in every aspect of cellular processes; 2) Results obtained at cell
population level are specific combinations of single-cell behaviour in a very complicated way; 3) Protein dynamics at single-cell level are meaningful, functional and manipulative, which correlates with and somehow defines cell fate (Loewer and Lahav, 2011).

When it comes to latest single-cell studies on p53, at both computational and experimental level they indeed demonstrated that p53 behaviour in response to stress in every individual cell almost fully conformed to those facts listed in the last paragraph. The major conclusions of those studies were concisely summarised as follows: 1) p53 dynamics in single cells was completely different from the results obtained at cell population level; 2) p53 dynamics at single-cell level showed substantial cell heterogeneity; 3) p53 dynamics at single-cell level was stimulus-dependent because it was dependent on the architecture of active DDR pathways; 4) Different patterns of p53 dynamics at single-cell level induced by different types of stimulation were correlated with the variation and diversity of cell fates in a particular population of cells (Loewer and Lahav, 2011, Purvis et al., 2012).

However, these studies have an obvious common drawback: the cell model used is merely based on two simple plasmids (please see Figure 4.1). This fact suggests that the behaviour of native p53 in response to stress is not able to be reproduced by the dual-plasmid-based fluorescence-labelled cassettes, weakening the reliability of their conclusions. To solve this problem, we developed a brand new BAC-based cell model, in which native p53 gene labelled with a fluorescent marker (DsRedXP) and driven by the native p53 promoter was incorporated into a BAC vector. Then this new BAC was introduced into original Lahav’s modified MCF7 cells to let fluorescence-labelled native p53 gene express in a completely natural genomic context. Nevertheless, we found that this BAC still did not meet our requirement because we cannot find BAC-transfected cells during time-lapse imaging. The reasons are as follows: 1) The level of p53 expression in unstressed cells is very low due to persistent MDM2 inhibition so fluorescence-labelled p53 cannot be used as a transfection marker; 2) There is no fluorescence-labelled cassette, which constitutively expresses at a high level, in this BAC vector as a transfection marker. To overcome this technical issue, a Lamin B1-eCFP fluorescent cassette, which encodes a constitutive component of nuclear inner membrane Lamin B1, was isolated from the plasmid pL451 and inserted into the BAC.
we previously constructed. Hence, cyan fluorescence can be detected in retrofitted BAC-transfected cells at normal growth condition while time-lapse imaging.

Our initially plan was to establish a stably BAC-transfected cell line based on original Lahav’s cell model, which aims to better mimic the natural cellular environment and the total copy number of p53 gene. However, despite our efforts, BAC stable transfection did not succeed and we speculated that in cancer cell lines p53 regulatory network was tuned to reflect a specific balance of its components so cells were not be able to bear ectopic level of p53 expression, even if BAC stable transfection theoretically only led to moderate, natural and stable over-expression. As a consequence, cells were prone to death or, if they managed to survive in this case, they must drive exogenous p53 gene off possibly by shearing the BAC vectors and degrading p53-DsRedXP cassette, leading to the results shown in Figure 5.3. Hence we had to use BAC transient transfection, which while more variable than stable transfection in terms of the total copy number and the expression level of p53 gene, was able to create a system that appeared to mimic native p53 signalling network over short periods of time. Because p53-DsRedXP fusion protein is partially encoded by native p53 gene and MDM2-YFP fusion protein is driven by native MDM2 promoter, p53-DsRedXP can activate the expression of MDM2-YFP, thus the level of two fluorescence-labelled proteins is directly correlated. Due to the same reason, p53-DsRedXP is able to induce many p53 downstream target genes and be regulated by miRNAs such as miR-125b. All of above advantages make this cell model a good system to investigate p53 and MDM2 dynamics simultaneously and the effect of miRNAs on p53-MDM2 dynamics in response to stress in single cells.

After clarifying the research background and cell model used, next we were going to address the most important objective we wanted to achieve in our project, which was to look at whether miRNAs can modulate p53-MDM2 dynamics in response to DSBs at single-cell level, whether their regulation can produce an impact on cell fate determination and how they make it. The reason why we were interested in this topic lies in the fact that different lines of evidence in literature have indicated that miRNAs were involved in the regulation of p53 and its signalling in many ways (Figure 1.9 and 1.10). Hence, miR-34a and miR-125b were chosen on the basis of the following facts: 1) Both of them have been previously described as the regulators of p53 and its network; 2) miR-125b directly targets to p53 mRNAs to perturb its translation process, whereas
miR-34a and p53 form a positive feedback loop via a NAD-dependent deacetylase SIRT1; 3) Previous investigations had demonstrated that miRNAs were involved in p53 regulation via positive/negative feedback loops or feed-forward pathways; 4) Previous computational studies also illustrated that tweaking the interaction between the feedback loops in p53 regulatory network can alter p53-MDM2 dynamics in single cells; 5) Cell fates can be affected by the manipulation of p53-MDM2 dynamics (Hermeking, 2012, Toettcher et al., 2010, Le et al., 2009, Yamakuchi et al., 2008, Purvis et al., 2012). According to these conclusions, we hypothesised that the over-expression of miR-34a and miR-125b may potentially influence the profiles of p53-MDM2 dynamics in individual cells and subsequent cell fate determination.

The BAC-based cell model described above is a much better platform to verify our hypothesis and achieve our research aims than the plasmid-based model because full-length p53 gene, together with its upstream and downstream regulatory components, were completely integrated into the BAC we developed and used, delivering native p53 behaviour in our cell model. And unlike the plasmid-based cell model, the effect of p53-related miRNAs on p53 dynamics at single-cell level can also be directly detected in the BAC-based cell model. Furthermore, we extended the total length of imaging period, thus most cells’ fates can actually be seen in a fluorescent microscope, which is much more convincing than the previous conclusions based on the results of the indirect experiments (Purvis et al., 2012). Therefore, in combination with more native genomic and cellular context created and the ability to capture actual fate of every single cell during imaging, the conclusion of what impact miR-34a and miR-125b produced on p53 behaviour at single-cell level, and subsequently on cell fates, drawn from our BAC-based model, is much more convincing than that from previous plasmid-based model.

However, this model also has a major disadvantage. As BAC transient transfection cannot overcome very low BAC transfection efficiency, it was still difficult to capture sufficient BAC-transfected cells in an imaging assay, which means that multiple repeats were needed to collect enough samples. For example, in Chapter 5 the results were obtained from 111 cells, which were collected from totally 8 independent runs of time-lapse microscopy. Despite of this, it is still necessary to collect more samples to make results of single-cell research more reliable statistically. Therefore, in Chapter 6 we got rid of the BAC vector and did the same experiments in original Lahav’s model and
collected much more samples (1044 cells) than any other previous studies. By performing the experiments in Chapter 6, we not only collected much more samples to produce more precise data and thus make the conclusions more convincing, but also provided an opportunity to compare the data with the ones obtained in Chapter 3 (population-based), Chapter 4 (control, miRNAs not involved) and Chapter 5 (BAC-based model). The analysis of the comparison, which will be addressed later in this chapter, is another brand new and innovative content of our project.

Going over all of the results from Chapter 3 to 6, we summarise the major conclusions of our project as follows:

In a population of cells:

1. p53 signaling, including the essential upstream and downstream components, were activated by NCS-induced DSBs (Figure 3.1 and 3.2).
2. In all cell lines we used, any of p53 isoforms was not detected by Western Blotting.
3. The damped oscillations of some key components of p53 signaling shown by Western Blotting in previous studies were not present in our study. Instead, the dynamics of p53 signaling we demonstrated were much similar to single-peak or single-bump profiles (Figure 3.2).
4. RT-qPCR assays indicated that miR-34a expression increased in response to NCS while miR-125b expression was down-regulated following NCS addition; both changes were time- and tissue-dependent (Figure 3.3 A).
5. miR-34a over-expression up-regulated the level of some key upstream and downstream components of p53 signaling in response to NCS whereas miR-125b transfection made an opposite impact under the same condition (Figure 3.4-3.6).

In single original Lahav's modified MCF7 cells (the plasmid-based model):

1. The level of NCS-induced DNA damage varied from cell to cell but overall it showed time- and concentration-dependent manner (Figure 4.2-4.3).
2. MDM2 dynamics showed great heterogeneity and so did some of their parameters. Specifically, from the statistical point of view, the profile of every different combination of transfection and treatment had its own traits but shared some common features as well.
3. Cell treatment alone (with no miRNAs transfected) cannot produce any significant impact on the proportion of each cell division in regard to the number of peaks (Figure 4.6, 4.8 and 4.10) and the timing of the first peak (Figure 4.7, 4.9 and 4.11) in MDM2 dynamics induced by NCS. It is not able to affect the periods of MDM2 oscillations as well (95% confidence interval) (Table 4.1 and 4.2).

4. In comparison with all results mentioned in the second point and all data obtained from the cells transfected with scrambled miRNAs, miR-34a over-expression increased the proportion of Type 3 cell division while decreased the proportion of Type 1 cell division regardless of different cell treatment (Figure 6.1); whereas miR-125b transfection made an opposite effect regardless of different cell treatment, which was miR-125b dose-dependent (Figure 6.1).

5. ZnCl₂ and pulsatile NCS addition can promote the proportion of Type 3 cell division while decreased the proportion of Type 1 cell division only in miR-34a transfection sets (Figure 6.1).

6. Any of miRNAs over-expression was not able to manipulate the timing of the first MDM2 peaks (95% confidence interval) (Table 6.2-6.5) and the periods of MDM2 oscillations (95% confidence interval) (Table 6.6-6.9).

In single BAC-transfected Lahav's modified MCF7 cells (the BAC-based cell model):

1. Again, the profiles of p53-MDM2 dynamics, as well as some of their specifications, were highly variable from cell to cell. However, they also shared common features.

2. Compared with the control, miR-34a over-expression greatly increased the percentage of cells with p53-MDM2 oscillations, which further facilitated cell survival; while miR-125b over-expression lowered this proportion and hence did not result in an increase of cell survival rate (Table 5.1).

3. Compared with the control, miR-34a over-expression overall made the first p53 and/or MDM2 peaks appear earlier in most cells while miR-125b over-expression led to an opposite consequence (Figure 5.6).

4. Compared with the control, any of miRNA over-expression cannot made significant change in the periods of both p53 and MDM2 oscillations (95% confidence interval) (Table 5.2, 5.3 and 5.5).
As these conclusions have been analysed and discussed individually in each chapter, here we only dissect the differences between them (especially between Chapter 5 and 6) and compare them with published ones as well. First of all, we found that in the plasmid-based model, miRNAs over-expression cannot change the timing of the first peaks; while in BAC-based model both miR-34a and miR-125b enabled it. Given the fact that the major differences between the two cell models were vectors and transfection methods, we speculate that both of the facts may contribute to this significant difference, especially the absence of many key regulatory elements such as miRNAs’ target sequence 3’-UTRs in p53-CFP plasmid compared to the retrofitted BAC. In other words, complete native genes in BACs allow and achieve more elaborate fine-tuning than mini-genes in plasmids. In addition, this result implies a new perspective of understanding the mechanisms of miRNAs regulation other than repressing the translation process of target mRNAs, as the timing of the first p53 and/or MDM2 peaks represents the sensitivity of p53 and MDM2 to stress and their accumulation rate in nuclei in response to stress. And this conclusion is reasonable because miR-34a advanced the timing of the first p53 and/or MDM2 peaks and thus increased the sensitivity of p53 signalling in response to NCS, which is consistent with the fact that miR-34a facilitates the formation of p53-MDM2 oscillations and cell survival. And so does miR-125b. Of course, this effect can only be seen at single-cell level, which indicates that single-cell studies deliver more perspectives than population-based research to look at a topic, as the latter can only show changes in terms of intensity, amount and function.

The second difference between the results obtained from the two cell models is that no matter which miRNA was transfected, the range of the periods of p53-MDM2 oscillations was tighter in BAC-transfected Lahav’s modified MCF7 cells than that in original Lahav’s modified MCF7 cells, although the requirement that the range of periods has to be between 4 and 7 hours is applicable to both cell models (see 4.5.1). Specifically, the actual range of periods of p53 and MDM2 oscillations in the BAC-based cell model was between 4.23 hours and 6.48 hours (p53 traces) and between 4.26 hours and 5.87 hours (MDM2 traces), respectively; as a comparison, in the plasmid-based model the range was from 4.15 hours to 6.94 hours (p53 traces) and from 4.19 hours to 6.73 hours, respectively (including the data from Chapter 4 and 6 both). According to these results, it can be concluded that the temporal order of p53-MDM2 dynamics was
regulated more precisely in the BAC-based cell model than in the plasmid-based cell model. And the regulation is potentially autonomous in that it is independent of miRNAs over-expression. Moreover, because the periods of p53-MDM2 oscillations largely depend on the turnover rate (including half-life, production rate and degradation rate) of p53 and MDM2, the tighter range of periods indicates a higher p53 and MDM2 turnover rate in the BAC based cell model than in the plasmid-based cell model, which perhaps lies in the fact that the temporal order of p53 and/or MDM2 gene expression was different between the two cell models.

Combined with the analysis above, it becomes evident that these differences better reflect the importance of choosing an appropriate transgenic system to investigate a complex biological process, such as the dynamics of p53 signalling in response to DNA damage.

Apart from discrepancies, in the meantime p53-MDM2 dynamics in the two cell models share some common characteristics as well, such as vastly heterogeneous profiles, highly variable, damage dose-independent peak amplitude (represents the highest level of p53 and/or MDM2 in a nucleus) and peak width (determined by the length of the period during which p53 and/or MDM2 express in a nucleus), miRNAs over-expression-independent oscillatory periods and the inevitable “natural peaks”, etc. These common traits suggest that p53-MDM2 dynamics are partially shaped by intrinsic properties of cells, such as cellular micro-environment, cell-cell contacts and basic metabolic rate, etc.

It is also noteworthy that in both cell models, the cells performing non-oscillatory p53-MDM2 dynamics (Type 1 and 2 divisions in Chapter 4 and 6) were more than those showing p53-MDM2 oscillations, no matter which miRNA over-expressed, or if any miRNA over-expressed in them, suggesting that it may be not necessary for every single cell to performed p53-MDM2 oscillations to maintain a steady state in the presence of 400ng/ml NCS (final concentration), a moderate stressed condition, till the end of imaging. The data in Chapter 5 partially supports this speculation because we did not find a direct causal relationship between non-oscillatory p53-MDM2 dynamics and cell fate determination in the BAC-based cell model, which was in contrast with the clear fact that p53-MDM2 oscillations facilitated cell survival in the BAC-transfected cells. Regretfully, due to the limitations of the plasmid-based cell model (please see 5.2.1 and
6.2), cell fates cannot properly correlate with p53-MDM2 dynamics and thus were not recorded; so the similar conclusion was not able to be drawn from Chapter 4 and 6 to testify this speculation.

After analysing the differences and common points between the two cell models, then we zoomed in on the plasmid-based cell model solely. The first point we were going to dissect was why MDM2 dynamics in single Lahav’s modified MCF7 cells were able to be manipulated by miRNAs, as both p53-CFP and MDM2-YFP plasmids had no 3’-UTR sequence. In fact, over-expressed miR-125b mimics first target to 3’-UTRs of p53 mRNAs transcribed from endogenous p53 genes, leading to the suppression of endogenous p53 expression. Then p53 down-regulation reduces expression level of endogenous MDM2 and MDM2-YFP, followed by another rise of endogenous p53 expression. Moreover, ectopic level of miR-34a promotes p53-MDM2 oscillations via a more indirect pathway: targets to 3’-UTRs of SIRT1 mRNAs first, then inhibits SIRT1 level, followed by the increase of endogenous p53 stability and trans-activity, hence results in the up-regulation of endogenous MDM2 and MDM2-YFP, and finally makes endogenous p53 level fall. That is to say, in plasmid-based cell model the impact of both miRNAs is indirect, thus giving rise to many differences from the BAC-based cell model where miR-125b can directly target to p53-DsRedXP and miR-34a can up-regulate p53-DsRedXP via a SIRT1-mediated positive feedback loop. Therefore, the BAC-based cell model much better mimics the actual behaviour in single cells and thus the results obtained from it are supposed to be more convincing.

The second interesting point is that different cell treatment (one-off NCS addition, one-off ZnCl₂ and NCS addition, and pulsatile NCS addition) can make an impact on p53-MDM2 dynamics in Chapter 6 but not in Chapter 4. Specifically, in Chapter 6 we found that this conclusion was only applicable to miR-34a transfection sets because: 1) For control sets and miR-125b transfection sets, the result of the comparison between Figure 6.1 B and C contradicted that of between Figure 6.1 B and A; 2) For control sets and miR-125b transfection sets, the result of the comparison between Figure 6.1 D and C contradicted that of between Figure 6.1 D and A. So here the discussion is only limited to miR-34a transfection sets. It can be seen that the only difference between the two experiments is that miR-34a were not introduced in Chapter 4, whereas in Chapter 6 it was over-expressed in the same cell model to look at its effect on p53-MDM2 dynamics.
in individual cells. However, we did not find any definite evidence, whether from our project or from previous research, to support the explanation that it is because of miR-34a over-expression that leads to the fact that pulsatile NCS addition and one-off ZnCl₂ and NCS addition promote p53-MDM2 oscillations in single cells transfected with miR-34a. Even though we speculate that the phenomenon probably results from miR-34a transfection-induced increase of p53 sensitivity to stress, which has been demonstrated by advancing the timing of the first p53 and/or MDM2 peaks in p53-MDM2 dynamics, further investigations and results are strongly required. However, the influence of different cell treatment on p53-MDM2 dynamics is not the main objective of our project so we did not invest too much on it; but we have been aware of its importance and put in into the list of our future plan.

Apart from this, our future work also includes the following items:

1. Continue to repeat the imaging assay using the BAC-based cell model to collect more samples and thus test the reliability of the conclusions in Chapter 5.
2. Try to develop a new BAC-based cell model in which the expression of fluorescence-labeled native p53 and MDM2 no longer relies on exogenous gene expression vectors. As mentioned in 5.5, this goal can be achieved either by a new genome-editing technique CRISPR/Cas9 or by stably transfecting cells with a BAC containing a fluorescence-labeled native MDM2 gene before p53-DsRedXP-Lamin B1-eCFP BAC stable transfection.
3. Since previous research indicated that p53-MDM2 dynamics was stimulus-dependent, it would be interesting to look at whether miRNAs can modulate p53-MDM2 dynamics in response to UV-induced SSBs at single-cell level, whether their regulation can produce an impact on cell fate determination and how they achieve it.
References


