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<th>The response of mouse mesenchymal stromal cells to radiation-induced DNA double-strand breaks</th>
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<td><strong>Author(s)</strong></td>
<td>Sugrue, Tara Kimberly</td>
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THE RESPONSE OF MOUSE MESENCHYMAL STROMAL CELLS TO RADIATION-INDUCED DNA DOUBLE-STRAND BREAKS

A thesis submitted to the National University of Ireland, Galway in fulfilment of the requirements for the degree

Doctor of Philosophy

By

Tara Sugrue, B.Sc. (Hons)

Immunology Group & Genome Stability Laboratory,
Regenerative Medicine Institute & Centre for Chromosome Biology,
National University of Ireland, Galway

Thesis Supervisor: Prof. Rhodri Ceredig
Thesis Co-supervisor: Prof. Noel F. Lowndes

September 2013
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Mesenchymal stromal cells (MSCs) are radio-resistant stem cell progenitors that support haematopoiesis in the bone marrow and contribute to the tumour microenvironment. The mechanisms that drive MSC radio-resistance are poorly understood. Ionising radiation (IR) negatively impacts on cell survival largely due to the generation of DNA lesions, particularly of highly genotoxic DNA double-strand breaks (DSBs). The DNA Damage Response (DDR) represents a network of signalling pathways that enable cells to activate biological responses to genotoxic stress, including DNA DSBs. In this study, the role of the DDR in mediating mouse MSC radio-resistance was investigated. Multiple DDR mechanisms synergistically contributed to MSC radio-resistance: robust DDR initiation; DNA damage checkpoint activation and efficient DNA DSB repair. Irradiated mouse MSCs could withstand IR-induced apoptosis; continued to proliferate and could differentiate along mesenchymal-derived lineages. MSCs reside in hypoxic niches within the bone marrow and tumour microenvironments. Herein, hypoxic MSCs exhibited (i) enhanced survival post irradiation; (ii) improved recovery from IR-induced cell cycle arrest and (iii) an increased DNA DSB repair capacity. In addition, HIF-1α was identified as an important mediator of the increased DNA DSB repair capacity of hypoxic MSCs.

Double negative II (DN2) thymocytes are radio-resistant T lymphocyte precursors that reside in the thymus. The mechanisms underlying DN2 radio-resistance are also un-described. Given the important role of the DDR in mediating MSC radio-resistance, the DDR of DN2 thymocytes to IR-induced DNA DSBs was also characterised in this study. Multiple DDR mechanisms were also found to contribute to DN2 radio-resistance including (i) rapid DDR initiation; (ii) induction of a radio-protective G1 checkpoint and (iii) activation of DNA DSB repair.

For the first time, this study demonstrates that (i) the DDR is fundamental for mediating mouse MSC resistance to IR-induced DNA DSBs; (ii) hypoxia alters the DDR of irradiated mouse MSCs and (iii) DN2 thymocytes activate the DDR to IR-induced DNA DSBs.
For my family, my grandmother Hannah & my grand-aunt Máire
ACKNOWLEDGEMENTS

I want to sincerely thank my supervisors, Rhodri and Noel. Rhodri, thank you for always having confidence in me and my ability, for your constant guidance and for sharing your wealth of knowledge with me. It has been a pleasure to be mentored by you. Noel, thank you for adopting me as a member of the Lowndes lab. Your advice and critique always stimulated me to do my best and, together with the expertise in your lab, have been indispensable for my project and for helping me become a scientist. I am truly grateful for your generosity. I wish to thank Ton Rolink for the fantastic opportunity to work in his laboratory in Basel. Thank you for showing me how enjoyable and exciting research can be and for sharing your immense knowledge and expertise. I also wish to thank Matt and Thomas for their unending support, enthusiasm and ideas. I am very grateful for your time and patience – particularly at lab meetings! I also wish to acknowledge the Irish Research Council, EMBO short-term fellowship scheme and the Thomas Crawford Hayes fund, NUI Galway, for funding my PhD work.

Words cannot describe my gratitude to my parents, Mary and Teddy, and my brother Rory. Your unfailing support has guided me through the best and worst of times and without you, none of this would have ever been possible. Thank you for always being there and I hope one day I can return the favour.

I want to thank everybody in REMEDI and the CCB who have helped me reach this goal – your time, help and advice have been invaluable. I particularly want to thank Michael Rainey and Alessandro Natoni for never saying ‘no’ – I hope to be able to help others as much as you have helped me in the coming years. I also want to say a big thank you to ‘the cloning experts’ – Marta, Emma, Janna, Louise, Danielle and Sylvie – for helping me so much during my PhD – I couldn’t have done it without you guys! Lilly, Anja, Matthias Martin and Flo – thank you so much for your unending help and for such a great time during my stay in Basel.

To all my friends and colleagues in REMEDI and the CCB – thank you for all the laughs, support and for never giving up on me in spite of my absence. You made every day in the lab an enjoyable one for me and I am so happy to know you all – stay as you are!
### ABBREVIATIONS

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>5’-FU</td>
<td>5’-fluorouracil</td>
</tr>
<tr>
<td>53BP1</td>
<td>p53 binding protein 1</td>
</tr>
<tr>
<td>α-SMA</td>
<td>alpha-smooth muscle actin</td>
</tr>
<tr>
<td>α-MEM</td>
<td>Minimum essential Eagle medium alpha</td>
</tr>
<tr>
<td>γ-H2AX</td>
<td>gamma-H2A.X(H2A.X Ser139 phosphorylation)</td>
</tr>
<tr>
<td>ACK</td>
<td>Ammonium-Chloride-Potassium</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>Apoptotic protease-activating factor 1</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell / Allophycocyanin</td>
</tr>
<tr>
<td>ARF</td>
<td>Alternative reading frame</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia telangiectasia-mutated</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-5’- triphosphate</td>
</tr>
<tr>
<td>ATR</td>
<td>Ataxia telangiectasia and Rad3 related</td>
</tr>
<tr>
<td>NH</td>
<td>NUP98-HOXB4</td>
</tr>
<tr>
<td>Bak</td>
<td>Bcl-2 antagonistic killer</td>
</tr>
<tr>
<td>Bax</td>
<td>Bcl-2 associated X protein</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B cell lymphoma-2</td>
</tr>
<tr>
<td>Bcl-XL</td>
<td>B cell lymphoma-extra long</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>BD</td>
<td>Becton Dickinson</td>
</tr>
<tr>
<td>BH3</td>
<td>Bcl-2 homology 3</td>
</tr>
<tr>
<td>Bim</td>
<td>Bcl-2 interacting mediator of cell death</td>
</tr>
<tr>
<td>BLM</td>
<td>Bloom syndrome protein</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>BMT</td>
<td>Bone marrow transplantation</td>
</tr>
<tr>
<td>BRCA1/2</td>
<td>Breast cancer type 1/2</td>
</tr>
<tr>
<td>BrdU</td>
<td>5’-bromo-deoxyuridine</td>
</tr>
</tbody>
</table>
BSA  Bovine serum albumin
BSC  Hair follicle bulge stem cell
CAF  Cancer-associated fibroblast
CAR  CXCL12-abundant reticular
CCL5 Chemokine (C-C motif) ligand 5
CD  Cluster of differentiation
CDC  Cell division cycle
CDK  Cyclin-dependent kinase
CFU-F/-S Colony forming unit-fibroblast/-spleen
CHK1/2 Checkpoint kinase 1/2
CHO  Chinese hamster ovary
CKI  CDK inhibitor
CLL  Chronic lymphocytic leukemia
CMJ  Corticomedullary junction
CML  Chronic myeloid leukemia
CSL  CBF-1 suppressor of hairless Lag1
CSR  Class switch recombination
CTBP  C-terminus binding protein of adenovirus E1A
cTEC  Cortical thymic epithelial cell
CtIP  CTBP-interacting protein
CXCL12 C-X-C motif chemokine ligand 12
D  Diversity gene segment / Displacement
DAPI  4',6-diamidino-2-phenylindole
DBM  Department of Biomedicine
DC  Dendritic cell
DDR  DNA Damage Response
DL1-4 Delta-like ligand 1-4
DL4-Fc Delta-like ligand 4 – IgG1 Fc fusion protein
DMEM Dulbecco’s modified Eagle’s medium
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>DN</td>
<td>Double negative</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNA-PK&lt;sub&gt;cs&lt;/sub&gt;</td>
<td>DNA-dependent protein kinase, catalytic subunit</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>DP</td>
<td>Double positive</td>
</tr>
<tr>
<td>DSB</td>
<td>Double-strand break</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-to-mesenchymal transition</td>
</tr>
<tr>
<td>ETP</td>
<td>Early thymus seeding progenitor</td>
</tr>
<tr>
<td>EYA</td>
<td>Eyes absent homolog 1</td>
</tr>
<tr>
<td>Fab</td>
<td>Fragment antigen-binding</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FAP</td>
<td>Fibroblast activation protein</td>
</tr>
<tr>
<td>Fc</td>
<td>Fragment crystallisable</td>
</tr>
<tr>
<td>FCGR1</td>
<td>Fc fragment of IgG high Affinity 1a receptor</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FTOC</td>
<td>Foetal thymus organ culture</td>
</tr>
<tr>
<td>G&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Gap phase 1</td>
</tr>
<tr>
<td>G&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Gap phase 2</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GVHD</td>
<td>Graft-versus-host disease</td>
</tr>
<tr>
<td>FIH</td>
<td>Factor inhibiting HIF-1</td>
</tr>
<tr>
<td>H2-K</td>
<td>Histocompatibility 2, K region</td>
</tr>
<tr>
<td>H2AX</td>
<td>Histone H2A.X</td>
</tr>
<tr>
<td>H3</td>
<td>Histone H3</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
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</tr>
<tr>
<td>H3Ser10</td>
<td>Histone H3 Ser10 phosphorylation</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HD</td>
<td>Host-derived</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia-inducible factor</td>
</tr>
<tr>
<td>HOXB4</td>
<td>Homeobox B4</td>
</tr>
<tr>
<td>HR</td>
<td>Homologous recombination</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HSC</td>
<td>Haematopoietic stem cell</td>
</tr>
<tr>
<td>HU</td>
<td>Hydroxyurea</td>
</tr>
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<td>HUF</td>
<td>Anti-human IgG1 Fc</td>
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<tr>
<td>ICN1</td>
<td>Intracellular Notch1</td>
</tr>
<tr>
<td>IDO</td>
<td>Indoleamine 2,3-dioxygenase</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-7Ra</td>
<td>Interleukin-7 receptor α chain</td>
</tr>
<tr>
<td>IMDM</td>
<td>Iscove’s modified Dulbecco’s medium</td>
</tr>
<tr>
<td>IR</td>
<td>Ionising radiation / irradiation</td>
</tr>
<tr>
<td>IRES</td>
<td>Internal ribosome entry site</td>
</tr>
<tr>
<td>IRIF</td>
<td>IR-induced foci</td>
</tr>
<tr>
<td>ISP</td>
<td>Immature single-positive</td>
</tr>
<tr>
<td>J</td>
<td>Joining gene segment</td>
</tr>
<tr>
<td>KO</td>
<td>Knock-out</td>
</tr>
<tr>
<td>KSC</td>
<td>Keratinocyte stem cell</td>
</tr>
<tr>
<td>Lepr</td>
<td>Leptin receptor</td>
</tr>
<tr>
<td>Lin</td>
<td>Lineage</td>
</tr>
<tr>
<td>LSK</td>
<td>Lin⁺ Sea-1⁺ c-kit⁺</td>
</tr>
<tr>
<td>LT</td>
<td>Long-term</td>
</tr>
<tr>
<td>M</td>
<td>Mitosis</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage colony stimulating factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>MDC1</td>
<td>Mediator of DNA damage checkpoint protein 1</td>
</tr>
<tr>
<td>MDM2</td>
<td>Mouse double minute 2</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse embryonic fibroblast</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MRE11</td>
<td>Meiotic recombination 11</td>
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<tr>
<td>MRN</td>
<td>Mre11-Rad50-Nbs1</td>
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<tr>
<td>MSC</td>
<td>Mesenchymal stromal cell</td>
</tr>
<tr>
<td>mTEC</td>
<td>Medullary thymic epithelial cell</td>
</tr>
<tr>
<td>N1</td>
<td>Notch1 receptor</td>
</tr>
<tr>
<td>NBS1</td>
<td>Nimegen breakage syndrome 1</td>
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<tr>
<td>NCBES</td>
<td>National Centre for Biomedical Engineering Science</td>
</tr>
<tr>
<td>NHEJ</td>
<td>Non-Homologous End Joining</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>Noxa</td>
<td>Named for ‘’damage’’</td>
</tr>
<tr>
<td>NUI</td>
<td>National University of Ireland</td>
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<tr>
<td>NUP-98</td>
<td>Nucleoporin-98</td>
</tr>
<tr>
<td>pRB</td>
<td>Retinoblastoma protein</td>
</tr>
<tr>
<td>pVHL</td>
<td>Von Hippel-Lindau tumour suppressor protein</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
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<tr>
<td>PAR</td>
<td>Poly-(ADP) ribose</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly-(ADP) ribose polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered Saline</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate buffered Saline / Tween®-20</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PDGFR</td>
<td>Platelet-derived growth factor receptor</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
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<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
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<td>PGE2</td>
<td>Prostaglandin E2</td>
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<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>PHD</td>
<td>Prolyl hydroxylase domain</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PI(3)K</td>
<td>Phosphatidylinositol-3-kinase</td>
</tr>
<tr>
<td>PMC</td>
<td>Perimedullary cortex</td>
</tr>
<tr>
<td>PTM</td>
<td>Post-translational modification</td>
</tr>
<tr>
<td>Puma</td>
<td>p53 up-regulated modulator of apoptosis</td>
</tr>
<tr>
<td>Rad</td>
<td>Recombinase radiation sensitive</td>
</tr>
<tr>
<td>RAG</td>
<td>Recombination activating gene</td>
</tr>
<tr>
<td>REMEDI</td>
<td>Regenerative Medicine Institute</td>
</tr>
<tr>
<td>RIF-1</td>
<td>Rap1-interacting factor</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNF</td>
<td>RING finger protein</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>S-MuLV</td>
<td>Soule murine leukemia virus</td>
</tr>
<tr>
<td>Sca-1</td>
<td>Stem cell antigen-1</td>
</tr>
<tr>
<td>SCF</td>
<td>Stem cell factor</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immuno-deficiency</td>
</tr>
<tr>
<td>SCZ</td>
<td>Sub-capsular zone</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SF-IMDM</td>
<td>Serum-free Isocove’s modified Dulbecco’s medium</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering ribonucleic acid</td>
</tr>
<tr>
<td>SNO</td>
<td>Spindle-shaped N-cadherin$^+$ CD45$^-$ osteoblasts</td>
</tr>
<tr>
<td>SP</td>
<td>Single positive</td>
</tr>
<tr>
<td>SSB</td>
<td>Single-strand break</td>
</tr>
<tr>
<td>ST</td>
<td>Short-term</td>
</tr>
<tr>
<td>T-ALL</td>
<td>T-acute lymphoblastic leukaemia</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline / Tween®-20</td>
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<tr>
<td>TBI</td>
<td>Total-body irradiation</td>
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12
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TEC</td>
<td>Thymic epithelial cell</td>
</tr>
<tr>
<td>Tg</td>
<td>Transgenic</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-beta</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra-violet</td>
</tr>
<tr>
<td>V</td>
<td>Variable gene segment</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<tr>
<td>WT</td>
<td>Wild type</td>
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<td>XLF</td>
<td>XRCC4-like factor</td>
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Chapter 1

Introduction
Introduction

1.1 MESENCHYMAL STROMAL CELLS: GUARDIANS OF BONE MARROW FUNCTION

In adults, the bone marrow (BM) is primarily responsible for haematopoiesis, the process of blood cell formation. Here, haematopoietic stem cells (HSCs) continuously give rise to haematopoietic progenitors that differentiate into specialised blood and immune cell types that circulate in the periphery. HSC self-renewal and differentiation must be tightly regulated to ensure that mature peripheral blood cells are continuously generated without exhausting the HSC pool. This delicate balance is achieved by developmental cues provided by the BM microenvironment known as the HSC niche. The HSC niche is comprised of multiple cell types which are distributed between two compartments: (i) the endosteal niche located between the bone and the marrow space and (ii) the perivascular niche located within the sinusoids of the BM cavity (Figure 1.1) [Li & Li, 2006; Celso & Scadden, 2011; Ehninger & Trumpp, 2011; Wang & Wagers, 2011]. Slowly cycling, long-term (LT) HSCs are hypothesised to be located in the endosteal niche whereas rapidly cycling, differentiating short-term (ST) HSCs are located within the sinusoids (Figure 1.1) [Calvi et al. 2003; Wilson & Trumpp, 2006; Wilson et al. 2008; van der Wath et al. 2009].

Mesenchymal stromal cells (MSCs) are a fundamental component of the HSC niche. MSCs are traditionally defined as BM-derived fibroblast-like adherent cells that generate colony-forming units (CFU-F) in vitro [Friedenstein et al. 1970 & 1974]. They are also multi-potent progenitors that differentiate along mesenchymal lineages to become adipocytes (fat cells), osteocytes (bone cells) and chondrocytes (cartilage cells) [Friedenstein et al. 1966; Grigoriadis et al. 1988; Kuznetsov et al. 1997; Pittenger et al. 1999; Morikawa et al. 2009]. MSCs are phenotypically distinguished by (i) the presence of cell surface markers including cluster of differentiation (CD)73, CD44, CD90, Stem cell antigen (Sca)-1 and Platelet-derived growth factor receptor (PDGFR)-α and (ii) by the absence of haematopoietic cell surface markers including CD45, CD4 and CD3 [Kode et al. 2009; Morikawa et al. 2009; Tolar et al. 2010]. Although clonal MSCs have been extensively used as a supportive system for studying haematopoiesis in vitro, particularly B lymphopoiesis [Dexter et al. 1977; Whitlock & Witte, 1982; Quesenberry, 1989], the lack of unique mouse MSC surface
markers has impaired extensive characterisation of MSCs in vivo. Consequently, unlike HSCs, the origin, functions and true lineage potential of MSCs in vivo remain ill-defined.

However, in recent years, advances in transgenesis have initiated in vivo characterisation of MSCs. These studies have revealed the existence of MSC sub-populations of varying differentiation potential and function within the HSC niche. These MSC sub-populations orchestrate haematopoiesis via direct cell contact and via the production of various cytokines, growth factors and extracellular matrix (ECM) molecules. For example, a population of osteo-lineage restricted MSCs expressing Mx1 (myxovirus resistance protein 1) give rise to osteoblasts in vivo and remodel the BM niche in response to injury [Park et al. 2012]. Osteoblasts, including spindle-shaped N-cadherin+ CD45- (SNO) cells, (i) maintain HSC pool size within the endosteal niche and (ii) produce ECM molecules [e.g. N-Cadherin and Vascular cell adhesion molecule-1 (VCAM-1)] that provide physical support for HSCs [Calvi et al. 2003; Zhang et al. 2003; Taichman, 2005]. Both osteo-chondrogenic Nestin+ (an intermediate filament protein) and Leptin receptor+ MSC populations maintain HSCs within the perivascular niche, preventing extramedullary haematopoiesis [Méndez-Ferrer et al. 2010; Ding et al. 2012].

Interestingly, several MSC sub-populations of differing functions which express the chemokine C-X-C motif chemokine ligand 12 (CXCL12) have also recently been identified. CXCL12 production by Osterix (transcription factor involved in osteogenesis)-expressing MSCs maintains HSC progenitors and B lymphopoiesis in the endosteal niche [Greenbaum et al. 2013]. This MSC population also consists of a subset of adipo-osteogenic progenitors known as CXCL12-abundant reticular cells (CAR) cells. CAR cells produce large amounts of CXCL12 and stem cell factor (SCF) and function to promote HSC self-renewal and to inhibit HSC differentiation [Omatsu et al. 2010; Greenbaum et al. 2013]. CXCL12-producing Nestin+ MSCs regulate HSC self-renewal in the perivascular niche [Ding & Morrison, 2013; Greenbaum et al. 2013]. In addition, PDGFRα+ Sca-1+ fibroblast activation protein-α (FAP)+ MSCs also produce CXCL12 and SCF and regulate B lymphopoiesis and erythropoiesis in the BM [Roberts et al. 2013; Tran et al. 2013]. Taken together, these findings suggest that interplay between MSC sub-populations in the HSC niche is likely required for orchestrating different stages of haematopoiesis in vivo.
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The BM niche is a relatively hypoxic (2-8% O₂) environment (Figure 1.1). Recent studies have demonstrated that hypoxia promotes HSC quiescence, differentiation potential and re-constitutive capacity [Eliasson & Jönsson, 2009; Guitart et al. 2010; Moyheldin et al. 2010; Winkler et al. 2010]. Although the effects of hypoxia on MSCs in vivo are largely unknown, in vitro studies indicate that hypoxia (i) increases the proliferation rate; (ii) suppresses senescence; (iii) induces pluripotency gene expression and telomerase activity; (iv) improves differentiation potential and (v) alters the cytokine and growth factor profile of MSCs [Jin et al. 2010; Tsai et al. 2011; 2012a & 2012b; Liu et al. 2013]. Therefore, it is likely that oxygen tension impacts on MSC and HSC proliferation, differentiation and function in vivo.

1.2 MESENCHYMAL STROMAL CELLS: IMMUNO-MODULATORS

In addition to their role in haematopoiesis, MSCs are also potent modulators of innate and adaptive immunity. MSCs are recruited to sites of inflammation where they suppress the immune response through various mechanisms including direct cell-cell contact and the production of immuno-modulatory factors. MSC interactions with Natural killer (NK) and dendritic cells (DCs) in the innate immune response are well-characterised. NK cells are cytotoxic lymphocytes that lyse cancerous and virally-infected cells and produce pro-inflammatory cytokines, e.g. tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), which stimulate the recruitment and activation of other immune cells, driving inflammation [Murphy et al. 2008]. MSCs inhibit (i) NK cell proliferation and activation (ii) induction of NK cytotoxicity and (iii) secretion of pro-inflammatory cytokines, via direct cell-cell interactions and secreted factors including indoleamine 2,3-dioxygenase (IDO) and prostaglandin E2 (PGE2) [Spaggiari & Moretta, 2013]. Dendritic cells (DCs) are phagocytes that engulf foreign pathogens and display antigenic peptides, bound by major histocompatibility complex (MHC) molecules, to T lymphocytes, promoting their activation [Murphy et al. 2008]. MSCs (i) inhibit the differentiation of myeloid precursors into DCs; (ii) suppress immature DC interactions with lymphocytes and (iii) down-regulate DC maturation markers (e.g. MHC class II, CD80 and CD86) [Nuata & Fibbe, 2007; English, 2013; Spaggiari & Moretta, 2013].
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MSCs modulate the adaptive immune response by regulating mature T and B lymphocyte functions. T lymphocytes are responsible for cell-mediated immunity. This involves direct interactions between the mature T cell receptor (TCR) and its target antigen, namely peptides presented by MHC molecules on the surface of antigen-presenting cells (APCs). This interaction stimulates T lymphocytes to exert antigen-specific cytotoxicity and to produce cytokines that stimulate other immune cell types, leading to the elimination of infected and cancerous cells [Murphy et al. 2008]. B lymphocytes are generated in the BM and mediate humoral immunity by producing antibodies, soluble forms of the B cell receptor (BCR) which recognise specific antigens expressed by foreign pathogens, leading to their destruction [Cooper et al. 1965 & 1966; Murphy et al. 2008].

MSCs suppress the proliferation of mature CD4⁺ helper and CD8⁺ cytotoxic T lymphocytes by secreting various paracrine factors including IDO, PGE2, nitric oxide (NO) and transforming growth factor-β (TGF-β) [Duffy et al. 2011b]. MSCs also (i) inhibit naïve CD4⁺ T lymphocyte differentiation into pro-inflammatory T helper (Th)1 and Th17 subsets; (ii) promote their differentiation into immuno-suppressive/regulatory Th2 and Treg subsets and (iii) modulate Th subset plasticity by stimulating a switch between (a) Th1 to Th2 phenotype and (b) Th17 to Treg phenotype [Duffy et al. 2011a & 2011b; English, 2013]. Some studies suggest that MSCs suppress mature B lymphocyte proliferation, activation and antibody production [Corcione et al. 2006; Chen et al. 2012] whereas others indicate that MSCs promote B lymphocyte function [Rasmusson et al. 2007; Ji et al. 2012]. Indeed, the potential immuno-suppressive effects of MSCs on mature B lymphocytes are paradoxical since MSCs are essential for promoting B lymphopoiesis in the BM. Further investigation is required to characterise the effects of MSCs on mature B lymphocytes.
Figure 1.1 A simplified view of the bone marrow niche. The BM niche is segregated into two distinct compartments: (i) the perivascular niche located proximal to the vascular sinusoids (far right) and (ii) the endosteal niche located proximal to bone, composed of osteoblasts and osteocytes (far left). The BM niche is a hypoxic environment (2-8% \(O_2\)) with \(O_2\) levels gradually decreasing with increasing proximity to the endosteal lining. Various MSC sub-populations of differing lineage potential, defined by specific cell surface marker expression, are distributed throughout the BM niche. These MSC sub-populations secrete various ECM components, cell adhesion molecules, growth factors and cytokines that regulate defined stages of haematopoiesis including HSC quiescence, self-renewal and mobilisation, and B lymphopoiesis. **Abbreviations:** Fibroblast activation protein (FAP); Haematopoietic stem cell (HSC); Leptin receptor (Lepr); LT-/ST-HSC (Long-term or dormant HSC; Short-term or cycling HSC – blue arrow indicates cycling activity); Mesenchymal stromal cell (MSC); Myxovirus resistance protein 1 (Mx1); Platelet-derived growth factor-\(\alpha\) (PDGFR\(\alpha\)); Stem cell antigen-1 (Sca-1).
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1.3 MESENCHYMAL STROMAL CELLS ARE RADIO-RESISTANT

The findings to date collectively indicate that MSCs are essential regulators of haematopoietic development and function. The haematopoietic system is one of the main cellular systems to fail following acute exposure to ionising radiation (IR) e.g. γ-rays and X-rays. Lymphocytes, in general, are highly radio-sensitive and die within 24 hrs following exposure to even low IR doses whereas granulocytes, erythrocytes and platelets are more radio-resistant [Fliedner et al. 2002; Dainiak et al. 2003]. HSCs and their progenitors are also radio-sensitive (D₀ of 0.6-1.6 Gy, i.e. the dose required to reduce the proportion of surviving cells by 37% less than its previous value), preventing continued renewal of functional blood and immune cell types in the periphery [Fliedner et al. 2002; Hall & Giaccia, 2006]. Without adequate BM transplantation (BMT), this ultimately leads to haematopoietic failure associated with anaemia, thrombocytopenia, agranulocytosis and lymphopenia [Fliedner et al. 2002; Dainiak et al. 2003].

However, unlike haematopoietic cells, MSCs are radio-resistant. The HSC was discovered by demonstrating that BM-derived haematopoietic cells injected intravenously into irradiated mice gave rise to colonies in the spleen, called CFU-S, consisting of multiple mature haematopoietic cell types [Till & McCulloch, 1961]. This finding also provided the first evidence that a radio-resistant stromal cell compartment capable of supporting haematopoiesis exists in vivo. In allogeneic BMT, myeloablative radiotherapy, with or without chemotherapy, is used to deplete host-derived HSC and their progenitors in the BM, creating a compartment in which donor HSCs can engraft and re-constitute the haematopoietic system [Copelan, 2006]. Numerous studies have demonstrated that MSCs isolated from BMT recipients are host-derived and can be subsequently expanded in vitro [Friedenstein et al. 1976, Laver et al. 1987; Simmons et al. 1987; Arematsu & Nakahori, 1991; Dickhut et al. 2005; Rieger et al. 2005; Bartsch et al. 2009]. Taken together, these findings demonstrate that MSCs are radio-resistant and can support haematopoietic reconstitution in vivo.
1.4 THYMOPOIESIS: FROM NEGATIVE TO POSITIVE

As previously mentioned, B lymphopoiesis occurs in the BM. However, T lymphopoiesis occurs in the thymus [Miller, 1961 & 2002]. The thymus is segregated into four distinct compartments, namely the sub-capsular zone (SCZ); the cortex; the corticomedullary junction (CMJ) and the medulla (Figure 1.2). Each compartment consists of a specialised microenvironment composed of specific stromal and immune cell types which direct defined stages of thymocyte development, i.e. thymopoiesis [Zúñiga-Pflücker, 2004; Petrie & Zúñiga-Pflücker, 2007; Koch & Radtke, 2011]. This process depends on a crosstalk between the thymic microenvironment and developing thymocytes in which (i) the production of soluble factors, ECM and adhesion molecules by thymic stromal cells guide thymocyte differentiation while (ii) developing thymocytes, in turn, regulate the differentiation of thymic epithelial cells (TECs), enabling thymic homeostasis to be established [Ritter & Boyd, 1993; Takahama, 2006; Petrie & Zúñiga-Pflücker, 2007].

Unlike the BM, the thymus does not possess self-renewing potential. Therefore, it is continually seeded by lymphoid-myeloid restricted HSC-derived progenitors that originate from the BM, known as early thymus seeding progenitors (ETPs) [Wallis et al. 1975; Mulder et al. 1988; Adolfsson et al. 2005; Balciunaite et al. 2005a & 2005c; Bell & Bhandoola, 2008; Ceredig, 2012]. ETPs enter the thymus at the CMJ where interactions with the thymic microenvironment stimulate them to lose B lymphocyte potential and to enter the first stage of T lineage commitment, defined as the double negative (DN) stage [Ceredig & Rolink, 2002; Balciunaite et al. 2005a; Koch & Radtke, 2011]. DN thymocytes are the earliest intra-thymic T cell precursors. They do not express CD4 or CD8 co-receptors, markers of maturing T lymphocytes, and are therefore classified as double negative (DN) [Ceredig et al. 1983a; Fowlkes et al. 1985; Godfrey et al. 1993; Ceredig & Rolink, 2002]. DN thymocytes represent approx. 5% of total thymocytes in the adult thymus and are sub-divided into four populations, known as DN1, DN2, DN3 and DN4 (Figure 1.3) [Godfrey et al. 1993; Ceredig & Rolink, 2002]. These populations represent sequential stages of early thymopoiesis and are phenotypically characterised based on differential surface expression of CD44, CD25, CD117.
and CD127 (Figure 1.3) [Ceredig et al. 1983b & 1985; Godfrey et al. 1992; Godfrey et al. 1993; Ceredig & Rolink 2002].

Notch signalling is an indispensable driver of early thymopoiesis. Notch receptors (Notch 1-4) interact with target transmembrane Notch ligands (Delta-like-ligand 1, -3, -4 and Jagged-1) on neighbouring cells, stimulating intracellular cleavage of the Notch receptor to generate the intracellular Notch (ICN) fragment [Radtke et al. 2010]. ICN subsequently interacts with the CSL (CBF1-suppressor of hairless Lag1) transcription factor in the nucleus, resulting in altered transcriptional regulation of cell type-specific genes that stimulate fate decisions in progenitor cells [Osborne & Minter, 2007; Radtke et al. 2010]. Interaction between the Notch1 receptor expressed by ETPs and DN thymocytes, and Delta-like ligand 4 (DL4) on TECs is essential for T lineage commitment and development. Ablation of Notch1 expression in BM-derived haematopoietic progenitors blocks early T lymphopoiesis and leads to an influx of immature B lymphocytes in the thymus [Radtke et al. 1999; Wilson et al. 2001]. Furthermore, expression of constitutively active Notch1 in HSCs results in extra-thymic T lymphopoiesis and suppression of B lymphopoiesis in the BM [Pui et al. 1999].

DN1 thymocytes (DN1) consist of a heterogenous population of ETPs and more committed pro-T lymphocytes that retain NK, DC and macrophage differentiation potential [Balciunaite et al. 2005a; Petrie & Zúñiga-Pflücker, 2007; Koch & Radtke, 2011]. DN1 extensively proliferate within the perimedullary cortex (PMC) and periodically migrate into the cortex. Here, DN1 interactions with TECs and fibroblasts, including Notch signalling, stimulate the up-regulation of CD25 and CD127, the α-chain of the Interleukin-7 receptor (IL-7Rα), generating DN2 thymocytes [Poritt et al. 2003; Ceredig & Rolink, 2002; Petrie & Zúñiga-Pflücker, 2007; Koch & Radtke, 2011]. CD117 is the receptor for SCF, a key cytokine involved in (i) regulating haematopoiesis, (ii) ETP T lineage commitment and (iii) in stimulating DN survival and proliferation [Godfrey et al. 1992; Rodewald et al. 1995; Massa et al. 2006].
Adapted from Crompton et al. 2007

**Figure 1.2. Architecture of the thymus.** The thymus is comprised of two lobes. Each thymic lobe consists of multiple thymic lobules segregated by connective tissue called trabeculae. Each thymic lobule is segregated into four major compartments known as (i) the sub-capsular zone (SCZ); (ii) the cortex; (iii) the corticomedullary junction and (iv) the medulla. Each compartment is distinguished by the presence of specific immune and stromal cell types which form distinctive microenvironments that direct defined stages of thymocyte differentiation. The SCZ mainly consists of cortical thymic epithelial cells (cTECs); the cortex is comprised of fibroblasts, MSCs, cortical TECs and macrophages; the CMJ contains a network of endothelial cells; and medullary TECs (mTECs) and dendritic cells comprise the medulla [Petrie & Zúñiga-Pflücker, 2007; Koch & Radtke, 2011]. **Abbreviations:** Double negative (DN); Thymic epithelial cell (TEC); Intermediate single positive (ISP); Double positive (DP) and Common lymphoid progenitor (CLP). CLP in the thymus have since been re-defined as ETPs.
The DN2 population can be sub-divided based on CD117 expression, namely DN2a (CD117\textsuperscript{high}) and DN2b (CD117\textsuperscript{int}) (Figure 1.3) [Ceredig & Rolink, 2002]. DN2a cells have DC and NK differentiation potential and have non-rearranged TCR-β loci. DN2b cells are restricted to NK potential and have rearranged TCR-β D and J segments, generated via recombination activating gene (RAG)-dependent V(D)J recombination, in preparation for pre-TCR expression at the DN3 stage [Ceredig & Rolink 2002; Masuda et al., 2007; Koch & Radtke, 2011]. IL-7 is a cytokine that is essential for lymphopoiesis and for DN thymocyte survival, proliferation and differentiation up to the DN3 stage [Murray et al. 1989; Peschon et al. 1994; von Freeden-Jeffry et al. 1995; Balciunaite et al. 2005b]. Interestingly, IL-7Rα expression level is thought to influence αβ versus γδ lineage commitment at the DN2 stage as IL-7Rα\textsuperscript{low} and IL-7Rα\textsuperscript{high} DN2 subsets are biased towards αβ and γδ lineages, respectively [Kang et al. 2001]. Although the mechanisms controlling αβ versus γδ lineage choice are unclear, studies suggest that IL-7 signalling regulates chromatin modifications that influence differential accessibility of TCR-γ and -β loci in thymocytes [Huang & Muegge, 2001; Kang et al. 2001].

DN2 thymocytes extensively proliferate and differentiate into DN3 thymocytes in the SCZ, phenotypically characterised by the down-regulation of CD25 and CD117 expression (Figure 1.3). The DN3 population can be further sub-divided according to CD27 expression levels: (i) DN3a (CD27\textsuperscript{low}) are small non-cycling cells that contain random TCR-β rearrangements and (ii) DN3b (CD27\textsuperscript{high}) are large, rapidly cycling cells that largely contain in-frame TCR-β rearrangements [Hoffman et al. 1996; Laurent et al. 2004; Koch & Radtke, 2011]. The DN3a-DN3b transition defines the stage of β-selection during T lymphocyte development [Möröy & Karsunky, 2000; Ceredig & Rolink 2002; Koch & Radtke, 2011]. β-selection eliminates DN3 thymocytes that have generated non-functional TCR-β chains. If TCR-β loci rearrangement is in-frame, the expressed functional TCR-β chain is associated with an un-rearranged pre-TCR-α chain and CD3 chains to form the pre-TCR complex. Upon reaching the cell surface, the pre-TCR complex initiates signal transduction via the CD3 complex to (i) stimulate intense proliferation of the β-selected DN3b thymocytes; (ii) to block further TCR-β gene rearrangement via allelic exclusion and (iii) to promote further differentiation into DN4 thymocytes [Möröy & Karsunky, 2000; Ceredig & Rolink, 2002].
By the DN4 stage, CD44 expression is down-regulated; thymocyte precursors have lost all non-T lineage potential and are now fully committed to becoming T lymphocytes. En route to the medulla, DN4 thymocytes progress through an immature CD8⁺ intermediate single positive (ISP) transition before up-regulating the expression of CD4 and CD8 co-receptors, which marks differentiation into CD4⁺ CD8⁺ double positive (DP) thymocytes [Ceredig & Rolink, 2002; Crompton et al. 2007; Koch & Radtke, 2011]. At the DP stage, V and J segments of the TCR-α locus are rearranged. The rearranged TCR-α chain replaces the pre-TCR-α chain on the cell surface and interacts with the TCR-β chain and the CD3 complex to generate a mature αβ TCR. [Möröy & Karsunky, 2000; Ceredig & Rolink, 2002; Germain, 2002].

DP thymocytes are quiescent and undergo a selection process in which their fate is dictated by the nature of the TCR interaction with MHC molecules expressed on antigen-presenting TECs. There are three main outcomes for DP thymocytes during this selection process: (i) Death by neglect occurs if low affinity interactions exist between the TCR and MHC, preventing TCR stimulation and resulting in apoptosis; (ii) Death by negative selection occurs when high affinity interactions exist between the TCR and MHC, resulting in over-active TCR signalling and rapid apoptosis of potentially auto-reactive T lymphocytes or (iii) Positive selection in which TCR-MHC interactions result in an appropriate intermediate level of TCR signalling that stimulates further maturation [Germain, 2002; Hernandez et al. 2010]. ~5% DP thymocytes are positively selected and subsequently mature into MHC class II-ligand binding CD4⁺ helper or regulatory T lymphocytes or MHC class I-ligand binding CD8⁺ cytotoxic T lymphocytes which migrate to the periphery to exert their immune functions [Germain, 2002].
Figure 1.3 An overview of thymopoiesis. During thymopoiesis, T lymphocyte precursors undergo a series of defined stages of differentiation, each defined by a unique cell surface marker profile (see text for details). CD4<sup>-</sup> CD8<sup>-</sup> double negative (DN) thymocytes are the earliest T lymphocyte precursors and are segregated into four main subsets (DN1-DN4) based on surface expression levels of CD117, CD25, CD44 and CD127. DN2 and DN3 subsets can be further divided based on CD117 and CD27 expression levels, respectively (see text for details). V(D)J rearrangement of the TCR-β locus occurs at DN2b & DN3a stages. DN3 thymocytes that generate in-frame TCR-β rearrangements are selected for expansion (i.e. β-selection) at the DN3b stage. β-selected DN thymocytes progressively differentiate into CD4<sup>+</sup> CD8<sup>-</sup> double positive (DP) thymocytes in which TCR-α rearrangement occurs, with in-frame rearrangements resulting in the generation of a mature αβ TCR. DP thymocytes subsequently undergo positive and negative selection resulting in the generation of mature CD4<sup>+</sup> and CD8<sup>+</sup> single positive (SP) T lymphocytes that recognise foreign antigens presented by MHC molecules on antigen-presenting cells in the periphery to exert their immune functions.

Adapted from Ceredig & Rolink, 2002; Koch & Radtke, 2011
1.5 LYMPHOCYTE RADIO-SENSITIVITY: EXCEPTIONS TO THE RULE

Irradiated B lymphocytes are rapidly depleted and B lymphopoiesis does not recover without subsequent BMT [Schrek, 1961; Anderson et al. 1974; Prosser, 1976; Nishnii et al. 1998; Bosco et al. 2010]. Similar to the BM, thymic cellularity is rapidly reduced following acute radiation exposure due to the extensive death of thymocytes [Kadish & Bash, 1975; Ceredig & MacDonald, 1982; Zúñiga-Pflücker & Kruisbeek, 1990]. However, approximately 5-7 days post IR, a single wave of increased cellularity occurs in the thymus, a phenomenon known as auto-reconstitution [Takada et al. 1969; Kadish & Bash, 1975; Ceredig & MacDonald, 1982; Zúñiga-Pflücker & Kruisbeek, 1990]. This was found to be due to the proliferation and differentiation of host-derived radio-resistant early T lymphocyte precursors [Ceredig & MacDonald, 1982; Zúñiga-Pflücker & Kruisbeek, 1990]. Recently, the DN2 thymocyte has been identified as the radio-resistant early T lymphocyte precursor exclusively responsible for thymic auto-reconstitution following irradiation [Bosco et al. 2010].

1.6 THE DNA DAMAGE RESPONSE: AN OVERVIEW

The cellular responses to IR are mostly due to its destructive effects on the genome [Kastan & Bartek, 2004; Ciccia & Elledge, 2010]. In general, IR-induced DNA damage can arise from energy deposited directly onto DNA or indirectly from free radicals generated by the ionisation of other molecules, such as oxygen and water, which modify and/or break chemical bonds within DNA strands [Ward et al. 1988; Hall & Giaccia, 2006]. The most genotoxic lesions caused by IR are DNA double-strand breaks (DNA DSBs). A DNA DSB can arise when breaks in sister DNA strands occur either directly opposite or within a few base pairs of each other, causing the chromatin to be segregated into two pieces. The lack of a DNA template at the damage site renders these lesions difficult to repair, increasing cellular susceptibility to chromosomal instability and to the induction of cell death mechanisms [Hall & Giaccia, 2006]. It is estimated that each gray of IR introduces 33 DSBs into the mammalian genome [Redon et al. 2003]. DSBs also occur endogenously during DNA replication and in other cellular processes such as V(D)J recombination in developing lymphocytes [Rothkamm et al. 2003]. The maintenance of genomic stability is essential for cell survival and tumour suppression. Therefore, cells have evolved an integrated circuit of
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signalling pathways, known as the DNA Damage Response, in order to mount a co-ordinated biological response to genotoxic stress [Harper & Elledge, 2007; Ciccia & Elledge, 2010]. Conceptually, the DDR consists of (i) sensor proteins, involved in recognising sites of damaged DNA; (ii) transducer proteins, which amplify the DNA damage signal, and (iii) effector proteins, required for the desired biological response(s) which include DNA repair, transient delays in cell cycle progression (termed checkpoints), transcriptional programmes, apoptosis, and senescence (Figure 1.4) [Kastan & Bartek, 2004; Harper & Elledge, 2007; Fitzgerald et al. 2009]. Therefore, execution of the DDR ultimately determines whether a damaged cell will survive or die following genotoxic insult.

1.6.1 DDR INITIATION: SENSING THE PROBLEM

The first step in DDR activation is to recognise DNA damage. In the initial response to IR-induced DNA DSBs, poly-(ADP) ribose polymerase 1 and 2 (PARP1/2) catalyse the addition of poly-(ADP) ribose (PAR) chains to histone tails flanking DSBs. These PAR chains provide a platform for various chromatin re-modelling proteins, e.g. histone deacetylases, which function to increase the accessibility of DNA damage sites to various sensor proteins [Ciccia & Elledge, 2010; Messner et al. 2010; Lukas et al. 2011]. PARP1 is also implicated in facilitating the recruitment of the Mre11-Rad50-Nbs1 (MRN) complex to DNA DSBs [Haince et al. 2008; Ciccia & Elledge, 2010]. Mre11 is a U-shaped nuclease that binds to short 3’OH overhangs at DNA DSB termini to form synaptic DNA complexes [Williams et al. 2007 & 2008; Lamarche et al. 2010; Rupnik et al. 2010]. Rad50, a member of the structural maintenance of chromosome (SMC) family of chromatin modelling proteins, contains an ATP-binding cassette which binds and unwinds DNA DSB termini [de Jager et al. 2001; Hopfner et al. 2001; Lamarche et al. 2010; Rupnik et al. 2010]. Nbs1 acts as a flexible adaptor between Mre11 and Rad50 and mediates the assembly and nuclease activity of MRN complexes at DNA DSB sites [Paull et al. 1999; Rupnik et al. 2010]. Together, the components of the MRN complex form a physical bridge between DNA DSB ends and act as a primary sensor of DNA DSBs.

Recruitment of the MRN complex to DNA DSBs is essential for activating DDR signalling pathways. The DDR is primarily mediated by members of the phosphatidylinositol 3-kinase (PI3K)-related family of Ser/Thr protein kinases, namely Ataxia telangiectasia-mutated
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(ATM) and Ataxia telangiectasia and Rad3 related (ATR). ATM is the master regulator of DNA DSB signalling pathways. ATR is primarily activated by DNA single-strand breaks (SSBs) which are generated during DNA replication and by various agents that inhibit DNA replication, such as ultra-violet (UV) light and hydroxyurea (HU). ATR is also activated by DNA DSBs that have undergone resection to generate single-stranded DNA tails [Osborn et al. 2002; Shiloh, 2003; Kumagai & Dunphy, 2006; Hurley & Bunz, 2007]. Another PI3K kinase, known as DNA-dependent protein kinase, catalytic subunit (DNA-PKcs) plays key roles in the repair of DNA DSBs by non-homologous end-joining (NHEJ), which will later be discussed in further detail [Ciccia & Elledge, 2010]. ATM is rapidly recruited to DNA DSBs by the MRN complex and physically interacts with Nbs1 [Lee and Paull, 2004 & 2005; Lamarche et al. 2010]. Interaction with the MRN complex stimulates ATM to be post-translationally modified on multiple residues that (i) activate (e.g. S1981 autophosphorylation) and (ii) modulate its function (e.g. TIP60 acetyltransferase-dependent K3016 acetylation) [Bakkenist & Kastan, 2003; Sun et al. 2007; Pellegrini et al. 2006; Shiloh & Ziv, 2013]. ATR is primarily activated by DNA single-strand breaks (SSBs) which are generated during DNA replication and by various agents that inhibit DNA replication, such as ultra-violet (UV) light and hydroxyurea (HU) [Osborn et al. 2002; Shiloh, 2003; Kumagai & Dunphy, 2006; Hurley & Bunz, 2007]. ATR is also activated by ssDNA tails generated by endonuclease-mediated resection of DNA DSB termini (discussed in more detail below) [Cuadrado et al. 2006; Jazayeri et al. 2006].

1.6.2 DDR AMPLIFICATION: SENDING AN SOS

Following the sensing of DNA DSBs by the MRN complex and ATM, the surrounding chromatin is further re-modelled in order to amplify the DDR signal downstream. In this process, histones are post-translationally modified to form open chromatin structures at damage sites and to create docking sites for a family of breast cancer type 1 (BRCA1) C-terminal (BRCT)-domain containing DDR scaffold or ‘mediator’ proteins [Kumar et al. 2013; Price & D’Andrea, 2013]. DDR mediators are essential for (i) retaining sensor proteins at DNA DSB sites; (ii) modulating ATM activation and function; and (iii) facilitating DNA repair. DDR mediators are being identified on a continuing basis and include mediator of DNA damage checkpoint protein 1 (MDC1); E3 ubiquitin ligase RING finger protein (RNRF)
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8; p53 binding protein 1 (53BP1) and BRCA1 [Harper & Elledge, 2007; Fitzgerald et al. 2009; Ciccia & Elledge, 2010; Lee et al. 2010; Lowndes, 2010].

A highly characterised histone modification generated in response to IR-induced DNA DSBs is ATM-dependent Ser139 phosphorylation of the histone variant, H2AX, termed γ–H2AX [Rogakou et al. 1998; Burma et al. 2001]. The γ–H2AX modification is an important signal for DDR activation in response to DNA DSBs and is generally recognised as a marker of DNA DSBs [Fernandez-Capetillo et al. 2004; Bonner et al. 2008]. H2AX Ser139 phosphorylation, combined with Tyr143 de-phosphorylation [via eyes absent homolog 1/3 (EYA1/3) phosphatases], enables MDC1 to dock onto chromatin at DNA DSB sites where it interacts with ATM and Nbs1. This stimulates the recruitment of additional ATM-MRN complexes and further ATM activation, resulting in H2AX phosphorylation over several megabases [Rogakou et al. 1999; Bonner et al. 2008; Savic et al. 2009; Shiloh & Ziv, 2013]. Other histone modifications, e.g. acetylation, ubiquitination and methylation, also stimulate the recruitment of other DDR mediators such as 53BP1 and BRCA1 [Lee et al. 2010; Sulli et al. 2012; Shiloh & Ziv, 2013]. This complex assembly of DDR sensors and mediators at DNA DSBs results in the formation of supramolecular structures known as DNA DSB-associated foci, or IR-induced foci (IRIF), which act to amplify the DDR signal downstream and facilitate DNA DSB repair [Lowndes & Toh, 2005; Lukas et al. 2011].

Crosstalk between DDR mediators and ATM at DNA DSBs orchestrates ATM-dependent phosphorylation of target substrates, including the DDR transducer, Checkpoint kinase 2 (Chk2). During DNA DSB resection, ATM mediates replication protein A (RPA) coating of DNA SSBs which triggers ATR activation and subsequent ATR-dependent phosphorylation of another DDR transducer, Chk1 [Zou & Elledge, 2003; Cuadrado et al. 2006; Jazayeri et al. 2006; Hurley & Bunz, 2007]. Therefore, the DDR is modulated by a crosstalk between ATM-Chk2 and ATR-Chk1 pathways which together transmit the damage signal to multiple effector proteins which orchestrate biological outcome(s) to DNA DSBs (Figure 1.4). These outcomes include DNA damage checkpoint activation, DNA DSB repair, apoptosis and senescence which will now be discussed in further detail.
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Figure 1.4 (overleaf) An overview of the DNA Damage Response. The DDR represents an integrated network of sensor, transducer and effector proteins whose activities induce biological responses to DNA damage. DNA DSBs are initially sensed by PARP and the MRN complex, which results in the recruitment and activation of the PI(3)K kinases, ATM and DNA-PKcs (see text for details). Post-translational modifications to histones surrounding DNA DSBs, including ATM-dependent H2AX Ser139 phosphorylation (γ-H2AX), stimulates the recruitment of DDR mediators, including MDC1, 53BP1, BRCA1, CtIP and RNF8 which act to amplify the DDR signal and to modulate ATM-dependent phosphorylation of target substrates. ATM-mediated DNA DSB resection to generate ssDNA tails also results in ATR activation. Activated ATM and ATR phosphorylate the DDR transducers, Chk2 and Chk1, respectively, which relay the damage signal downstream to effector proteins involved in various biological responses. These include p53 and p21 (DNA damage checkpoints, DNA damage-induced transcription; DNA repair; apoptosis and cellular senescence); Cdc25a (DNA damage checkpoints); DNA ligase IV and Rad51 (DNA DSB repair) and Caspase-3 (Apoptosis) (see text for details). **Abbreviations:** Double-strand break (DSB); H2AX Ser139 phosphorylation (γ-H2AX).
Figure 1.4 An overview of the DNA Damage Response.
1.6.3 DNA DAMAGE CHECKPOINTS: TIME TO SLOW DOWN

The cell cycle is segregated into four distinct phases: (i) Gap phase 1 (G₁) in which cells prepare for DNA synthesis; (ii) S phase in which DNA synthesis occurs; (iii) G₂ in which cells prepare for mitosis and (iv) M phase in which cell division occurs [Norbury & Nurse, 1992]. The cell cycle is regulated by cyclin-dependent kinases (Cdks). Cdk activity is dependent on the binding of specific cyclins whose expression levels fluctuate throughout the cell cycle. (i) Cyclin D-Cdk4/6 and cyclin E-Cdk2 complexes promote G₁ progression into S phase; (ii) cyclin A-Cdk2 complexes are required for DNA synthesis and (iii) cyclin A- and cyclin B-Cdk1 complexes are required for G₂/M progression and mitosis, respectively [Vermeulen et al. 2003; Massagué, 2004]. Cdk activity is also modulated by (i) phosphorylation on conserved Thr and Tyr residues which induce conformational changes that either promote or inhibit cyclin binding and (ii) Cdk inhibitors (CKIs) which can bind Cdks alone or cyclin-Cdk complexes, impairing Cdk activity.

Activation of the DDR signal cascade can induce transient delays at specific cell cycle transitions (G₁/S or G₂/M) or phases (intra-S), known as the DNA damage checkpoints [Kastan and Bartek, 2004]. The G₁/S checkpoint prevents the initiation of DNA synthesis in the presence of DNA damage and can be instigated via two different pathways. The slowly activated G₁/S checkpoint response is mediated by the ATM-p53-p21 signalling axis [Bartek & Lukas, 2001; Deckbar et al. 2010 & 2011] (Figure 1.5). p53 and p21 are two key transcription factors that inhibit the proliferation of genetically unstable cells by regulating the expression of genes involved in multiple DDR mechanisms including cell cycle arrest, DNA repair and apoptosis. Under normal conditions, p53 function is constitutively maintained at low levels by the E3 ubiquitin ligase, mouse double minute 2 (Mdm2), which targets p53 for proteasomal degradation. However, in response to DNA DSBs, p53 and Mdm2 become post-translationally modified by various DDR proteins, including ATM and Chk2, leading to p53 stabilisation [Bartek & Lukas, 2001; Fei & El-Deiry, 2003]. p53 positively regulates the expression of the CKI, p21, which inhibits (i) cyclin D-Cdk4/6 and cyclin E-Cdk2 complexes required for the G₁/S transition and (ii) proliferating cell nuclear antigen (PCNA) activity required for DNA replication, thereby blocking S phase entry [Fei & El-Deiry, 2003; Gartel & Radhakrishnan, 2005].

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Although the p53-dependent G1/S checkpoint is sensitive to even low dose irradiation, DNA synthesis can be initiated in damaged cells during a 4-6 hr window post IR due to the length of time required to induce p53 stabilisation and p21 expression [Deckbar et al. 2010 & 2011]. Therefore, a rapid, transcription-independent, G1/S checkpoint can also be induced via the ATM-Chk2-Cdc25a signalling axis (Figure 1.5). Cell division cycle 25a (Cdc25a) phosphatase removes inhibitory phosphate moieties on Cdk2, promoting Cdk2 activation. Cyclin E-Cdk2 complexes load Cdc45, a component of the pre-initiation complex, onto DNA replication origins, which is required for subsequent recruitment of DNA polymerase α and the firing of DNA replication origins [Grallert & Boye, 2008; Willis & Rhind, 2009]. However, in response to DNA DSBs, ATM-dependent Chk2 phosphorylation of Cdc25a targets it for ubiquitin-dependent proteasomal degradation, preventing Cdk2 activation, and thereby inhibiting the initiation of DNA replication [Mailand et al. 2000; Bartek & Lukas, 2001; Lukas et al. 2004; Deckbar et al. 2011].

The intra-S-phase checkpoint is characterised by a reduction in the rate of DNA synthesis. This occurs due to (i) inhibition of replication origin firing and (ii) stalling of replication fork progression [Grallert & Boye, 2008]. Blockade of replication origin firing in response to DNA DSBs is primarily mediated by the ATM-Chk2-Cdc25a signalling axis described above (Figure 1.5) [Falck et al. 2001]. The mechanism underlying replication fork stalling in response to DNA DSBs is not fully understood. However, the protein complex, Cohesin, has emerged as an important substrate of ATM signalling in this process. Cohesin, a protein complex consisting of SMC1, SMC3, Rad21 and Scc1, forms a ring around sister chromatids as they are being synthesised during DNA replication [Sherwood et al. 2010]. The pairing of sister chromatids, i.e. cohesion, is essential for accurate chromosome condensation and segregation in mitosis and for DNA DSB repair by homologous recombination (HR) [Bauerschmidt et al. 2010; Sherwood et al. 2010]. Nbs1/BRCA1/ATM-dependent phosphorylation of SMC1 and ATM/Chk2-dependent phosphorylation of SMC3 are required for replication fork stalling in response to DNA DSBs [Kitagawa et al. 2004; Luo et al. 2008]. The molecular mechanism linking ATM-mediated post-translational modification of Cohesin and replication fork stalling remains to be investigated in detail.
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The G2/M checkpoint prevents G2 cells harbouring DNA damage from entering mitosis. This process is essential (i) for preventing chromosomal instability and (ii) for ensuring that mutations are not inherited by daughter progeny. The G2/M checkpoint is rapidly activated in irradiated G2 cells in an ATM-dependent manner whereas prolonged ATM-independent G2/M arrest is activated when irradiated S phase cells with persisting DNA damage progress into G2 [Xu et al. 2002]. In response to DNA DSBs, ATM/Chk2-dependent phosphorylation and subsequent ubiquitin-dependent proteasomal degradation of Cdc25c prevents de-phosphorylation of Cdk1, preventing Cdk1 activation, and thereby blocking mitotic entry (Figure 1.5). The G2/M checkpoint can also be activated via ATM/Chk2-dependent phosphorylation of Che-1, a RNA polymerase binding II protein that induces p53 and p21 transcription, of which the latter also potently inhibits Cdk1 activity [Lukas et al. 2004; Gartel & Radhakrishnan, 2005; Bruno et al. 2006]. ATR-dependent Chk1 phosphorylation and subsequent degradation of Cdc25c also contributes to G2/M arrest in response to resected DNA DSBs as previously described [Abraham, 2001; Lukas et al. 2004]. The G2/M checkpoint is maintained via two MDC1/53BP1-dependent pathways, depending on the nature of the DNA DSB. 53BP1 and MDC1 sustain activated ATM-Chk2 signalling in response to persisting DNA DSBs whereas they promote the activation of ATR-Chk1 signalling in response to resected DNA DSBs [Shibata et al. 2010].

1.6.4 DNA REPAIR: RESOLVING THE ISSUE

A key function of cell cycle checkpoints is to provide time for DNA repair. Failure to repair DSBs, or their mis-repair, can result in chromosomal aberrations, chromosome loss and/or cell death. DNA DSBs are primarily repaired via two pathways known as non-homologous end-joining (NHEJ) and homologous recombination (HR). The majority of DNA DSBs are repaired via NHEJ which occurs throughout the cell cycle, whereas ~15-20% DNA DSBs are repaired via HR which is restricted to late S and G2 phases [Rothkamm et al. 2003; Beucher et al. 2009]. In NHEJ, DNA DSBs are bound by the Ku70/Ku80 ring-shaped heterodimeric complex which (i) tethers broken DNA ends together and (ii) recruits DNA-PKcs to DNA DSB termini, forming the DNA-PK complex in which DNA-PKcs is activated [Weterings & Chen, 2008]. Phosphorylation of DNA-PKcs stimulates the recruitment of multiple processing factors including Artemis 5’-3’ endonuclease, polynucleotide kinase and DNA polymerase X.
family members which process DNA DSBs to generate 5’ and 3’ ligatable DNA ends. The X4-L4 complex, consisting of X-ray repair cross-complementing protein 4 (XRCC4), XRCC4-like factor (XLF) and DNA ligase IV is then recruited to DNA DSBs where XRC44 and XLF create a scaffold for Ligase IV and stimulate its ligase activity, resulting in DNA DSB repair (Figure 1.6, left panel) [Lieber et al. 2003; Jeggo & Löbrich, 2006; Hartlerode & Scully, 2009; Hammel et al. 2011]. Interestingly, 53BP1 has been shown to interact with Rap1-interacting factor (RIF1) at DNA DSBs to block 5’ end resection, thereby promoting DNA DSB repair by NHEJ and inhibiting HR [Chapman et al. 2013; Zimmerman et al. 2013]. 53BP1 is also involved in long-range end-joining of antigen receptor loci segments in V(D)J recombination and in class switch recombination (CSR) [Bothmer et al. 2011; Rybanska-Spaeder et al. 2013].

Figure 1.5 DNA damage checkpoints (overleaf). In response to DNA double-strand breaks, ATM and Chk2 activation leads to downstream p53 stabilisation and subsequent induction of p21 transcription. p21 is a potent Cdk inhibitor that prevents the activation of cyclin D-Cdk4/6, cyclin E-Cdk2 and cyclin B-Cdk1 complexes, resulting in (i) a block in S phase entry (i.e. the G1/S checkpoint); (ii) a reduction in the rate of DNA synthesis (i.e. the intra-S-phase checkpoint) and (iii) a block in mitotic entry (i.e. the G2/M checkpoint). Both ATM-Chk2 and ATR-Chk1 signalling pathways lead to the phosphorylation and targeted degradation of Cdc25a and Cdc25c phosphatases. Cdc25a and Cdc25c degradation prevent the downstream activation of cyclin E-Cdk2 and cyclin B-Cdk1 complexes, respectively, inhibiting DNA replication (i.e. the intra-S-phase checkpoint) and blocking entry into mitosis (M) (i.e. the G2/M checkpoint) (See text for further details). Abbreviations: Double-strand break (DSB); H2AX Ser139 phosphorylation (γ-H2AX).
Figure 1.5 DNA damage checkpoints.
Unlike NHEJ, HR employs a complementary DNA strand within a sister chromatid as a homologous template for DNA DSB repair. In this process, CTBP (C-terminus binding protein of adenovirus E1A)-interacting protein [CtIP] and BRCA1 interact with the MRN complex at DNA DSBs. The CtIP-BRCA1-MRN complex carries out 5’-to-3’ DNA DSB resection, generating 3’ ssDNA tails that are subsequently bound by RPA oligomers, unwinding the DNA and making it accessible to recombination proteins [Sartori et al. 2007; Chen et al. 2008; Hartlerode & Scully, 2009; Wang H et al. 2012]. Various mediators including BRCA1, BRCA2, and XRCC3 subsequently co-ordinate the assembly of Rad51 molecules along the ssDNA strands, forming a nucleoprotein filament that displaces RPA [Bishop et al. 1998; Rijkers et al. 1998; Li & Heyer, 2008; Gildemeister et al. 2009; Wang H et al. 2012]. BRCA2-dependent loading of Rad51 at processed DNA DSBs results in Rad51 IRIF formation which can be used experimentally as a marker of DNA DSB repair by HR [Haaf et al. 1995; Yuan et al. 1999; Paull et al. 2000]. ATM and Artemis have also recently been implicated in regulating DNA DSB resection, and RPA and Rad51 recruitment, during HR in G2 cells [Beucher et al. 2009]. The Rad51 nucleoprotein filament captures the duplex DNA of the sister chromatid and invades a single DNA strand to search for a homologous template, generating a displacement (D) loop intermediate within the sister chromatin. The sister DNA strand located at the DSB is proposed to be captured by annealing to the D loop intermediate and subsequent DNA synthesis initiated along both broken DNA strands results in the formation of four-stranded DNA structure known as a double Holliday junction. Subsequent resolution of the Holliday junction by resolvase complexes, including the product of Bloom syndrome gene (BLM)-topoisomerase IIIa complex, results in the generation of repaired non-crossover or crossover DNA products (Figure 1.6, right panel) [Rothkamm et al. 2003; Li & Heyer, 2008; Hartlerode & Scully, 2009].
Figure 1.6 (overleaf) DNA double-strand break repair pathways. In Non-Homologous End-Joining (left panel), DNA DSBs are recognised by the Ku70/Ku80 complex (Step 1) which form a synaptic complex with DNA DSB termini and recruit DNA-PKcs to form the DNA-PK holoenzyme (Step 2). DNA-PKcs is activated and recruits the 5’-3’ endonuclease Artemis (Step 3). Subsequent processing of DNA DSB ends facilitates the recruitment of the X4-L4 complex (Step 4) in which DNA ligase IV is activated and ligates the processed DNA DSB ends, resolving the DSB (Step 5). In Homologous Recombination (right panel), DNA DSBs are resected by the 5’-3’ endonuclease activity of the MRN/CtIP/BRCA1 complex, generating ssDNA tails that are subsequently bound by replication protein A (RPA) (Step 1). RPA unwinds DNA secondary structure, facilitating further resection and the recruitment of HR proteins, including BRCA2, XRCC3, Rad52 and Rad54 (Step 2). These proteins assemble Rad51 molecules along ssDNA tails, generating a Rad51 nucleofilament that displaces RPA oligomers (Step 2). The Rad51 nucleofilament invades the sister chromatid and facilitates complementary binding between the resected DNA strand (generated at the DNA DSB) and the unprocessed sister DNA strand, resulting in the formation of a displacement (D)-loop DNA intermediate (Step 3). Subsequent annealing of the un-resected DNA strand at the DNA DSB to the D loop and DNA synthesis generates a four-stranded DNA structure known as a double Holliday junction (Step 4). These Holliday junctions can be repaired by either (i) sister chromatid dissociation, forming a non-crossover product or (ii) cleavage and subsequent resolution of the cleaved products, generating a crossover product (Step 5).
Figure 1.6 DNA double-strand break repair pathways. Abbreviations: BRCA1/2 (Breast cancer type 1/2); DSB (Double-strand break); DNA-PK (DNA-dependent) protein kinase; HR (homologous recombination); MRN (Mre11/Rad50/Nbs1) complex; NHEJ (non-homologous end-joining); RPA (replication protein A); XRCC3/4 (X-ray repair cross-complementing protein 3/4).
**1.6.5 APOPTOSIS AND CELLULAR SENESCENCE: DEADLY DECISIONS**

DNA damage checkpoints and DNA repair mechanisms co-operate to restore genomic stability following genotoxic insult. However, when genomic stability cannot be restored, cell death mechanisms can be activated to destroy cells harbouring potentially harmful genetic defects. Apoptosis is a type of programmed cell death that eliminates damaged and stressed cells and is executed by a family of cysteine proteases known as caspases [Degterev et al. 2003; Strasser et al. 2011]. DNA damage can activate the intrinsic, or mitochondrial, apoptotic pathway regulated by the B cell lymphoma-2 (Bcl-2) family [Strasser et al. 1995 & 2011]. The Bcl-2 family acts as a ‘life / death’ switch that is orchestrated via interactions between (i) the anti-apoptotic members which include Bcl-2 and Bcl-extra long (Bcl-XL); (ii) the pro-apoptotic Bcl-2 associated X protein (Bax)-like sub-family which includes Bax and Bcl-2 antagonistic killer (Bak) and (iii) the pro-apoptotic Bcl-2 homology 3 (BH3)- only members which include Bcl-2 interacting mediator of cell death (Bim), p53 promoter up-regulated modulator of apoptosis (Puma) and named for ‘damage’ (Noxa) [Willis & Adams, 2005; Adams & Cory, 2007; Ola et al. 2011].

Under normal conditions, anti-apoptotic Bcl-2 proteins sequester Bax and Bak, preventing the induction of apoptosis. However, under stress, activated BH3-only proteins insert their BH3 domain into the hydrophobic groove of anti-apoptotic Bcl-2 proteins, releasing Bax and Bak molecules. In response to DNA DSBs, ATM/Chk2-dependent p53 stabilisation can lead to the transcription of the p53 target genes, Puma and Noxa. Although Bim is not a direct transcriptional target of p53, it also plays a key role in activating apoptosis in response to DNA DSBs [Bouillet et al. 1999; Erlacher et al. 2005]. Bax and Bak aggregate on the outer mitochondrial membrane, stimulating the cytosolic release of various pro-apoptotic mediators, particularly cytochrome c. Cytochrome c subsequently associates with apoptotic protease-activating factor 1 (Apaf-1) and deoxyadenosine triphosphate (dATP) to form the apoptosome [Willis & Adams, 2005; Ola et al. 2011; Happo et al. 2012]. Formation of the apoptosome initiates a signalling cascade in which a sequential series of initiator caspases (e.g. caspase-9) and downstream executioner caspases (e.g. caspase-3) cleave and inactivate target proteins. For example, caspase-3 can cleave PARP (normally involved in DNA SSB repair) in the presence of extensive DNA damage, preventing PARP-dependent recruitment
of other DNA repair proteins, ultimately resulting in DNA degradation. Other targets include nuclear lamina, cytoskeletal proteins and cellular adhesion molecules, altogether culminating in cellular disassembly [Oliver et al. 1999; Hengartner, 2000, Ola et al. 2011].

Cellular senescence is defined as an irreversible arrest in growth in which cells remain metabolically active but are unable to respond to mitogenic stimuli [Campisi & d’Adda di Fagagna, 2007]. Activation of cellular senescence coincides with dramatic alterations in gene expression, resulting in multiple cellular changes including enlarged and defective mitochondria; increased β-galactosidase production in lysosomes; protein aggregation in the endoplasmic reticulum and an enlarged, flattened morphology [Rayess et al. 2012]. The causes of cellular senescence are still under investigation but include (i) telomere dysfunction; (ii) excessive mitogenic signals, e.g. the over-activation of an aggressive oncogene, and (iii) unrepaired DNA lesions [Campisi & d’Adda di Fagagna, 2007; d’Adda di Fagagna, 2008; Evan & d’Adda di Fagagna, 2009]. In response to DNA DSBs, cellular senescence can be activated via ATM/Chk2-dependent p53 phosphorylation and stabilisation [d’Adda di Fagagna, 2008]. This leads to the induction of p21 which inhibits cyclin D-Cdk4/6; cyclin E-Cdk2 and PCNA activity, halting G1 progression into S phase as previously described (Section 1.6.3). Cellular senescence can also be activated by the tumour suppressor, p16\(^{INK4a}\), a CKI encoded by the the ink4a/arf locus [Wang et al. 2006; Campisi & d’Adda di Fagagna, 2007; Rayess et al. 2012]. p16\(^{INK4a}\) prevents Cdk-dependent phosphorylation and inactivation of the retinoblastoma protein (pRB) via inhibition of cyclin D-Cdk4/6 complex activities. Activated pRB subsequently suppresses the activity of E2F, a transcription factor that stimulates the expression of genes required for passage through the restriction point in late G1, thereby halting cell cycle progression [Ivanchuk, 2001; Massagué, 2004]. Overall, apoptosis and cellular senescence are key tumour-suppressive mechanisms that prevent the survival and/or growth of cells harbouring persistent DNA damage.
1.7 STEM CELLS: A MIXED RESPONSE TO DNA DAMAGE

The maintenance of genomic integrity in stem cells is indispensable for development and for maintaining and regenerating tissues throughout adult life. The deleterious effects of DNA damaging agents, such as IR, on tissue viability and regeneration, have fuelled mounting interest in the DDR of stem cells and their progenitors. Interestingly, these studies have collectively demonstrated that the level of radio-sensitivity varies widely between different stem cell types. For example, embryonic stem cells, HSCs and intestinal stem cells are highly radio-sensitive and undergo extensive apoptosis following irradiation [Meijne et al. 1991; Filion et al. 2009; Harfouche & Martin, 2010]. However, other stem cell types such as keratinocyte stem cells (KSCs) and hair follicle bulge stem cells (BSCs) are relatively resistant to IR-induced cell death [Tibeiro et al. 2002; Sotiropoulou et al. 2010]. Interestingly, radio-resistant stem cells, e.g. BSCs and KSCs, can repair IR-induced DNA DSBs whereas radio-sensitive stem cells, e.g. HSCs, have a poor DNA DSB repair capacity [Harfouche et al. 2010; Milyavsky et al. 2010; Sotiropoulou et al. 2010]. IR-induced DNA damage can also stimulate the differentiation of certain stem cell types, for example, (i) of melanocyte stem cells into mature melanocytes; (ii) of HSCs into lymphocytes and (iii) of neural stem cells into astrocytes [Inomata et al. 2009; Wang J et al. 2012; Schneider et al. 2013]. However, the differentiation of other stem cell types, including BSCs and KSCs, is unaffected by irradiation [Tibeiro et al. 2002; Sotiropoulou et al. 2010]. These studies collectively indicate that the execution of the DDR plays an important role in the response of individual stem cell types to DNA damaging agents.
1.8 HYPOTHESIS AND AIMS

The DNA Damage Response is fundamental for enabling cells to respond to the deleterious effects of DNA damaging agents including ionising radiation. The mechanisms underlying the radio-resistance of mouse MSCs are un-characterised. As previously described, MSCs reside in hypoxic niches \textit{in vivo}. Hypoxia-mediated alterations in the DDR are highly implicated in the increased radio-resistance of hypoxic cancer cells. However, whether hypoxia affects the DDR of irradiated MSCs is also currently unknown. In addition, with respect to the haematopoietic system, the mechanism(s) that account for the unique radio-resistance of DN2 thymocytes, in comparison with other haematopoietic cells, are also undescribed. Given the importance of the DDR in modulating the cellular response to irradiation, we hypothesised that (i) the execution of the DDR may play an important role in mediating the radio-resistance of mouse MSCs and DN2 thymocytes and that (ii) hypoxia may impact on mouse MSC radio-resistance by altering DDR signalling in this cell type.

Therefore, the overall goals of my Ph.D. studies were:

(i) To investigate the role of the DDR in mediating the radio-resistance of primary and cloned mouse MSCs \textit{in vitro} (Chapter 2).

(ii) To determine the effects of hypoxia on DDR signalling pathways executed by $\gamma$-irradiated mouse MSCs \textit{in vitro} (Chapter 3).

(iii) To characterise the DDR executed by $\gamma$-irradiated primary DN2 thymocytes (Chapter 4).

The experimental work performed to achieve these goals will now be discussed in detail.
Introduction
Chapter 2

The DNA Damage Response contributes to the radio-resistance of mouse MSC lines
2.1 INTRODUCTION

Haematopoietic homeostasis, or haemostasis, is the physiological state in which the rates of haematopoietic cells entering and leaving the peripheral blood are balanced. The maintenance of haemostasis is dependent on interactions between HSCs and the BM niche. Within the BM niche, MSCs provide soluble factors, contact-dependent interactions and physical support to control HSC self-renewal, quiescence and differentiation (Section 1.1) [Li & Li, 2006; Marigo & Dazzi, 2011]. When the haematopoietic system is exposed to IR e.g. γ-rays and X-rays, haemostasis is disturbed. Myeloablative radiation exposure results in the rapid depletion of haematopoietic stem and progenitor cells, causing the pools of differentiating and maturing blood cells to become exhausted, ultimately leading to haematopoietic failure [Mauch et al. 1995; Fliedner et al. 2002]. BMT is currently the only treatment available for haematopoietic failure following total body irradiation [Copelan, 2006]. The destruction of the host haematopoietic system by a lethal radiation dose creates a compartment within the BM in which donor HSCs and their progenitors can engraft and give rise to a new haematopoietic system. Several studies have shown that host-/patient-derived MSCs support the reconstitution of the haematopoietic system following allogeneic BMT [Arematsu & Nakahori, 1991; Dickhut et al. 2005; Rieger et al. 2005; Bartsch et al. 2009]. These findings indicate that MSCs are able to survive doses of radiation that are lethal to the haematopoietic system.

The major trigger of the cellular response to IR is its destructive impact on genome integrity. Cells can mount a co-ordinated response to genotoxic stresses, including IR, by activating a network of interacting signalling pathways, collectively known as the DNA Damage Response (DDR). The DDR signalling pathways consist of (i) Sensor proteins which recognize sites of damaged DNA and activate signal cascades by interacting with (ii) Transducer proteins which relay the signal to other downstream transducer proteins and, in turn, to (iii) Effector proteins which act on various genes and other proteins to induce a biological response [Kastan & Bartek, 2004; Harper & Elledge 2007; Bekker-Jensen & Mailand, 2010]. These effector proteins are involved in mechanisms that can cause cells to undergo cell cycle arrest, initiate DNA repair, become senescent or in the presence of irreparable DNA damage, to undergo apoptosis [Harper & Elledge, 2007]. Therefore, the ability of cells to effectively execute DDR signalling is essential for restoring genomic stability and for promoting survival following DNA damage.
The mechanisms underlying the radio-resistance of MSCs are currently poorly understood. A major element hindering advancement in this area is the use of bulk MSC populations which are heterogeneous in nature and vary in phenotype between isolations. Therefore, I have compared primary bulk mouse MSCs with two cloned mouse MSC lines, MS5 and ST2, which are widely used as supportive stroma for HSCs and their progenitors in vitro [Hardy et al. 1987; Itoh et al. 1989; Tong et al. 1999]. In recent years, they have been confirmed to be authentic MSC lines which express MSC cell surface markers and differentiate along mesenchymal-derived lineages [Yasuda et al. 1996; Ding et al. 2003; Durand et al. 2006; Zhou et al. 2012]. The objective of this study was to examine the role of the DDR in mediating the resistance of MSCs to gamma radiation (γ-radiation). I have compared in detail the DDR of the mouse MSC lines, MS5 and ST2, with that of the radio-sensitive mouse CD4⁺ CD8⁺ DP thymocyte cell line, ST4.5, to determine key aspects of the DDR that contribute to the radio-resistance of MSCs. This is the first study where the DDR of cloned mouse MSCs has been investigated in detail.

2.2 MATERIALS AND METHODS

2.2.1 Cell culture and treatment

Multiple independent isolates of C57BL/6 primary bulk mouse MSCs were provided by Prof. Matthew D. Griffin (REMEDE, NUI Galway) and cultured from passage 5-8 as previously described [Duffy et al. 2011a] (Appendix II). MS5 and ST2 MSC lines (Appendix I) were provided by Prof. Antonius Rolink (Department of Biomedicine, University of Basel) and were re-cloned prior to use. Primary bulk MSCs and MSC lines were CD45⁻, CD44⁺ and CD29⁺ (Figure 2.1A). ST4.5 CD4⁺ CD8⁺ DP thymocyte line (Appendix I) was provided by Dr. Anne Wilson (Ludwig Institute of Cancer Research, Lausanne). ST4.5 were CD4⁺ CD8⁺ double positive (DP) and expressed cytoplasmic TCR-β and CD3ε (Figure 2.1C). J774A.1 monocyte/macrophage cell line (Appendix I) was provided by Prof. Benjamin Bradley (Department of Orthopaedics, University of Bristol). All cell lines were cultured in high glucose DMEM (Sigma-Aldrich) supplemented with 10% FCS (Lonza) and 1% penicillin/streptomycin sulphate solution (Gibco) (Appendix II). All cell types were cultured in a humidified incubator at 37°C containing 5% CO₂. Cells were γ-irradiated at the indicated doses using a Mainance Millennium Sample Irradiator containing a ¹³⁷Cs source at a dose rate of approximately 102 cGy/min. Cells were treated with 25 µM Etoposide (Sigma-Aldrich)
The DNA Damage Response contributes to the radio-resistance of mouse mesenchymal stromal cell lines and with 1 μM Staurosporine solution (Cell Signaling Technologies) and harvested at the indicated time-points post treatment.

2.2.2 Clonogenic Survival Assay

Adherent cells were irradiated at the indicated doses and seeded into 6 well plates (Nunc) at a concentration of 200-500 cells/well, depending on cell type. Cells were incubated for 7-14 days until colonies of each cell type were clearly visible. Colonies were stained with Coomassie Blue (Sigma-Aldrich) and counted. Non-adherent ST4.5 cells were seeded into T25 flasks (Nunc) at a concentration of 5,000 cells/ml; harvested 5 days post irradiation and cell numbers counted in duplicate using a haemocytometer. The percentage survival of each cell type was determined by normalising the number of colonies/cells generated by irradiated cultures to the number of colonies/cells generated by control cultures. The plating efficiency of each cell type was MS5 (56.4 ± 10%), ST2 (60.8 ± 12.5%), J774A.1 (47 ± 4.85%) and bulk MSCs (27.9 ± 1.1%).

2.2.3 Differentiation Assays

Control and irradiated (10 Gy) cells were seeded into 24 well plates (Nunc) at a concentration of 30,000 cells/well and of 5,000 cells/well for osteogenic and adipogenic differentiation assays, respectively (see Appendix II for media preparation). Cells were continuously cultured in osteogenic induction medium and in adipogenic induction and maintenance media (alternated every 3 days) for 14 days. Osteogenic and adipogenic differentiation capacities were determined using Alizarin Red staining of calcium deposits and Oil Red O staining of lipid droplets, respectively, as previously described [Birmingham et al. 2012]. All images were captured using an Olympus IX71 Inverted Fluorescent Microscope with Olympus Cell^P Software® (Olympus, Hamburg, Germany).
Figure 2.1 The cell surface phenotype of MSC lines is comparable to bulk mouse MSCs. (A): Representative cytograms of MS5, ST2 and ST4.5 cells and primary bulk MSCs stained for CD45, CD44 and CD29 expression. MS5 cells stained for CD127 (IL-7Rα) expression were used as an isotype control. (B): Photograph of primary bulk C57BL/6 mouse MSCs in culture captured using 10X magnification. (C): Representative cytograms of ST4.5 thymocytes stained for CD4 and CD8 expression (left and centre panels) and surface / intracellular (i.c.) TCR-β and CD3ε staining (right panel). Unstained ST4.5 thymocytes were used as a negative control. All cytograms and images are representative of two independent experiments.
2.2.4 Antibodies

For Western Blotting, anti-phospho-Histone H2A.X (Ser139) mouse monoclonal antibody (Millipore), anti-H2AX rabbit polyclonal antibody, anti-ATM [2C1(1A1)] mouse monoclonal antibody, anti-DNA Ligase IV rabbit polyclonal antibody, anti-Ku70 [N3H10] mouse monoclonal antibody, anti-Ku80 [5C5] mouse monoclonal antibody, anti-β-Tubulin rabbit polyclonal antibody (Abcam), anti-ATR (N-19) goat polyclonal antibody, anti-Chk2 (H-300) rabbit polyclonal antibody (Santa Cruz Biotechnology Inc.), anti-p53 1C12 mouse monoclonal antibody, anti-PARP rabbit polyclonal antibody, anti-Caspase-3 rabbit polyclonal antibody, anti-Bcl-xL (54H6) rabbit monoclonal antibody, anti-Bcl-2 (D17C4) rabbit monoclonal antibody, anti-Bim (C34C5) rabbit monoclonal antibody, anti-Puma rabbit polyclonal antibody (Cell Signaling Technology), anti-β-Actin rabbit polyclonal antibody (Sigma-Aldrich), anti-DNA-PKcs Ab-4 mouse monoclonal antibody, Pierce® horseradish peroxidase (HRP) conjugated rabbit anti-mouse IgG antibody and ImmunoPure® HRP conjugated goat anti-rabbit IgG antibody (Thermo Scientific) were used. For Immunofluorescence Staining, anti-phospho-Histone H2A.X (Ser139) mouse monoclonal antibody (Millipore), anti-Rad51 rabbit polyclonal antibody (Abcam) were used. Secondary Fluorescein (FITC)-conjugated AffiniPure F(ab')2 Fragment goat anti-mouse IgG antibody and Texas Red®-conjugated AffiniPure F(ab')2 Fragment goat anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories Inc.) were used. For Flow Cytometry, rat anti-mouse CD45 PE-Cy7 conjugated, rat anti-mouse CD44 APC conjugated, anti-BrdU mouse monoclonal antibody (BD Biosciences), anti-mouse/rat CD29 (Integrin β1 chain) APC conjugated, anti-mouse CD127 (IL-7Rα) PE-Cy7 conjugated (Isotype control) (eBioscience) and FITC-conjugated anti-mouse IgG (whole molecule) antibody (Sigma-Aldrich) were used. Annexin-V-FITC conjugate was a gift from Prof. Corrado Santocanale (Centre for Chromosome Biology, NCBES, NUI Galway). Antibody preparations used are described in Appendix IV.

2.2.5 Western Blotting

Whole cell extracts were prepared from harvested control or irradiated cells at the indicated time-points post IR by re-suspending cell pellets in 4X SDS-loading buffer (Appendix III). Samples were heated and sonicated prior to separation using SDS-PAGE gels and transferred
The DNA Damage Response contributes to the radio-resistance of mouse mesenchymal stromal cell lines to nitrocellulose membranes. Chemiluminescence was detected using SuperSignal West Pico Chemiluminescent Substrate® (Thermo Scientific) and medical X-ray film (Konica Minolta).

2.2.6 Immunofluorescence Staining

MSCs were cultured on glass coverslips (Fisher Scientific) prior to irradiation. Irradiated ST4.5 thymocytes were transferred to poly-L-lysine coated microscope slides (Fischer Scientific) using a cytopsin. All cultures were fixed in 4% paraformaldehyde (Sigma-Aldrich), permeabilised in 0.1% Triton®-X 100 solution (Sigma-Aldrich), and incubated with the indicated primary antibodies at 4°C overnight. Following incubation with the indicated secondary antibodies at 37°C for 1 hr, cells were mounted in Vectashield containing Hoechst solution (Sigma-Aldrich). All images were captured using 100X magnification on an IX71 Olympus fluorescent microscope with ImageProPlus® 6.0 software (MediaCybernetics, MD, USA). Manual quantification of γ-H2AX IRIF per cell (total of 50 cells per time-point) was performed blind.

2.2.7 Flow Cytometry

2.2.7a Cell cycle analysis using BrdU–Propidium Iodide Staining

Cells were pulsed for 15 minutes - 4 hrs (depending on cell type) with 25 µM 5’-bromo-deoxyuridine (BrdU) (Sigma-Aldrich), washed in PBS and re-suspended in growth medium. Cells were harvested at the indicated time-points post irradiation and fixed in ice-cold 70% ethanol. Following acid denaturation using HCl, the cells were blocked in PBS containing 0.1% Triton-X solution and 0.5% BSA (Sigma-Aldrich). The cells were sequentially incubated with anti-BrdU antibody (BD Biosciences) and FITC-conjugated anti-mouse IgG antibody (Sigma-Aldrich) for 45 minutes each at room temperature (RT), separated by a washing step, and then re-suspended in Propidium Iodide (PI)/RNase staining buffer (BD Biosciences). The progression of G1, S and G2/M cells through the cell cycle was analysed by measuring the percentage of cells in each phase until 72 hrs post IR.

2.2.7b Analysis of Apoptosis using Annexin-V–Propidium Iodide Staining
Cells were irradiated and harvested at the indicated time-points post irradiation. Adherent cells were incubated at 37°C for 5 minutes. All cell types were then re-suspended in Annexin-V binding buffer (Appendix III) and incubated in FITC-conjugated Annexin-V solution for 15 minutes on ice. 5 µl of 20 µg/l PI solution (Sigma-Aldrich) was added to each cell suspension prior to analysis.

All FACS samples were analysed using a BD FACS Canto® Flow Cytometer and FlowJo® software (TreeStar Inc., OR, USA).

2.2.8 In Vitro DNA Double-Strand Break End-Joining Assay

Briefly, nuclear extracts were prepared according to manufacturer’s instructions (Active Motif). The pmaxFP-Green-N (pMAX-GFP) plasmid (Amaxa) was linearised by cleavage with XmnI. Nuclear extracts were incubated with digested plasmid in NHEJ sample buffer (Appendix III) at 37°C as previously described [Sotiropoulou et al. 2010]. Samples were separated on 0.8% agarose gels following proteinase K (0.65 mg/ml) (Fermentas) digestion.

2.2.9 Statistical Analysis

Statistical significance between the % survival of (i) MSCs (MS5, ST2 and bulk MSCs) and J774A.1 monocytes and of (ii) MSCs and ST4.5 thymocytes was calculated using Student’s t test using GraphPad Prism 6® software; p < 0.05 was considered statistically significant.

2.3 RESULTS

2.3.1 MSCs are resistant to IR treatment

To compare the radio-sensitivity of MSCs with haematopoietic cells, MSC lines (MS5 and ST2), primary bulk MSCs and haematopoietic cell lines (J774A.1 and ST4.5) were irradiated at 2-10 Gy and cultured at low density for a period of 5-14 days (depending on cell type). At the highest IR dose of 10 Gy, ~21% MS5 cells, ~34% ST2 cells and ~12% primary bulk MSCs formed colonies in contrast with 0.65% J774A.1 and 0% ST4.5 cells (Figure 2.2). No viable ST4.5 thymocytes were detected above 6 Gy irradiation (* in Figure 2.2). The LD50 dose was 2 Gy for ST4.5, 4 Gy for J774A.1 and 6 Gy for MSCs (Figure 2.2). Overall, these...
results indicate that MSCs were substantially more radio-resistant than haematopoietic cells. They also confirm that the MSC lines, MS5 and ST2, exhibited levels of radio-resistance similar to that of primary bulk MSCs.

Figure 2.2 Mouse MSCs are resistant to γ-radiation treatment. Clonogenic survival assay of mouse MSC lines (MS5 and ST2), primary bulk mouse MSCs, J774A.1 and ST4.5 cell lines γ-irradiated at 2-10 Gy and cultured for 5-14 days (depending on cell type) as previously described. Error bars represent mean ± SD, n=3. \( p < 0.05 \) (paired Student’s \( t \) test) for difference in % cell survival between * MSCs and J774A.1 and # MSCs and ST4.5 thymocytes. Viable ST4.5 thymocytes were not detected above 6 Gy.
2.3.2 MSCs rapidly activate the IR-induced DNA Damage Response

Confirming previous studies [Harris & Lowenthal, 1982; Radford, 1991], ST4.5 thymocytes were found to be sensitive to IR treatment. Therefore, using ST4.5 as a model for radiosensitivity, I examined the contribution of DDR mechanisms to the radio-resistance of MSCs. The ability of MSCs and ST4.5 thymocytes to activate the DDR was determined by analysing H2AX Ser139 phosphorylation (γ-H2AX – marker of DNA DSBs) at 0-24 hrs post IR using Western Blotting. Maximal H2AX phosphorylation was detected in MS5 and ST2 cells within 1 hr post 1 and 10 Gy irradiation whereas it was at 2 hrs in ST4.5 thymocytes (Figure 2.3A). I next examined whether the difference in γ-H2AX induction kinetics between irradiated MSC lines and ST4.5 thymocytes was correlated with varying expression levels of several key DDR proteins. ATM, DNA-PKcs and Chk2 were expressed at higher levels in MSCs than in ST4.5 thymocytes (Figure 2.3B and 2.3C). No difference in the expression levels of H2AX and ATR was detected between cell types (Figure 2.3B and 2.3C).

2.3.3 MSCs undergo cell cycle recovery following activation of IR-induced DNA damage checkpoints

I analysed the ability of MS5 and ST4.5 cells to activate IR-induced DNA damage checkpoints using a flow cytometry-based BrdU incorporation assay. Since BrdU is only incorporated into replicating DNA (i.e. S phase cells) at the time of BrdU treatment, BrdU-PI staining enabled clear identification of cells in G1 (BrdU negative), S (BrdU positive) and G2/M (BrdU negative) phases (Figure 2.4A, upper left panel). Dramatic differences in the response of the BrdU positive populations of MS5 and ST4.5 were observed following 10 Gy treatment. The irradiated BrdU positive MS5 population exhibited delayed cell cycle progression, indicative of the activation of intra-S-phase and G2 checkpoint mechanisms, as the proportion of BrdU positive cells remained essentially unchanged until 12 hrs post IR in comparison with untreated cells (Figure 2.4B). In contrast, the BrdU positive ST4.5 population reduced from ~47.9% to ~32.1% within 12 hrs post IR compared with ~53% to ~23.7% in the control (Figure 2.4B). A major factor contributing to this reduction in the ST4.5 thymocyte line was the generation of sub-G1 cells beginning at 2 hrs post IR which originated from both the BrdU negative and positive populations (Figure 2.4A, arrowheads in lower panels). Irradiated BrdU labelled MS5 and ST4.5 cells that progressed through S phase
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Figure 2.3 Mouse MSCs rapidly activate the DNA Damage Response. (A): Western blot analysis of H2AX Ser139 phosphorylation (γ-H2AX – marker of DNA double-strand breaks); total H2AX and β-Actin expression in MS5, ST2 and ST4.5 cells at 0-24 hrs post 1 and 10 Gy irradiation. Western blot analysis of (B): ATM, ATR and DNA-PKcs expression and (C): of H2AX and Chk2 expression in control MS5, ST2, bulk MSCs and ST4.5 cells. β-Actin and β-Tubulin expression were used as loading controls. All Western blot images are representative of one of three independent experiments.
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subsequently accumulated as a cohort in late S/G₂ until 12 hrs post IR (Figure 2.4A, right panels and Figure 2.4B) and exhibited delayed re-entry into G₁, indicating activation of the G₂/M accumulation checkpoint (Figure 2.4C) [Xu et al. 2002]. Following low dose irradiation (1 Gy), transient delays in S and G₂/M phase progression followed by a 2 hr delay in G₁ re-entry were observed in both MS5 and ST4.5 cells (Figure 2.4D and 2.4E), indicating that the duration of DNA damage checkpoint activation was dose-dependent in both cell types.

The BrdU labelled MS5 population remained arrested in G₂ until 12 hrs post IR (Figure 2.4A, *upper 12 hr time-point panel and 2.4B) whereas the BrdU labelled ST4.5 population was maintained at this checkpoint until 24 hrs post IR (Figure 2.4B). Approximately 42% total BrdU labelled MS5 cells were present in G₁/early S at 24 hrs post IR whereas BrdU labelled ST4.5 thymocytes entered the sub-G₁ population (Figure 2.4C and 2.4A). In addition, BrdU labelled G₁ MS5 cells re-initiated DNA synthesis, indicated by a reduction in BrdU signal intensity at 72 hrs post IR (Figure 2.4A, upper 72 hr time-point panel). ST2 cells could not be analysed by flow cytometry due to the high auto-fluorescence of this cell type. However, irradiated (10 Gy) BrdU labelled primary bulk MSCs also progressed as a cohort through S phase, accumulated in G₂ and exhibited delayed re-entry into G₁, indicative of the activation of intra-S and G₂/M DNA damage checkpoints (Figure 2.4F and 2.4G). In addition, approximately 48% total BrdU labelled MSCs were present in G₁/S at 36 hrs post IR with only low levels of sub-G₁ cells detected (Figure 2.4F and 2.4G).

To examine the G₁/S checkpoint response of these cell types, p53 stabilisation was analysed at 0-4 hrs post IR. p53 was only transiently stabilised by irradiated MSC lines and bulk MSCs whereas p53 stabilisation was maintained in irradiated ST4.5 thymocytes (Figure 2.5A). After 10 Gy irradiation, a dose-dependent increase in p53 stabilisation was detected in MSCs while p53 stabilisation kinetics remained unaltered in ST4.5 thymocytes (Figure 2.5A). However, prolonged p53 stabilisation and downstream induction of p21 and Puma expression were detected in all cell lines in the presence of etoposide (Figure 2.5B), indicating that p53 is functional in MSC lines.
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**Figure 2.4 (overleaf)** Mouse MSCs continue to cycle following DNA damage checkpoint activation. *(A)*: Representative cytograms of MS5 and ST4.5 cells harvested at 0-72 hrs post 10 Gy irradiation and stained for BrdU incorporation and DNA content using PI. Black boxes indicate G1 and G2/M (BrdU negative) and S (BrdU positive) populations. Black arrowheads indicate sub-G1 cells originating from BrdU negative and positive populations. * indicates cohort of BrdU labelled cells accumulated in late S/G2 and † indicates BrdU labelled G1/early S phase cells. *(B)*: Graph of the percentage BrdU labelled S phase MS5 and ST4.5 cells at 0-72 hrs post 10 Gy irradiation. *(C)*: Graph of the percentage BrdU labelled G1 / early S phase MS5 and ST4.5 cells at 0-72 hrs post 10 Gy irradiation. *(D)*: Graph of the percentage BrdU labelled S phase MS5 and ST4.5 cells at 0-72 hrs post 1 Gy irradiation. *(E)*: Graph of the percentage BrdU labelled G1/early S phase MS5 and ST4.5 cells at 0-72 hrs post 1 Gy irradiation. *(F)*: Representative cytograms of bulk MSCs harvested at 0-72 hrs post 10 Gy irradiation and stained for BrdU incorporation and for DNA content using PI. *(G)*: Graph of the percentage BrdU labelled G1/early S phase bulk MSCs at 0-72 hrs post 10 Gy irradiation. All cytograms are representative of one of three independent experiments. Error bars represent mean ± SD, n=2 (Control) or n=3 (Irradiated). * SD in bulk MSC control samples represents 2 independent experiments.
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Figure 2.4 Mouse MSCs continue to cycle following DNA damage checkpoint activation.
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Figure 2.5 Mouse MSCs stabilise p53 in response to γ-radiation and Etoposide treatment. Western blot analysis of (A): p53 stabilisation in MSC lines, bulk MSCs and ST4.5 thymocytes harvested at 0-4 hrs post IR at 1 and 10 Gy and (B): p53 stabilisation and p21 and Puma expression in MSC lines and ST4.5 thymocytes harvested at 0-8 hrs post Etoposide (25 µM) treatment. β-Tubulin expression was used as a loading control. All Western blot images are representative of one of three independent experiments.
2.3.4 MSCs are resistant to IR-induced apoptosis

The rapid appearance of sub-G1 cells and prolonged p53 stabilisation by irradiated ST4.5 thymocytes, in contrast to MSCs, indicated that these cell types may execute different apoptotic responses following IR treatment. Annexin-V/PI staining was performed to confirm whether irradiation induced apoptosis in MSCs and ST4.5 thymocytes. At only 4 hrs post IR, ~84.6% ST4.5 thymocytes were Annexin-V positive compared with ~1.7% MS5 cells (Figure 2.6A and 2.6B). At 36 hrs post IR, only ~4.7% MS5 cells were Annexin-V positive in comparison with ~96% ST4.5 thymocytes (Figure 2.6B). To determine whether MSCs activated the intrinsic apoptotic pathway in response to IR-induced DNA damage, cleavage of pro-caspase-3 and PARP was analysed. Minimal levels of caspase-3 and cleaved PARP were detected in irradiated (10 Gy) MS5 and ST2 cells (Figure 2.6C, left panels). In contrast, caspase-3 was detected in irradiated ST4.5 thymocytes beginning at 4 hrs post IR with levels remaining high for the duration of the experiment (Figure 2.6C, left panels). PARP cleavage was also detected in untreated ST4.5 thymocytes, likely due to the normal presence of a proportion of apoptotic cells correlated with the rapid turnover of this cell type. Nevertheless, a striking increase in PARP cleavage was detected in ST4.5 thymocytes following irradiation (Figure 2.6C, left panels).

To confirm that MSCs contained a functional intrinsic apoptotic pathway, all three cell lines were treated with 1 μM Staurosporine which potently induced pro-caspase-3 and PARP cleavage in all cell types (Figure 2.6C, right panels). To further examine the apparent resistance of MSCs to IR-induced apoptosis, I analysed the basal expression levels of pro- and anti-apoptotic proteins in MSCs and ST4.5 thymocytes. MSCs expressed higher levels of Bcl-2 and Bcl-XL than ST4.5 thymocytes (Figure 2.6D). In addition, MSCs expressed low levels of Bim and Puma in comparison with ST4.5 thymocytes (Figure 2.6E).
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Figure 2.6 (overleaf) Mouse MSCs are resistant to IR-induced apoptosis. (A): Representative cytograms of MS5 and ST4.5 cells harvested at 0-36 hrs post 10 Gy irradiation and stained with Annexin-V and for DNA content using PI. (B): Graph of the percentage Annexin-V positive MS5 and ST4.5 cells at 0-36 hrs post 10 Gy irradiation. Error bars represent mean ± SD, n=3. (C): Western blot analysis of Caspase-3 and PARP cleavage in MS5, ST2 and ST4.5 cells harvested at 0-36 hrs post IR (10 Gy) and Staurosporine treatment (1 μM). (D): Western blot analysis of Bcl-2 and Bcl-XL and (E): of Bim isoforms and Puma expression in control MS5, ST2, bulk MSCs and ST4.5 cells. β-Tubulin expression was used as a loading control. All cytograms and western blot images are representative of one of three independent experiments.
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Figure 2.6 Mouse MSCs are resistant to IR-induced apoptosis.

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2.3.5 MSCs resolve IR-induced DNA double-strand breaks

The ability of irradiated MSCs to de-phosphorylate H2AX and to resume proliferation without significant apoptosis suggested that MSCs may have a high capacity for repairing IR-induced DNA damage. To examine whether irradiated MSCs could potentially repair DNA DSBs, the resolution of γ-H2AX IRIF in MSCs and ST4.5 thymocytes was analysed following 1 and 10 Gy irradiation (Figure 2.7A – 2.7D). Greater numbers of γ-H2AX IRIF were detected in MS5 and ST2 cells than in ST4.5 thymocytes at 1 hr post IR (Figure 2.7B and 2.7D). This corresponded with the faster induction of maximal H2AX phosphorylation detected in irradiated MSC lines relative to ST4.5 thymocytes (Figure 2.3A). γ-H2AX IRIF were largely resolved in MS5 and ST2 cells at 24 hrs post 1 and 10 Gy irradiation (Figure 2.7A – 2.7D). In contrast, γ-H2AX IRIF persisted in irradiated ST4.5 thymocytes (Figure 2.7A).

In contrast to the MSC lines, highly variable numbers of γ-H2AX IRIF were detected in irradiated bulk MSCs at 1 hr post high dose (10 Gy) irradiation (Figure 2.8A and 2.8B). In addition, conflicting data was found between the kinetics of γ-H2AX IRIF formation and γ-H2AX expression by irradiated bulk MSCs. Following 1 Gy irradiation, low γ-H2AX signal was detected at 1 hr post IR by Western Blotting whereas increased numbers of γ-H2AX IRIF were observed at this time-point (Figure 2.8A - 2.8C). In addition, following 10 Gy irradiation, strong γ-H2AX signal was still present at 24 hrs post IR whereas γ-H2AX IRIF numbers were reduced at this time-point (Figure 2.8A - 2.8C). Thus, bulk MSCs displayed a heterogeneous response to IR-induced DNA DSBs. Importantly, bulk MSC cultures were devoid of contaminating haematopoietic (CD45+) cells (Figure 2.1A and 2.1B). Rad51 IRIF, which co-localised with γ-H2AX IRIF, were also detected in all cell types following IR treatment (Figure 2.7A and 2.7C; Figure 2.8A), suggestive of DNA DSB repair by HR. Interestingly, MSCs also expressed higher levels of Rad51 than ST4.5 thymocytes (Figure 2.7E).

To investigate the ability of MSCs to repair DNA DSBs using end-joining reactions, I used an established in vitro assay to examine the capacity of control nuclear extracts to re-ligate digested plasmid DNA containing a single DNA DSB [Sotiropoulou et al. 2010]. Nuclear extracts from MS5 and ST2 cells converted monomeric DNA fragments into multimers more efficiently than nuclear extracts from ST4.5 thymocytes (Figure 2.7F). Successful end-joining
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was inhibited by heat denaturation of nuclear extracts and was not detected in the presence of cytoplasmic extracts (Figure 2.7F). DNA Ligase IV and β-Tubulin expression were enriched in nuclear and cytoplasmic extracts, respectively, demonstrating nuclear extract purity (Figure 2.7G). Higher expression levels of DNA Ligase IV and DNA-PKcs were detected in both nuclear and whole cell extracts of MS5 and ST2 compared with ST4.5 cells, suggesting that MSCs performed end-joining reactions using NHEJ (Figure 2.7H and 2.7I). Taken together, these results suggest that MSCs may have a higher capacity to repair IR-induced DNA DSBs than ST4.5 thymocytes which is likely to contribute to their greater ability to survive IR treatment.

2.3.6 MSCs retain their differentiation potential following IR treatment

Finally, we determined whether irradiation affects the adipogenic and osteogenic differentiation potential of MSCs. Adipogenic differentiation, characterised by the formation of large spherical-shaped cells containing lipid droplets stained with Oil Red O, was detected in control and irradiated MS5 and ST2 cultures (Figure 2.9A) [Gregoire et al. 1998]. Similar staining for calcium deposition (detected using Alizarin Red S), indicative of osteogenic differentiation, was found between control and irradiated cultures for each MSC line (Figure 2.9B). Overall these results indicate that MSCs retained the ability to differentiate in vitro following irradiation.
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Figure 2.7 Mouse MSCs repair DNA double-strand breaks (continued overleaf).
Representative immunofluorescent images of MS5, ST2 and ST4.5 cells at 0-24 hrs post (A): 1 Gy and (C): 10 Gy irradiation and stained for DNA content (DAPI), γ-H2AX (green) and Rad51 (red) IRIF captured using 100X or 10X (ST4.5 in panel C) magnification. Graph of the average number of γ-H2AX IRIF per cell (50 cells in total per time-point) at 0, 1 and 24 hrs post (B): 1 Gy and (D): 10 Gy irradiation. Error bars represent mean ± SD, n=2 (1 Gy) or n=3 (10 Gy). (E): Western blot analysis of Rad51 expression in control MS5, ST2, Bulk MSCs and ST4.5 cells.
Figure 2.7 (continued) Mouse MSCs repair DNA double-strand breaks. (F): Analysis of efficiency in end-joining reactions containing linearised plasmid and nuclear extracts/nuclear extracts denatured at 95°C for 5 minutes / cytoplasmic extracts derived from control MS5, ST2 and ST4.5 cells. (G): Western blot analysis of DNA ligase IV and β-Tubulin expression in nuclear and cytoplasmic extracts of MS5, ST2 and ST4.5 cells. Western Blot analysis of DNA ligase IV, DNA-PKcs, Ku70 and Ku80 expression in (H): nuclear and (I): whole cell extracts of control MS5, ST2 and ST4.5 cells. β-Tubulin expression was used as a loading control. Agarose gel, immunofluorescent and Western blot images are representative of one of three independent experiments.
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Figure 2.8 Primary bulk mouse MSCs display a heterogeneous response to IR-induced DNA damage. **(A):** Representative immunofluorescent images of bulk MSCs 0-24 hrs post 1 and 10 Gy irradiation stained for DNA content (DAPI), γ-H2AX (Green) and Rad51 (Red) IRIF and captured using 100X magnification. **(B):** Graph of the average number of γ-H2AX foci per cell (50 cells in total per time-point) at 0, 1 and 24 hrs post 1 and 10 Gy irradiation. Error bars represent mean ± SD, n=2. **(C):** Western blot analysis of γ-H2AX and H2AX expression in primary bulk mouse MSCs at 0-24 hrs post 1 Gy and 10 Gy irradiation using β-Actin as a loading control. Immunofluorescent and Western blot images are representative of one of three independent experiments.
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Figure 2.9 MSCs retain the capacity to differentiate following IR treatment. (A): Oil Red O staining of control and irradiated (10 Gy) MS5 and ST2 cells cultured in adipogenic induction and maintenance media for 17 days as previously described. White arrows indicate spherical-shaped adipocytes containing triglycerides stained as red droplets using Oil Red O. (B): Alizarin Red S staining of calcium deposition by control and irradiated (10 Gy) MS5 and ST2 cells cultured in osteogenic induction medium for 14 days as previously described. Images were captured using 4X and 10X magnification and are representative of one of two independent experiments.
2.4 DISCUSSION

IR causes high levels of genotoxic stress due to the production of reactive oxygen species (ROS) and of a wide range of genomic lesions including DNA DSBs. The execution of an effective DDR is critical for promoting cell survival following IR exposure. Previous studies examining the role of the DDR in driving the radio-resistance of MSCs have been performed on primary, un-cloned, bulk MSC cultures [Chen et al. 2006; Damek-Propawa et al. 2010; Prendergast et al. 2011]. These bulk MSC cultures are heterogeneous in nature, making detailed analysis difficult. Similarly, I found that primary bulk mouse MSCs displayed a heterogeneous response to IR-induced DNA damage (Figure 2.8). The use of two widely used clonal authentic mouse MSC lines, MS5 and ST2, has enabled a detailed in vitro study of the IR response of mouse MSCs to be performed. This is the first study where the DDR of cloned mouse MSCs has been investigated in detail. Only by studying cloned MSC lines can we understand the DDR mechanisms of MSCs at the molecular level. The MS5 cell line was originally established from irradiated long-term BM cultures of C3H/HeNSlc mice whereas the ST2 cell line was established from non-irradiated BALB/c mouse foetal liver [Hardy et al. 1987; Itoh et al. 1989]. Despite their differing sources, both MSC lines executed highly comparable responses to IR treatment. These two MSC lines were also as radio-resistant as primary bulk mouse MSCs (Figure 2.2). In addition, their ability to differentiate was maintained following high dose irradiation (Figure 2.9). By comparing the radio-biology of MSCs and ST4.5 thymocytes, this study has clearly demonstrated that the DDR is differentially executed by different cell types.

DNA DSBs are the most genotoxic lesions caused by IR [Ward et al. 1988; Ciccia & Elledge, 2010]. The activation of the DDR in response to DNA DSBs is characterised by ATM-dependent H2AX Ser139 phosphorylation (γ-H2AX) [Rogakou et al. 1998; Shiloh, 2003; Fernandez-Capetillo et al. 2004]. Analysis of H2AX phosphorylation demonstrated that irradiated MSC lines activated the DDR more rapidly than the radio-sensitive CD4+ CD8+ thymocyte line, ST4.5 (Figure 2.3A). However, currently it is unknown whether similar numbers of DNA DSBs are generated in these cell types when irradiated at the same dose. Thus, given more time, it would be interesting to analyse irradiated ST4.5 and MSC nuclei via the neutral comet assay to compare DNA DSB generation in these cell types. In addition, the increasing γ-H2AX signal in ST4.5 extracts from 1-2 hrs post IR (Figure 2.3A) could be as a consequence of the rapid activation of apoptosis in this cell type (Figure 2.6A and 2.6B).
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Therefore, it would be interesting to determine whether blocking apoptosis in ST4.5 thymocytes, e.g. via a caspase inhibitor, alters H2AX phosphorylation kinetics in this cell type post irradiation. Furthermore, MSCs expressed higher levels of ATM and DNA-PKcs than ST4.5 thymocytes which mediate γ-H2AX formation in response to DNA DSBs (Figure 2.3B and 2.3C) [Bartek & Lukas, 2003; Shiloh, 2003]. This may suggest that high levels of ATM and DNA-PKcs in MSCs could promote efficient DDR activation via rapid γ-H2AX induction, enabling rapid activation of mechanisms such as DNA repair and DNA damage checkpoints, enhancing survival following IR exposure. Therefore, given more time, it would also be interesting to over-express ATM and / or DNA-PKcs in ST4.5 thymocytes to determine whether altering the expression levels of these proteins could influence H2AX phosphorylation kinetics in this cell type.

DNA damage caused by IR can activate DNA damage checkpoints which transiently delay or arrest cell cycle progression to facilitate DNA repair before entering the next phase of the cell cycle. By monitoring the progression of BrdU labelled S phase MSCs, I found that MSCs activated robust intra-S-phase and G2/M checkpoint mechanisms in response to high dose irradiation (Figure 2.4A and 2.4B). In addition, irradiated (10 Gy) BrdU labelled MS5 cells recovered from cell cycle arrest faster than ST4.5 thymocytes and re-initiated DNA synthesis (Figure 2.4C). This suggested that MSCs have a high capacity for repairing IR-induced DNA damage. In addition, I found that p53 was transiently stabilised in irradiated MSCs (Figure 2.5A). Transient p53 stabilisation by irradiated mouse hair follicle BSCs has been correlated with their high NHEJ capacity [Sotiropoulou et al. 2010]. Using an in vitro DNA DSB end-joining assay applied in this study, I demonstrated that MSC nuclear extracts were more proficient at end-joining than extracts from ST4.5 thymocytes (Figure 2.7F) [Sotiropoulou et al. 2010]. Analysis of γ-H2AX IRIF kinetics and detection of Rad51 IRIF formation also suggested that MSCs could repair DNA DSBs using HR (Figure 2.7A – 2.7D). Moreover, MSCs expressed higher levels of the DNA DSB repair proteins - DNA ligase IV, DNA-PKcs and Rad51 - than ST4.5 thymocytes (Figure 2.7E – 2.7I). The high DNA DSB repair capacity of MSCs is likely to enhance their ability to overcome DNA damage checkpoint activation and to survive γ-radiation treatment.

Previous studies have shown that the balance between the levels of pro- and anti-apoptotic proteins plays an important role in mediating the radio-resistance of other stem cells such as HSCs and BSCs [Domen et al. 1998; Sotiropoulou et al. 2010]. Correspondingly, MSCs
expressed high levels of the anti-apoptotic proteins, Bcl-2 and Bcl-\textsuperscript{XL} (Figure 2.6D), and low levels of the potent pro-apoptotic proteins, Bim and Puma (Figure 2.6E). In addition, despite containing functional apoptotic machinery, irradiated MSCs did not immediately activate the intrinsic apoptotic pathway (Figure 2.6A – 2.6C). The bias of pro- and anti-apoptotic proteins towards those that favour survival is likely to enhance the ability of MSCs to activate pro-survival mechanisms following DNA damage.

By comparing the radio-biology of MSCs and ST4.5 thymocytes, this study has demonstrated that the DDR is differentially executed by independent cell types. Although ST4.5 thymocytes served as an important radio-sensitive control in this study, they represent fully committed T lymphocyte precursors undergoing selection in the thymus and therefore, are intrinsically primed to undergo apoptosis (Section 1.4). Therefore, future investigations into DDR mechanisms underlying MSC radio-resistance may be strengthened by directly comparing the DDR of MSCs with that of (i) the other BM-derived stem cell, i.e. the HSC and (ii) other known radio-resistant stem cell types, such as BSCs and KSCs.

Clinically, the radio-resistance of MSCs has multiple implications. Total body irradiation (TBI) is frequently used prior to allogeneic BMT. Depletion of MSC sub-types within mouse bone marrow impedes HSC maintenance and function indicating that host MSCs are essential for promoting HSC engraftment and haematopoietic reconstitution [Sugiyama et al. 2006; Méndez-Ferrer et al. 2010; Ding et al. 2012; Roberts et al. 2013; Tran et al. 2013]. Therefore, understanding MSC radio-biology could impact on the development of pre-conditioning regimens for allogeneic BMT. MSCs are also important components of the tumour stroma to which they have been recruited [Marigo & Dazzi, 2011]. Tumour cells can secrete various stimulatory signals (e.g. VEGF, IL-8 and TGF-\textbeta) which induce MSC homing and subsequent conversion into cancer-associated fibroblasts (CAFs) [Berfeld & DeClerck, 2010; Kraman et al. 2010; Kidd et al. 2012]. CAFs promote tumour growth, angiogenesis and metastasis and suppress anti-tumour immune responses [Bergfeld & DeClerck, 2010; Kraman et al. 2010]. In addition, irradiated MSCs produce factors, including tumour necrosis factor (TNF)-\textalpha, transforming growth factor (TGF)-\textbeta, IL-6 and fibronectin, implicated in tumour formation and tumour stem cell maintenance [Greenberger et al. 1996b; Ayala et al. 2009]. Intratumoural MSCs may thus survive conventional radiotherapy. Therefore, our understanding of MSC radio-biology also has important implications for understanding cancer progression and for developing new strategies for cancer treatment.
This study has clearly shown that multiple aspects of the DDR play key roles in promoting the radio-resistance of MSCs. Firstly I have shown that MSCs retained their capacity to proliferate and to differentiate following IR exposure. Secondly, while irradiated ST4.5 thymocytes failed to execute effective DDR mechanisms and undergo apoptosis, MSCs activated robust DNA damage checkpoints and DNA repair mechanisms that enhance their ability to survive, thereby suppressing apoptosis. Finally, I have demonstrated that MSCs appeared to be intrinsically programmed with the ability to effectively execute the DDR following IR treatment by expressing high levels of key DDR proteins such as ATM, Chk2, DNA-PKcs and DNA Ligase IV; high levels of the anti-apoptotic proteins Bel-2 and Bel-XL and low levels of pro-apoptotic proteins such as Bim and Puma. MSCs are responsible for supporting and monitoring haematopoiesis; for modulating immune responses and for promoting tumourigenesis. Therefore, our understanding of how MSCs behave in response to irradiation has direct implications on future advances in improving the success rates of allogeneic BMT and in cancer research and treatment.
Chapter 3

Hypoxia enhances the radio-resistance of mouse MSCs
3.1 INTRODUCTION

The BM is essential for adult haematopoiesis and for regulating haemostasis in the periphery. These key processes are tightly controlled by a specialised microenvironment known as the BM niche which consists of a network of immune and stromal cell types, including MSCs. MSCs provide physical support for HSCs and regulate haematopoiesis by controlling HSC self-renewal, differentiation and retention [Wilson & Trumpp, 2006; Celso & Scadden, 2011; Wang & Wagers, 2011; Mercier et al. 2012]. MSCs are also an integral component of the tumour microenvironment [Hanahan & Weinberg, 2011; Hanahan & Coussens, 2012]. Signals from the tumour mass stimulate MSC exodus from the BM to the tumour where they develop into CAFs. CAFs are characterised by the expression of specific fibroblast markers including α-smooth muscle actin (α-SMA) and FAP [Bergfeld & De Clerk, 2010; Kraman et al. 2010; Kidd et al. 2012; Hanahan & Coussens, 2012]. CAFs support tumourigenesis in multiple ways including (i) physical tumour support (ii) secretion of growth (e.g. EGF) and pro-angiogenic factors (e.g. VEGF); (iii) suppression of anti-tumour immune responses and (iv) inhibition of tumour cell apoptosis [Bergfeld & De Clerk, 2010; Kraman et al. 2010; Marigo & Dazzi, 2011; Hanahan & Coussens, 2012].

Hypoxia is a key feature of the BM and tumour microenvironments [Vaupel, 2004; Vaupel et al. 2004; Eliasson & Jönsson, 2009; Ruan et al. 2009; Moyheldin et al. 2010]. Cellular adaptation to hypoxia is primarily mediated by the transcriptional activator, hypoxia inducible factor-1 (HIF-1). HIF-1 consists of a constitutively expressed HIF-1 β-subunit and an oxygen-regulated HIF-1α subunit (or its paralogues HIF-2α and HIF-3α). HIF-1α is stabilised in hypoxia due to inhibited proteasomal degradation, a pathway involving PHDs (prolyl hydroxylase domain-containing proteins); FIH (factor inhibiting HIF) and an E3 ubiquitin ligase termed, pVHL (product of the von Hippel-Lindau tumour suppressor). This enables dimerisation with β subunits in the nucleus and downstream activation of hypoxia-responsive genes [Semenza, 2000; Ke & Costa, 2006; Benizri et al. 2008; Ruan et al. 2009]. Intra-tumoural hypoxia and HIF-1α over-expression are associated with increased malignancy and metastatic potential, and with poor patient outcome [Unruh et al. 2003; Vaupel, 2004]. This prognosis is highly correlated with the
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increased therapeutic resistance of hypoxic cancer cells [Vaupel, 2004; Vaupel et al. 2004; Bertout et al. 2008].

Radiotherapy destroys cells by inducing extensive DNA damage, particularly highly genotoxic DNA DSBs [Ward, 1988; Ciccia & Elledge, 2010]. This genotoxic stress activates a network of interacting signalling pathways, collectively termed the DNA Damage Response (DDR). Conceptually, the DDR consists of (i) sensor proteins which recognise sites of damaged DNA; (ii) transducer proteins which amplify the DNA damage signal and (iii) effector proteins which execute appropriate biological response(s) such as DNA repair, transient delays in cell cycle progression (i.e. checkpoints), transcription and apoptosis [Kastan & Bartek, 2004; Harper & Elledge, 2007]. Cellular exposure to severe hypoxia (<1% O2) can activate the DDR independently of DNA damage, indicating an important role for the DDR in adaptation to low oxygen environments [Hammond et al. 2002 & 2003; Gibson et al. 2005 & 2006; Bouquet et al. 2011; Wrann et al. 2013].

There is mounting evidence that hypoxia-mediated modulation of the DDR contributes to the resistance of hypoxic cancer cells to DNA damaging agents, including IR [Bristow & Hill, 2008; Rohwer et al. 2012]. MSCs are highly radio-resistant and can support haematopoietic re-constitution following TBI [Anklesaria et al. 1987; Dickhut et al. 2005; Rieger et al. 2005; Chen et al. 2006; Mugurama et al. 2006; Bartsch et al. 2009; Sugrue et al. 2013a & 2013b]. I have previously shown that execution of the DDR is fundamental to the radio-resistance of mouse MSCs (Chapter 2) [Sugrue et al. 2013a]. Despite their important roles in supporting BM re-constitution and tumourigenesis, the effects of hypoxia on MSC radio-resistance are currently unknown. In this study, I compared the effects of normoxia (21% O2) and hypoxia (5% O2) on mouse MSC radio-resistance using primary bulk MSCs, two clonal authentic mouse MSC lines, MS5 and ST2, and the mouse CD4+ CD8+ DP thymocyte line, ST4.5, as a radio-sensitive control. This study demonstrates, for the first time, that hypoxia enhances mouse MSC radio-resistance and this is due to alterations in their DDR that occur in a HIF-1α dependent manner.
3.2 MATERIALS AND METHODS

3.2.1 Cell culture and treatment

C57BL/6 primary bulk mouse MSCs were provided by Prof. Matthew D. Griffin (REMEDI, NUI Galway) and cultured from passage 6-8 as previously described (Section 2.2.1) [Duffy et al. 2011a; Sugrue et al. 2013a]. BM was isolated from Fox Chase SCID® Beige mice, provided by Dr. Maoija Xu (REMEDI, NUI Galway) by crushing femurs in sterile DMEM using a mortar and pestle. The resulting BM suspension was filtered through 70 µm nylon mesh, centrifuged and re-suspended in ammonium-chloride-potassium (ACK) buffer (Appendix III) for 5 minutes to lyse erythrocytes. Following centrifugation, the cells were re-suspended in growth medium, transferred to 10 cm culture dishes (Nunc) and maintained at passage 0 for 4 weeks in 5% O2, with frequent media changes to remove contaminating haematopoietic cells. MS5 and ST2 MSC lines (Appendix I) were provided by Prof. Antonius Rolink (Department of Biomedicine, University of Basel) and were re-cloned prior to use. All MSC cultures were CD45−, CD44+ and CD29+ as previously described (Figure 2.1A) [Duffy et al. 2011a; Sugrue et al. 2013a]. ST4.5 CD4+ CD8+ DP thymocyte line (Appendix I, Figure 2.1C) was provided by Dr. Anne Wilson (Ludwig Institute for Cancer Research, Lausanne). All cell types were cultured in humidified incubators at 37°C containing 21% O2 (normoxia) or 5% O2 (hypoxia).

Cells were γ-irradiated at the indicated doses using a Mainance Millennium Sample Irradiator containing a 137Cs source at a dose rate of approximately 102 cGy/min.

3.2.2 Growth curve analysis

Cells were seeded into 6 well plates (Nunc) at a concentration of 30,000 (ST4.5) or 50,000 (MSCs) cells/well. Individual cultures were harvested daily for 5 (ST4.5) or 7 days (MSCs) and cell counts were performed in duplicate in a haemocytometer using Trypan blue exclusion.
3.2.3 Clonogenic survival assay

MSCs were irradiated at 2-10 Gy and seeded into 6 well plates (Nunc) at a concentration of 200 (MSC lines) or 1,000 (bulk MSCs) cells/well. Cells were cultured for 7 (MSC lines) or 14 (bulk MSCs) days in 5% or 21% O2 until colonies were clearly visible. Colonies were stained with Coomassie Blue solution and counted as previously described (Section 2.2.2) [Sugrue et al. 2013a]. Non-adherent ST4.5 thymocytes were irradiated (2-10 Gy) and seeded into 6 well plates (Nunc) at a concentration of 50,000 cells/ml and cell numbers were counted 5 days post irradiation using trypan blue exclusion. The percentage survival of each cell type was determined by normalising the number of colonies/cells generated in irradiated cultures to the number of colonies/cells generated in control cultures. Average colony size (sq. mm) per well was calculated using Kodak MI 4.5 software.

3.2.4 Antibodies

For Flow Cytometry, anti-BrdU mouse monoclonal antibody (BD Biosciences); anti-phospho histone H3 (Ser10) rabbit polyclonal antibody (Merck Millipore); FITC-conjugated anti-mouse IgG (whole molecule) antibody (Sigma-Aldrich) and FITC-conjugated AffiniPure F(ab’)2 Fragment goat anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories Inc.) were used. Annexin-V-FITC conjugate was a gift from Prof. Corrado Santocanale (Centre for Chromosome Biology, NCBES, NUI Galway). For Immunofluorescence Staining, anti-phospho-Histone H2A.X (Ser139) mouse monoclonal antibody (Millipore); anti-Rad51 rabbit polyclonal antibody (Abcam); FITC-conjugated AffiniPure F(ab’)2 Fragment goat anti-mouse IgG antibody and Rhodamine Red®-conjugated AffiniPure F(ab’)2 Fragment goat anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories Inc.) were used. For Western Blotting, anti-phospho-Histone H2A.X (Ser139) mouse monoclonal antibody (Merck Millipore), anti-H2AX rabbit polyclonal antibody, anti-DNA Ligase IV rabbit polyclonal antibody, anti-Rad51 rabbit polyclonal antibody, anti-β-Tubulin rabbit polyclonal antibody (Abcam), anti-p53 1C12 mouse monoclonal antibody, anti-HIF-1 alpha rabbit polyclonal antibody, anti-HIF-2 alpha rabbit polyclonal antibody (Novus Biologicals), anti-DNA-PKcs Ab-4 mouse monoclonal antibody, Pierce® HRP conjugated rabbit anti-mouse IgG antibody
Hypoxia enhances the radio-resistance of mouse MSCs and ImmunoPure® HRP conjugated goat anti-rabbit IgG antibody (Thermo Scientific) were used. Antibody preparations used are described in Appendix IV.

3.2.5 Flow Cytometry

3.2.5a Cell cycle analysis using BrdU–Propidium Iodide Staining

Cells were labelled for 15 minutes (ST4.5) or 45 minutes (MS5) with 25 µM BrdU (Sigma-Aldrich), washed in PBS and re-suspended in growth medium. Cells were harvested at the indicated time-points post irradiation (10 Gy or 0.5 Gy), fixed in ice-cold 70% ethanol and stained with anti-BrdU and FITC-conjugated anti-mouse IgG antibodies and PI/RNase staining buffer (BD Biosciences) as previously described (Section 2.2.7a) [Sugrue et al. 2013a]. The progression of G₁, S and G₂/M cells through the cell cycle was analyzed by measuring the percentage of cells in each phase until 36 hrs post IR.

3.2.5b G₂/M checkpoint analysis using phospho-Histone H3 Ser10-Propidium Iodide Staining

MS5 and ST4.5 cells were harvested at the indicated time-points post irradiation (10 Gy or 0.5 Gy), washed in PBS and fixed in ice-cold 70% ethanol. Following permeabilisation in PBS/0.25% Triton X-100, cells were sequentially stained with anti-phospho-histone H3(Ser10) and FITC-conjugated anti-rabbit IgG antibodies for 2 hrs and 30 minutes at RT, respectively, separated by a washing step in PBS/1% BSA. Cells were then re-suspended in PI/RNase staining buffer (BD Biosciences) and mitotic index was analysed up to 24 hrs post irradiation.

3.2.5c Analysis of Apoptosis using Annexin-V–Propidium Iodide Staining

MS5 and ST4.5 cells were harvested at the indicated time-points post irradiation (10 Gy or 0.5 Gy); incubated at 37ºC for 5 minutes and then re-suspended in Annexin-V binding buffer (Appendix III) and stained with FITC-conjugated Annexin-V solution and PI as previously described (Section 2.2.7b) [Sugrue et al. 2013a].
3.2.5d In vivo Homologous Recombination Assay

MS5 cells were stably transfected with DR-GFP HR reporter [Pierce et al. 1999], provided by Prof. Ciaran Morrison (Centre for Chromosome Biology, NUI Galway) using Lipofectamine 2000® (Invitrogen) according to manufacturer’s instructions. Individual MS5 clones were selectively expanded in Puromycin (10 µg/ml). Positive clones were screened by amplifying a ~500 bp fragment encompassing the Sce-gfp sequence with 1 µg genomic DNA using the following PCR primers: Forward: 5’-GTGAGCAAGGGCGAGGAG-3’ and Reverse: 5’-ATCTTGAAGTTCACCTTGAT-3’ (Figure 3.7A and 3.7B). DSBs were generated by transfecting cells with I-Sce I endonuclease expressing vector, pCBA Sce, and HR efficiency was measured 2 days later by analysing GFP expression using flow cytometry [Pierce et al. 1999; Nakanishi et al. 2011]. Clone MS5 HR4.4 was selected based on screening of transfected clones for efficient GFP expression and expanded in 21% and 5% O₂ prior to transfection.

All FACS samples were analysed using BD FACS Canto® and Accuri™ C6 flow cytometers (BD Biosciences) and FlowJo® software (TreeStar Inc., OR, USA).

3.2.6 Western Blotting

Whole cell extracts were prepared from harvested control or irradiated cells at the indicated time-points post irradiation as previously described (Section 2.2.5) [Sugrue et al., 2013a]. Samples were separated using 4-15% SDS-PAGE gels and transferred to nitrocellulose membranes. Chemiluminescence was detected using SuperSignal West Pico Chemiluminescent Substrate® (Thermo Scientific) and medical X-ray film (Konica Minolta).

3.2.7 Immunofluorescence staining

Cells were cultured on glass coverslips in 21% or 5% O₂ for at least 24 hrs prior to irradiation. All cultures were fixed in 4% paraformaldehyde (Sigma-Aldrich), permeabilised in 0.1% Triton®-X 100 solution and nuclei stained for γ-H2AX and Rad51 IRIF as previously described (Section 2.2.6) [Sugrue et al. 2013a]. All images were captured using 100X magnification on an IX51 Olympus fluorescent microscope using
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Olympus xcellence® software (Olympus, Hamburg, Germany). The number of γ-H2AX and Rad51 IRIF per nucleus was manually quantified in a blind manner in a total of 50 cells per time-point in each experiment.

3.2.8 In Vitro DNA Double-Strand Break End-Joining Assay

Briefly, nuclear extracts were prepared according to manufacturer’s instructions (Active Motif). The pMAX-GFP plasmid (Amaxa) was linearised by cleavage with XmnI. End-joining efficiency of nuclear extracts prepared from MS5 cells cultured in 21% O₂ and 5% O₂ and of passage 0 bulk MSC cultures (5% O₂) derived from Fox Chase SCID® Beige mouse femurs was analysed as previously described (Section 2.2.8) [Sotiropoulou et al. 2010; Sugrue et al. 2013a]. Ligated DNA fragment intensity was quantified using ImageJ® software. Ligated band intensity was determined by normalising the intensity values of ligated DNA fragments detected in normoxic and hypoxic MS5 samples to the background intensity detected in SCID bulk samples.

3.2.9 Small interfering (si)RNA-mediated knockdown of HIF-1α expression

MSC lines cultured in 5% O₂ in Opti-MEM® (Invitrogen) were transfected with 5 nM Silencer® select negative control mouse siRNA or with 50 nM Silencer® select pre-designed HIF-1α siRNAs (s67532 and n414564) (Ambion®) using Oligofectamine® according to manufacturer’s instructions (Invitrogen). High glucose DMEM (Sigma-Aldrich) supplemented with 30% FCS was added to cultures 3 hrs post transfection. Irradiation and harvesting of transfected cultures for required experiments was performed 2 days post transfection. ~60-80% HIF-1α knockdown efficiency was achieved in MSC lines (Figure 3.9E).

3.2.10 Neutral Comet Assay

MS5 cells cultured in 21% or 5% O₂ were harvested immediately following 10 Gy irradiation. Single gel electrophoresis of lysed cells embedded in Comet LMAagarose® on poly-L-lysine coated microscopy slides (Fisherbrand) was performed according to manufacturer’s instructions (Trevigen). Slides were subsequently stained with SYBR
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Green® following DNA precipitation and images of 50 cells on average per slide were captured using 10X magnification on an IX51 Olympus fluorescent microscope using Olympus xcellence® software (Olympus, Hamburg, Germany). Average comet tail moment was calculated using CometScore™ software (TriTek Corp., VA, USA).

3.2.11 Statistical Analysis

Statistical significance between (i) normoxic (21% O₂) and hypoxic (5% O₂) samples and (ii) MSCs transfected with scrambled siRNA or HIF-1α siRNA was calculated using paired Student’s t test using Graph Pad Prism 6® software; p < 0.05 was considered statistically significant.

3.3 RESULTS

3.3.1 Hypoxia enhances mouse MSC radio-resistance in vitro

Growth curves of MSC lines (MS5 and ST2), primary bulk C57BL/6 MSCs and CD4⁺ CD8⁺ DP thymocytes (ST4.5 cell line) cultured in normoxia (21% O₂) and hypoxia (5% O₂) were first analysed to determine the effects of hypoxia on cell growth. MSC growth rate was measurably increased in hypoxia (Figure 3.1A and 3.1B). For example, on day 5, ~136 x 10⁴ MS5 cells; ~134 x10⁴ ST2 cells and ~25 x 10⁴ bulk MSCs were detected in normoxia compared with ~160 x 10⁴ MS5 cells; ~152 x 10⁴ ST2 cells and ~46 x 10⁴ bulk MSCs in hypoxia (Figure 3.1A and 3.1B). Unlike MSCs, the growth of ST4.5 thymocytes was un-altered in hypoxia (Figure 3.2). Clonogenic survival assays revealed a 2-3 fold increase in MSC survival post irradiation, particularly at higher IR doses (6-10 Gy) (Figure 3.1C and 3.1D). The LD₅₀ dose was increased from 6 Gy (MS5 and ST2) and 4 Gy (bulk MSCs) in normoxia to 8 Gy for all in hypoxia (Figure 3.1C and 3.1D). Furthermore, MSC colony size was increased in hypoxia under control and irradiated conditions (Figure 3.1E - 3.1H). These results demonstrate that MSC radio-resistance was enhanced in hypoxia. In contrast to MSCs, hypoxic ST4.5 thymocytes were more sensitive to irradiation and this sensitivity increased in a dose-dependent manner (Figure 3.1C).
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I have previously demonstrated that unlike MSC lines, irradiated bulk MSCs exhibit a highly variable DDR, likely due to the inherent heterogeneity of primary un-cloned MSC cultures (Figure 2.8) [Sugrue et al. 2013a]. MS5 and ST2 cells responded to irradiation in a similar manner to bulk MSCs (Figure 2.2) [Sugrue et al. 2013a]. Furthermore, my results indicated that the effects of hypoxia on the radio-resistance of these MSC lines were also comparable to bulk MSCs (Figure 3.1A – 3.1F). Therefore, for detailed molecular investigation of the effects of hypoxia on the DDR of MSCs, we focused upon cloned MS5 and ST2 MSC lines. In addition, as my previous characterisation of irradiated mouse MSCs in normoxia was largely carried out using 10 Gy irradiation (Chapter 2) [Sugrue et al. 2013a], this IR dose was used to directly compare the DDR of normoxic and hypoxic MSCs throughout this study.

**Figure 3.1 (overleaf) Hypoxia enhances mouse MSC survival post γ-irradiation.**

Growth curves of (A): MSC lines (MS5 and ST2) and (B): primary bulk C57BL/6 MSCs cultured in 21% O$_2$ (normoxia) or 5% O$_2$ (hypoxia) for 7 days. Dose response curves of (C): MS5, ST2 and ST4.5 (CD$^4^+$ CD$^8^+$ thymocyte) cells and of (D): primary bulk C57BL/6 MSCs irradiated at 2-10 Gy and cultured in 21% or 5% O$_2$ for 5-14 days, depending on cell type. Representative images of (E): MS5 and (F): bulk MSC colonies generated in clonogenic survival assays in 21% or 5% O$_2$ and stained with Coomassie Blue 7 (MS5) or 14 (bulk MSC) days post 0, 4 and 8 Gy irradiation. Quantification of average size (sq. mm) of (G): MS5 and (H): bulk MSC colonies post 0 (Control) and 4 Gy irradiation. All images are representative of one of three independent experiments. Error bars represent mean ± SD, n=3. * denotes MS5 samples; # denotes ST2 samples, */# $p < 0.05$ ** $p < 0.01$ compared with normoxic samples, paired Student’s $t$ test.
Figure 3.1 Hypoxia enhances mouse MSC survival post γ-irradiation.
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Figure 3.2 Hypoxia does not affect growth rate of ST4.5 thymocytes. Growth curves of ST4.5 thymocytes cultured in 21% or 5% O₂ for 5 days. Error bars represent mean ± SD, n=3.

3.3.2 Hypoxia accelerates mouse MSC recovery from IR-induced cell cycle arrest

Increased MSC survival and colony size post IR in hypoxia suggested that hypoxia could affect DNA damage checkpoints activated by MSCs. Irradiated mouse MSCs activated robust intra-S-phase and G₂ checkpoints in normoxia (Figure 2.4A – 2.4C) [Sugrue et al. 2013a]. Here, I further characterised the G₂/M checkpoint response of irradiated MSCs by analysing mitotic index using intracellular phosphorylated histone H3(Ser10) (pH3S10) staining by flow cytometry [Xu et al. 2002] (Figure 3.3A and 3.3B). The absence of mitotic (pH3S10 positive) cells at early time-points (2-8 hrs) post IR confirmed that irradiated MS5 cells activated the G₂/M checkpoint in both normoxia and hypoxia (Figure 3.3A and 3.3B). However, recovery from G₂/M arrest occurred earlier in hypoxia, indicated by the presence of mitotic MS5 cells at 8 hrs post IR in hypoxia (Figure 3.3A, black arrowhead, lower 8 hr panel; Figure 3.3B), which were absent until 12 hrs post IR in normoxia (Figure 3.3A and 3.3B).

Due to the intrinsic radio-sensitivity of ST4.5 thymocytes (Figure 3.1C; Figure 2.2) [Sugrue et al. 2013], 0.5 Gy irradiation was used to study the effects of hypoxia on DNA damage checkpoints in this cell line. In contrast to MS5 cells, irradiated ST4.5
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Thymocytes did not arrest at the G2/M checkpoint in either normoxia or hypoxia, indicated by the continued presence of mitotic cells post irradiation (Figure 3.4A and 3.4B). The reason(s) for this G2/M checkpoint deficiency are currently unknown but may be linked to the low levels of ATM and Chk2, key proteins involved in the G2/M checkpoint, expressed in this cell type (Figure 2.3B and 2.3C) [Xu et al. 2002; Bartek & Lukas, 2003; Sugrue et al. 2013a].

To analyse intra-S-phase and G2 checkpoints, the cell cycle progression of irradiated BrdU labelled MS5 cells (S phase population) (Figure 3.3C, black box in upper left panel), cultured in normoxia and hypoxia, was analysed using a flow cytometry-based BrdU incorporation assay (Section 2.2.7a) [Xu et al. 2002; Sugrue et al. 2013a]. Following irradiation, BrdU labelled MS5 cells accumulated as a cohort in late S/G2 under both oxygen tensions (Figure 3.3C; 8 hr time-point) and the proportions of these BrdU labelled populations remained largely unchanged up to 8 hrs post IR whereas the proportions of control BrdU labelled populations steadily declined (Figure 3.5A). Cell cycle progression of un-irradiated (control) BrdU labelled MS5 populations was comparable in normoxia and hypoxia (Figure 3.3D & Figure 3.5A). These findings demonstrate that DNA damage checkpoints were intact in hypoxic MSCs and were activated in a similar manner under both oxygen tensions (Figure 3.3A – 3.3D; Figure 3.5A).

Interestingly, in comparison with normoxia, increased proportions of BrdU labelled G1/S cells were present at 12 hrs (~7.6% in 21% O2 versus ~22.4% in 5% O2) and at 24 hrs (~18.4% in 21% O2 versus ~32.6% in 5% O2) post IR in hypoxia (Figure 3.3C, black arrowheads; Figure 3.3D). This indicated that MSC recovery from IR-induced cell cycle arrest was accelerated under hypoxic conditions. In contrast to MS5 cells, irradiated BrdU labelled ST4.5 thymocytes recovered from cell cycle arrest at similar rates under both oxygen tensions (Figure 3.4C and 3.4D). Similar proportions of apoptotic (Annexin-V positive) MS5 and ST4.5 thymocytes were detected post IR in normoxia and hypoxia (Figure 3.5B and 3.5C). As previously shown in normoxia (Figure 2.5A) [Sugrue et al. 2013a], p53 was transiently stabilised in irradiated MSCs (Figure 3.3E) whereas it was maintained in irradiated ST4.5 thymocytes (Figure 3.5D) and similar kinetics occurred in hypoxia (Figure 3.3E and Figure 3.5D).
3.3.3 DNA double-strand break repair is enhanced in mouse MSCs exposed to hypoxia

Improved MSC recovery from IR-induced cell cycle arrest in hypoxia, in combination with no effect on the extent of apoptosis or on p53 stabilisation kinetics, indicated that the DNA DSB repair capacity of MSCs may be altered in hypoxia. Analysis of the DNA DSB marker, γ-H2AX [Rogakou et al. 1998; Fernandez–Capetillo et al., 2004; Bonner et al. 2008], demonstrated that (i) H2AX Ser139 phosphorylation (Figure 3.6A) and (ii) γ-H2AX IRIF (Figure 3.6B - 3.6D) were resolved at a faster rate in irradiated MSCs cultured in hypoxia than in normoxia. For example, at 2 hrs, ~41 (21% O2) versus ~33 (5% O2) γ-H2AX IRIF were present per MS5 nucleus (Figure 3.6D, upper panel) and ~33 (21% O2) versus ~23 (5% O2) IRIF per ST2 nucleus (Figure 3.6D, lower panel). Similar to previous findings (see Figure 2.3A) [Sugrue et al. 2013a], maximal H2AX phosphorylation in ST4.5 thymocytes was delayed until 2-4 hrs post IR and its resolution was not improved in hypoxia (Figure 3.5E).

Figure 3.3 (Overleaf) Hypoxia accelerates mouse MSC recovery from IR-induced cell cycle arrest. (A): Representative cytograms of MS5 cells cultured in 21% or 5% O2; harvested at 0-24 hrs post 10 Gy irradiation and stained for histone H3(Ser10) phosphorylation (pH3S10) (mitotic marker) and DNA content using PI. Black arrowhead indicates appearance of mitotic MS5 cells in hypoxia at 8 hrs post IR. (B): Quantification of % mitotic index (i.e. phosho-histone H3Ser10 stained MS5 cells) at 0-24 hrs post IR in normoxia and hypoxia. (C): Representative cytograms of MS5 cells cultured in 21% or 5% O2; harvested at 0-36 hrs post 10 Gy irradiation and stained for BrdU incorporation and DNA content using PI. Black box represents BrdU labelled S phase population and black arrowheads indicate appearance of BrdU labelled G1/early S phase cells following G2/M arrest. (D): Quantification of the percentage BrdU labelled G1/early S phase MS5 cells in 21% or 5% O2 at 0-36 hrs post 10 Gy irradiation. (E): Western blot analysis of total p53 and
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Figure 3.3 Hypoxia accelerates mouse MSC recovery from IR-induced cell cycle arrest (continued): and β-Tubulin (loading control) expression in MSC lines cultured in 21% or 5% O₂ at 0-4 hrs post 10 Gy irradiation. All images are representative of one of three independent experiments. Error bars represent mean ± SD, n=3. * p < 0.05 compared with normoxic samples, paired Student’s t test.
Figure 3.4 Hypoxia does not affect DNA damage checkpoints in ST4.5 thymocytes. 

(A): Representative cytograms of ST4.5 thymocytes cultured in 21% or 5% O₂; harvested at 0-24 hrs post 0.5 Gy irradiation and stained for histone H3(Ser10) phosphorylation (pH3S10) (mitotic marker) and DNA content using PI. 

(B): Quantification of % mitotic index (i.e. pH3S10 positive cells) in 21% or 5% O₂ at 0-24 hrs post 0.5 Gy irradiation. 

(C): Representative cytograms of ST4.5 thymocytes cultured in 21% or 5% O₂; harvested at 0-24 hrs post 0.5 Gy irradiation and stained for BrdU incorporation and DNA content using PI. 

(D): Quantification of the percentage BrdU labelled G₁/early S phase ST4.5 thymocytes in 21% or 5% O₂ at 0-36 hrs post 0.5 Gy irradiation. All images are representative of one of three independent experiments. Error bars represent mean ± SD, n=3.
Figure 3.5 Hypoxia does not affect DNA damage checkpoint activation, apoptosis or p53 stabilisation in irradiated MSCs and ST4.5 thymocytes. (A): Quantification of the percentage BrdU labelled S phase MS5 cells in 21% or 5% O₂ at 0-36 hrs post 10 Gy irradiation. Representative cytograms of (B): MS5 cells and (C): ST4.5 thymocytes cultured in 21% or 5% O₂; harvested at 0-36 hrs post 10 or 0.5 Gy irradiation and stained with Annexin-V (apoptotic marker) and for DNA content using PI. Western blot analysis of (D): total p53 and β-Tubulin (loading control) and (E): γ-H2AX; total H2AX and β-Tubulin (loading control) expression in ST4.5 thymocytes cultured in 21% or 5% O₂ at 0-4 hrs post 0.5 Gy irradiation. All images are representative of one of three independent experiments. Error bars represent mean ± SD, n=3.
Interestingly, Rad51 IRIF (indicative of DNA DSB repair by HR) formed at a faster rate in MSCs cultured in hypoxia than in normoxia (Figure 3.6C and 3.6E). For example, at 1 hr, ~2 (21% O₂) versus ~5 (5% O₂) Rad51 IRIF were present per MS5 nucleus (Figure 3.6E, upper panel) and ~2 (21% O₂) versus ~6 (5% O₂) per ST2 nucleus (Figure 3.6E, lower panel). The extent of γ-H2AX IRIF formation and H2AX phosphorylation at 1 hr post IR was comparable in normoxic and hypoxic MSC nuclei (Figure 3.6B and 3.6C, 1 hr panels). However, in addition to DNA DSB formation, γ-H2AX can also be induced during other biological processes such as replication stress and cellular senescence [Ward & Chen, 2001; d'Adda di Fagagna, 2008]. Therefore, γ-H2AX only serves as an indirect marker of DNA DSBs. To overcome this issue, irradiated MSC nuclei were thus analysed in the neutral comet assay to directly assess DNA DSB generation. No significant difference in comet tail moment was detected between normoxic and hypoxic MSCs, indicating that DNA DSBs were extensively generated under both oxygen tensions (Figure 3.7A and 3.7B).

To analyse the HR efficiency of MSCs, MS5 cells were stably transfected with the DR-GFP HR reporter to generate the MS5 sub-clone, MS5 HR4.4 (Section 3.2.5d; Figure 3.8A and 3.8B). Transfection with pCBASce vector expressing the rare cutting endonuclease, I-Sce I, generates DSBs within the GFP reporter substrate and successful HR-mediated repair results in GFP expression (Figure 3.8A) [Pierce et al. 1999; Nakanishi et al. 2011]. At 2 days post transfection, ~4.6% GFP-positive cells were detected in normoxia whereas this increased to ~6.6% in hypoxia (Figure 3.9A and 3.9B). A comparable transfection efficiency of ~35-40% was obtained in normoxic and hypoxic MS5 cells (data not shown). These results demonstrate that mouse MSCs could repair DNA DSBs using HR and that their HR efficiency was increased in hypoxia.

NHEJ efficiency was measured using a previously established in vitro assay in which the capacity of control nuclear extracts to re-ligate digested plasmid DNA containing a single DNA DSB is analysed (Figure 2.7F) [Sotiropoulou et al. 2010; Sugrue et al. 2013a]. End-joining of monomeric DNA fragments into multimers was more efficient in nuclear extracts of MS5 cells cultured in hypoxia than in normoxia (Figure 3.9C and 3.9D). End-joining was completely absent in the presence of SCID-derived bulk MSC nuclear extracts (SCID bulk), used as negative control, demonstrating that plasmid re-ligation was
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NHEJ-dependent (Figure 3.9C). Western blot analysis of control whole cell extracts revealed that DNA ligase IV and DNA-PKcs were expressed at higher levels in MSCs cultured in hypoxia than in normoxia whereas Rad51 expression levels were comparable (Figure 3.9E). The expression levels of these proteins in ST4.5 thymocytes, although low, were unaltered by oxygen tension (Figure 3.9E).

**Figure 3.6 (overleaf) Hypoxia accelerates resolution of DNA double-strand breaks by irradiated mouse MSCs.** (A): Western blot analysis of H2AX Ser139 phosphorylation (γ-H2AX - marker of DNA double-strand breaks) and of total H2AX and β-Tubulin (loading control) expression in MSC lines cultured in 21% or 5% O2 and harvested 0-24 hrs post 10 Gy irradiation. (B): Representative immunofluorescent images (100X magnification) of MS5 and ST2 nuclei in 21% or 5% O2 at 0-4 hrs post 10 Gy irradiation and stained for DNA content (DAPI), γ-H2AX (green) and Rad51 (red) IRIF. (C): Representative immunofluorescent images (100X magnification) of ST2 nuclei in 21% or 5% O2 at 1-8 hrs post 10 Gy irradiation and stained for γ-H2AX and Rad51 IRIF. Quantification of the average number of (D): γ-H2AX IRIF and (E): Rad51 IRIF per MSC nucleus (50 nuclei analysed per time-point per experiment) in 21% or 5% O2 at 0-24 hrs post 10 Gy irradiation. All images are representative of one of three independent experiments. Error bars represent mean ± SD, n=3. * p < 0.05; ** p < 0.01 compared with normoxic samples, paired Student’s t test.
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Figure 3.6 The resolution of DNA double-strand breaks by irradiated mouse MSCs is accelerated in hypoxia.
Figure 3.7 DNA double-strand break generation is unaffected by oxygen tension in MSCs. (A): Representative images of MS5 comet tails stained with SYBR Green® and captured using 10X magnification. (B): Quantification of the average tail moment (50 cells per slide per experiment) in control and irradiated (10 Gy) MS5 samples. N.S. denotes non-significant ($p > 0.05$) compared to normoxic samples, paired Student’s $t$ test.
Figure 3.8 Generation of MS5 HR4.4 stable cell line. (A): Diagram of in vivo Homologous Recombination assay. DR-GFP plasmid encodes two mutated gfp genes (i) Scegfp in which the gfp gene encodes the restriction site for I-SceI endonuclease and (ii) a 3’ and 5’ truncated gfp gene, termed the internal GFP fragment (igfp). **Step 1:** Transfection with pCBA.Sce encoding I-SceI endonuclease results in the generation of a DNA DSB within Scegfp. **Step 2:** Correct repair of the DNA DSB via HR using igfp sequence as a homologous template leads to the generation of an intact gfp sequence which when transcribed, results in GFP expression (**Step 3**). (B): PCR screening of genomic DNA isolated from puromycin-selected MS5 clones for stable integration of Sce-gfp sequence (Section 3.2.5d).
Figure 3.9 (overleaf) Hypoxia enhances DNA double-strand break repair in mouse MSCs. (A): Representative cytograms of GFP positive MS5 HR4.4 cells (50,000 cells recorded per sample) detected in 21% or 5% O₂ 2 days post transfection with Lipofectamine 2000® alone (Mock) or with 10 µg I-SceI endonuclease expression vector, pCBASce, to induce DNA double-strand breaks. (B): Quantification of GFP positive MS5 HR4.4 cells detected in 21% or 5% O₂ 2 days post transfection. (C): Representative image of end-joining efficiency in reactions containing linearised plasmid DNA and nuclear extracts of control SCID-derived bulk MSCs (SCID bulk) and MS5 cells cultured in 21% or 5% O₂ and corresponding Western blot analysis of DNA-PKcs and Rad51 expression levels in these nuclear extracts. (D): Quantification of ligated fragment (dimeric, trimeric and multimeric fragments) intensity normalised to the background intensity detected in SCID bulk nuclear extracts. (E): Western blot analysis of DNA ligase IV, DNA-PKcs, Rad51 and β-Tubulin (loading control) expression levels in control MS5, ST2 and ST4.5 cells cultured for >3 days in 21% or 5% O₂. All images are representative of one of three independent experiments. Error bars represent mean ± SD, n=3, * p < 0.05 compared with 21% O₂, paired Student’s t test.
Figure 3.9 Hypoxia enhances DNA double-strand break repair in mouse MSCs.
3.3.4 HIF-1α is a mediator of enhanced MSC radio-resistance in hypoxia

To confirm that this increased DNA ligase IV and DNA-PKcs expression was hypoxia-dependent, MSCs continuously cultured in normoxia were transferred to hypoxia and their expression levels were analysed over a 24 hr time-course (Figure 3.10A). In both MSC lines, DNA ligase IV and DNA-PKcs expression levels began to increase at 4 and 8 hrs, respectively, in hypoxia whereas Rad51 expression levels remained stable (Figure 3.10A). HIF-1α and HIF-2α were stabilised in MSC lines, beginning at 4 hrs and at 24 hrs, respectively, in hypoxia (Figure 3.10A). Stabilisation of HIF-1α and HIF-2α was detected at 24 hrs in hypoxic bulk MSCs (Figure 3.11A). HIF-1α stabilisation coincided with increasing levels of DNA ligase IV and DNA-PKcs in MSCs exposed to hypoxia (Figure 3.10A). This indicated a potential role for HIF-1α in regulating DNA DSB repair in hypoxic MSCs. siRNA-mediated knockdown of HIF-1α caused a ~1.5 fold and a ~2 fold reduction in the radio-resistance of MS5 and ST2 cells, respectively, in hypoxia (Figure 3.10B). It also reduced the LD50 dose from 8 Gy to 6 Gy in MS5 cells and to 4 Gy in ST2 cells (Figure 3.10B). MSC colony size was unaffected by HIF-1α knockdown (data not shown).

In correlation with increased radio-sensitivity, (i) H2AX phosphorylation (Figure 3.10C) and (ii) γ-H2AX IRIF (Figure 3.10D; Figure 3.11B) persisted for longer in irradiated HIF-1α depleted MSCs than in irradiated control MSCs. For example, at 4 hrs, ~19 (control siRNA) versus ~24 (HIF-1α siRNA) γ-H2AX IRIF were present per MS5 nucleus (Figure 3.10D, upper panel) and ~22 (control siRNA) versus ~30 (HIF-1α siRNA) IRIF were present per ST2 nucleus (Figure 3.10D, lower panel). In comparison with control irradiated MSCs, Rad51 IRIF formed less efficiently and persisted for longer in irradiated HIF-1α depleted MSC nuclei (Figure 3.11B – 3.11D). For example, at 1 hr, MS5 nuclei contained ~6 (control siRNA) versus ~3 (HIF siRNA) Rad51 IRIF (Figure 3.11C) and at 8 hrs, ST2 nuclei contained ~7 (control siRNA) versus ~11 (HIF siRNA) Rad51 IRIF (Figure 3.11D). Finally, HIF-1α knockdown prevented hypoxia-dependent increases in DNA ligase IV and DNA-PKcs expression in MSCs, whereas Rad51 expression levels were unaltered (Figure 3.10E). These results confirm that HIF-1α contributed to enhanced MSC radio-resistance in hypoxia by altering their DNA DSB repair capacity.
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Figure 3.10 (overleaf) HIF-1α contributes to enhanced DNA double-strand break repair in mouse MSCs in hypoxia. (A): Western blot analysis of HIF-1α, HIF-2α, DNA ligase IV, DNA-PKcs, Rad51 and β-Tubulin (loading control) expression levels in MS5 and ST2 cells transferred from 21% to 5% O2 and harvested over a 24 hr time-course. (B): Dose response curves of hypoxic MSC lines irradiated at 2-10 Gy post transfection with scrambled (Control) or HIF-1α siRNA (HIF-1α) and cultured in 5% O2 for 7 days. (C): Western blot analysis of γ-H2AX; total H2AX and β-Tubulin (loading control) expression in hypoxic MSC lines transfected with scrambled (Control) or HIF-1α siRNA (HIF-1α) and harvested at 0-24 hrs post 10 Gy irradiation. (D): Quantification of the average number of γ-H2AX IRIF per nucleus (50 nuclei analysed per time-point per experiment) at 0-24 hrs post 10 Gy irradiation, transfected with scrambled (Control) or HIF-1α siRNA (HIF-1α). (E): Western blot analysis of HIF-1α, DNA ligase IV, DNA-PKcs, Rad51 and β-Tubulin (loading control) expression in MSC lines at 0-24 hrs in 5% O2, transfected with scrambled (Control) or HIF-1α siRNA (HIF-1α). All images are representative of one of three independent experiments. Error bars represent mean ± SD, n=3. * denotes MS5 samples; # denotes ST2 samples, */# p < 0.05 compared with control (scrambled siRNA) samples, paired Student’s t test.
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Figure 3.10 HIF-1α contributes to enhanced DNA double-strand break repair in hypoxic mouse MSCs in hypoxia.
Figure 3.11 (overleaf) HIF-1α contributes to the resolution of DNA double-strand breaks by irradiated hypoxic MSCs. (A): Western blot analysis of HIF-1α and HIF-2α stabilisation in bulk MSCs at 0-24 hrs following transfer from 21% to 5% O₂. (B): Representative immunofluorescent images (100X magnification) of hypoxic MS5 (upper panels) and ST2 nuclei (lower panels); transfected with scrambled (Control) or HIF-1α siRNA (HIF-1α) and stained for γ-H2AX (green) and Rad51 (red) IRIF at 1-8 hrs post 10 Gy irradiation. Quantification of the average number of Rad51 IRIF per (C): MS5 and (D): ST2 nucleus (50 nuclei analysed per time-point per experiment) at 0-24 hrs post 10 Gy irradiation. All images are representative of one of three independent experiments. Error bars represent mean ± SD, n=3.
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Figure 3.11 HIF-1α contributes to the resolution of DNA double-strand breaks by irradiated hypoxic MSCs.
3.4 DISCUSSION

MSCs are radio-resistant progenitors that reside in hypoxic niches in vivo, particularly in the BM [Wilson & Trumpp, 2006; Eliasson & Jönsson, 2009; Celso & Scadden, 2011; Sugrue et al. 2013b]. However, the effects of hypoxia on MSC radio-resistance are currently unknown. I have demonstrated that hypoxia enhances the radio-resistance of mouse MSCs in vitro. Hypoxia increased the (i) proliferation rate; (ii) colony size and (iii) long-term survival post irradiation of both primary and cloned mouse MSCs (Figure 3.1A – 3.1H). Activation of intra-S and G2/M DNA damage checkpoints is important for MSC survival post IR in normoxia (Figure 2.4A – 2.4C) [Sugrue et al. 2013a]. I found that these DNA damage checkpoints were also activated by MSCs in hypoxia (Figure 3.3A – 3.3D; Figure 3.5A). Interestingly, MSCs recovered from G2/M arrest at a faster rate in hypoxia than in normoxia (Figure 3.3A – 3.3D). This improved recovery was not due to potential hypoxia-induced changes in MSC cell cycle distribution or alterations in either IR-induced p53 stabilisation or apoptosis (Figure 3.3; Figure 3.5A and 3.5B). This indicated that hypoxia may affect DNA DSB repair in irradiated MSCs.

DNA DSB repair has been reported to be impaired in hypoxic cancer cells, thereby promoting genomic instability and increased therapeutic resistance [Bindra et al. 2007; Bristow & Hill, 2008; Ruan et al. 2009]. However, in our study, γ-H2AX resolution, indicative of DNA DSB repair, occurred at a faster rate in hypoxic MSCs (Figure 3.6A – 3.6D). Suppression of HR in hypoxia has been previously reported in multiple cancer cell types [Bindra et al. 2004; Meng et al. 2005; Chan et al. 2008]. However, (i) increased HR efficiency (Figure 3.9A and 3.9B), and (ii) accelerated Rad51 IRIF formation (Figure 3.6B/C/E) was detected in hypoxic MSCs. This indicates that hypoxia up-regulated, rather than suppressed, HR in mouse MSCs. Reports on the effects of hypoxia on NHEJ are variable [Meng et al. 2005; Sprong et al. 2006; Wirthner et al. 2008]. NHEJ was also up-regulated in hypoxic MSCs, demonstrated by (i) increased in vitro end-joining efficiency (Figure 3.9C and 3.9D) and (ii) increased expression levels of DNA ligase IV and DNA-PKcs in hypoxic nuclear and whole cell extracts (Figure 3.9C and 3.9E; Figure 3.10A). Overall, my results demonstrate that hypoxia up-regulated DNA DSB repair in mouse MSCs. This study also suggests that NHEJ and HR can be simultaneously up-regulated in response to hypoxia in MSCs and whether other cell types behave similarly requires further investigation.
HIF-1α can modulate the DDR in response to hypoxia [Unruh et al. 2003; Um et al. 2004; Kohsiji et al. 2004; Wirthner et al. 2008]. HIF-1α depletion in MSCs resulted in (i) increased radio-sensitivity (Figure 3.10B); (ii) delayed γ-H2AX resolution (Figure 3.10C and 3.10D) and (iii) altered Rad51 IRIF formation and resolution (Figure 3.11B – 3.11D). In addition, HIF-1α regulated the expression of the key NHEJ proteins, DNA-PKcs and DNA ligase IV, in mouse MSCs exposed to hypoxia (Figure 3.10E). Although HIF-1α depletion did not affect Rad51 expression levels, the observed alterations in Rad51 IRIF kinetics suggest that HIF-1α may influence MSC HR activity by affecting the recruitment of repair proteins, such as Rad51, to DNA DSBs. These results collectively indicate that HIF-1α is a mediator of enhanced DNA DSB repair in hypoxic mouse MSCs.

The effects of hypoxia on MSC radio-biology have numerous important implications. Conditional deletion of MSC sub-populations in hypoxic BM results in defective haematopoiesis [Sugiyama et al. 2006; Méndez-Ferrer et al. 2010; Omatsu et al. 2010; Park et al. 2012; Tran et al. 2013]. TBI is commonly used in preparative regimens preceding allogeneic BMT in order to destroy the radio-sensitive host haematopoietic system [Mauch et al. 1995; Copelan, 2006]. Therefore, increased MSC radio-resistance in hypoxia is likely to promote host-/patient-derived MSC survival in the BM post TBI, thereby improving haematopoietic reconstitution. MSCs are capable of homing to tumours where they integrate into the hypoxic tumour microenvironment and contribute to tumourigenesis and metastasis [Direkze et al. 2004; Kucerova et al. 2008; Wang et al. 2009; Quante et al. 2011; Kidd et al. 2012; Jung et al. 2013]. MSCs produce increased amounts of pro-angiogenic factors (e.g. VEGF) and pro-tumourigenic cytokines (e.g. CXCL12) in hypoxia [Tsai et al. 2012b; Liu et al. 2013]. Furthermore, MSCs promote the therapeutic resistance of multiple haematological malignancies, including chronic myeloid leukaemia (CML) and chronic lymphocytic leukaemia (CLL) [Ramasamy et al. 2007; Jin et al. 2008; Vianello et al. 2010]. In combination with the finding that MSC radio-resistance is increased in hypoxia, it is likely that MSC-derived CAFs will be more resistant to radio-therapy within the tumour microenvironment and thereby support tumour survival.

Recent studies indicate that biological response(s) modulated in hypoxia are cell type-dependent [Gammon et al. 2013; Sermeus et al. 2013; Strese et al. 2013]. By comparing
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the radiation response of MSCs and DP thymocytes (ST4.5), I have also shown that hypoxia-mediated modulation of the DDR is cell type-dependent. Therefore, our understanding of how hypoxia influences the radio- and chemo-resistance of not only cancer cells, but also of their supportive stroma, including MSCs, will also likely be essential for developing effective strategies to overcome hypoxia-induced therapeutic resistance in cancer.
Chapter 4

DN2 thymocytes activate a robust DNA Damage Response to IR-induced DNA double-strand breaks
4.1 INTRODUCTION

T lymphopoiesis primarily occurs in the thymus [Miller, 1961 & 2002]. T lymphocytes are a fundamental component of the adaptive immune response in which they orchestrate antigen-specific destruction of infected and cancerous cells and secrete soluble factors or express surface molecules that modulate, or help, the functions of other immune cell types. The thymus is under constant turnover and in the adult mouse generates ~10^6 mature thymocytes per day but is incapable of self-renewal [Wallis et al. 1975; Miller, 2002]. Therefore, it is continually seeded by BM-derived, lymphoid-myeloid restricted HSC-derived progenitors known as ETPs (Section 1.4) [Adolfsson et al. 2005; Balciunaite et al. 2005a & 2005c; Ceredig, 2012]. Interactions between ETPs and the thymic microenvironment activate signalling pathways that stimulate ETPs to lose non-T lineage potential and to become committed along the T lineage [Ceredig & Rolink, 2002; Balciunaite et al. 2005a & 2005c; Koch & Radtke, 2011].

Double negative (DN) (CD4^- CD8^-) thymocytes are the earliest intra-thymic T lymphocyte precursors [Ceredig et al. 1983a & 1983b; Fowlkes et al. 1985]. They are sub-divided into four major subsets, known as DN1-DN4, which represent sequential stages of T lineage commitment and are phenotypically divided based on differential surface expression of CD44, CD25 and CD117 (Section 1.4, Figure 1.3). [Ceredig et al. 1983b & 1985; Godfrey et al. 1992 & 1993; Ceredig & Rolink, 2002]. During thymopoiesis, DN thymocytes undergo an orchestrated sequence of differentiation and selection stages to ultimately generate MHC-restricted, TCR-expressing, CD4^+ helper and CD8^+ cytotoxic T lymphocytes that recognise non-self peptide antigens presented by cells in the periphery. Therefore, tight regulation of DN survival and differentiation within the thymus is important for regulating T lymphocyte homeostasis.

Thymic cellularity is rapidly reduced in lethally irradiated mice [Kadish & Bash, 1975; Ceredig & MacDonald, 1982; Zúñiga-Pflücker & Kruisbeek, 1990]. However, following BMT, the irradiated thymus is first re-constituted by single wave of host-derived (HD) thymocytes, beginning at 5-7 days post IR, a process defined as auto-
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reconstitution [Kadish & Bash, 1975; Ceredig & MacDonald, 1982; Zúñiga-Pflücker & Kruisbeek, 1990; Bosco et al. 2010]. In recent years, HD DN2 thymocytes have been identified as the radio-resistant thymocyte population exclusively responsible for initiating thymic auto-reconstitution [Bosco et al. 2010]. These HD DN2 thymocytes persist in the thymus post IR; generate functional mature T lymphocytes with a polyclonal TCR repertoire and are exclusively capable of re-constituting ~35% of the normal T cell compartment [Bosco et al. 2010]. However, the mechanisms that account for the unique radio-resistance of DN2 thymocytes are currently unknown.

Investigating molecular pathways in individual DN thymocyte subsets \textit{in vivo}, particularly in DN1 and DN2, is technically difficult due to their low numbers (approximately $10^4$) in the thymus. Therefore, various culture systems that aim to mimic the thymic microenvironment have been designed to study DN thymocyte biology \textit{in vitro}, including the foetal thymus organ culture (FTOC) system and the OP9-DL1 system [Ceredig & Rolink, 2002]. However, the extent of interactions between DN thymocytes and their supportive stroma in these systems is heterogeneous and unknown signals that may alter DN biology \textit{in vitro} cannot be controlled [Ceredig & Rolink, 2002]. Notch signalling, particularly through the Notch1 receptor, is indispensable for DN thymocyte survival, proliferation and differentiation until the CD4$^+$CD8$^+$ DP stage [Radtke et al. 1999; Wilson et al. 2001; Wolfer et al. 2002; Ciofani et al. 2004; Balciunaite et al. 2005b; Tussiwand et al. 2011]. \textit{In vivo}, interaction between Notch1 expressed on DN thymocytes and the Notch ligand, Delta-like ligand-4 (DL4), expressed on TECs, is essential for early thymopoiesis [Koch et al. 2008]. In addition, production of IL-7 and stem cell factor (SCF) by TECs is essential for promoting DN thymocyte survival, expansion and T lineage commitment [Murray et al. 1989; Massa et al. 2006; Ceredig & Rolink, 2012].

The laboratory of Prof. Antonius Rolink [Department of Biomedicine (DBM), Basel] recently established a stroma-free culture system that supports DN proliferation and differentiation, termed ‘‘\textit{the plastic thymus}’’ [Tussiwand et al. 2011]. In this system, a homogeneous Notch-based microenvironment is generated by binding DL4-human IgG$_1$-Fc (DL4-Fc) fusion protein to tissue culture plates pre-coated with anti-human
IgG$_1$-Fc antibody (Section 4.2.1; Figure 4.1A) [Tussiwand et al. 2011]. By supplemental addition of recombinant IL-7 and SCF, purified DN2 thymocytes can be maintained long-term in vitro (A. Rolink, unpublished data). Therefore, this culture system uniquely enables the study of DN2 thymocytes at the molecular level.

The study of HSCs at the molecular level is also impaired due to their (i) low numbers in the bone marrow and (ii) poor expansion in vitro. However, in vitro expansion of bona-fide clonal HSCs has now been accomplished by genetically engineering HSCs to express the NUP98-HOXB4 (NH) fusion gene [Ruedl et al. 2008]. Homeobox B4 (HOXB4) belongs to the Hox family of transcription factors which regulate the expression of numerous genes involved in embryonic development and haematopoiesis. Specifically, HOXB4 regulates the expression of genes that promote HSC self-renewal and expansion [Thorsteinsdottir et al. 1999; Antonchuk et al. 2002; Ohno et al. 2010]. Through an unknown mechanism, fusion of HOXB4 with nucleoporin 98 (NUP98) uniquely enables long-term HSC self-renewal in vitro [Ruedl et al. 2008]. In vitro expanded NH-HSCs (i) maintain the LSK (Lin$^-$/Sca-1$^+$ c-kit$^+$) phenotype; (ii) fully re-constitute lethally irradiated mice and (iii) unlike fusions with other HOXB members, do not give rise to haematological malignancies, e.g. leukaemia, in re-constituted mice [Antonchuk et al. 2002; Pineault et al. 2004; Ruedl et al. 2008].

During a three-month EMBO short-term fellowship placement in the laboratory of Prof. Rolink, I used the ‘‘plastic thymus’’ culture system described herein to perform a novel study of DN2 radio-biology in vitro. I have previously shown that execution of the DDR plays a key role in mediating the radio-resistance of mouse MSCs (Chapters 2 & 3) [Sugrue et al. 2013a]. Using the toolkit of techniques I optimised to study MSC radio-biology, I compared the DDR of irradiated DN2 thymocytes and of NH-HSCs (radio-sensitive control) to investigate the DDR pathways that contribute to DN2 radio-resistance. This is the first time that the role of the DDR in DN2 radio-resistance has been studied.
4.2 MATERIALS AND METHODS

4.2.1 *In vitro* expansion of CD4<sup>-</sup> CD8<sup>-</sup> II (DN2) thymocytes using the ‘’plastic thymus’’ culture system.

DN2 thymocytes were isolated from thymi of C57BL/6 mice in the Rolink laboratory (DBM Basel), sorted using a BD FACS Aria® cell sorter and characterised as CD4<sup>-</sup> CD8<sup>-</sup> CD44<sup>+</sup> CD25<sup>+</sup> c-kit<sup>+</sup> CD127<sup>+</sup> as previously described (Figure 1.3) [Ceredig & Rolink, 2002; Bosco *et al.* 2010]. To prepare the ‘’plastic thymus’’ culture system, each well of a 6 well plate was coated with 2 ml of 10 µg/ml mouse monoclonal anti-human IgG<sub>1</sub> Fc antibody (HUF) solution prepared in sterile PBS (Sigma-Aldrich) and stored overnight at 4°C. PBS was removed and the wells were washed twice with 5 ml of growth medium per well. A final concentration of 2 µg/ml DL4-Fc was added to 2 ml of growth medium per well and incubated overnight at 4°C. The wells were then washed twice with growth medium and DN2 thymocytes were cultured in 2 ml of growth medium per well (Figure 4.1A). DN2 thymocytes were maintained in Serum Free-Iscove’s Modified Dulbecco’s Medium (SF-IMDM) (Gibco) supplemented with 5% HyClone FCS (Thermo Scientific), 10% IL-7 supernatant, 0.1 µg/ml SCF and 0.2% Ciproxin® (Bayer Pharmaceutical) (Appendix II) at 37°C in 21% O<sub>2</sub>.

4.2.2 *In vitro* expansion of NUP98-HOXB4 (NH) HSC and HSC-Bcl-2 lines.

NH HSCs (HSC) and HSCs over-expressing Bcl-2 (HSC-Bcl-2) were previously isolated from C57BL/6 CD3ε<sup>-/-</sup> and H-2K.BCL-2 transgenic (Tg) mice, respectively, in the Rolink laboratory (DBM Basel) (Appendix I) [von Münchow, 2013]. Briefly, to generate the HSC lines, mice were intra-peritoneally injected with 300 µl of 10 mg/ml 5'-fluorouracil (5'-FU) prepared in PBS to selectively induce death of cycling HSCs, thereby enriching for primitive quiescent HSCs [Ruedl *et al.* 2008]. After 4 days, BM was flushed from mouse femurs; erythrocytes were lysed using ACK buffer and the remaining cell suspension was cultured for 4 days. HSCs were then retrovirally transduced with pMYc-Nup98-HoxB4-IP vector [Ruedl *et al.* 2008] produced in Phoenix-Eco cells and selectively expanded in Puromycin (1 µg/ml) (Sigma-Aldrich).
DN2 thymocytes activate a robust DNA Damage Response to IR-induced DNA double-strand breaks [von Münchow, 2013]. HSCs were maintained in SF-IMDM (Gibco) supplemented with 5% HyClone FCS (Thermo Scientific), 5% IL-6 supernatant, 0.1 µg/ml SCF and 0.2% Ciproxin® (Bayer Pharmaceutical) (Appendix II) at 37°C in 21% O₂. HSC and HSC-Bcl-2 lines were previously confirmed to be CD45⁺ Lin⁻ c-kit⁺ Sca-1⁺ CD11c⁻ CD19⁻ B220⁻ CD4⁺ CD8⁻ and successfully re-constituted all haematopoietic lineages in sub-lethally (4 Gy) irradiated mice [von Münchow, 2013].

4.2.3 Cell treatment

Cells were γ-irradiated at the indicated doses using a Gammacell 40 irradiator containing a $^{137}$Cs source at a dose rate of approximately 80 cGy/minute. A final concentration of 25 µM BrdU was added to cells at 50% confluency in 6 well plates (Nunc) for 45 minutes.

4.2.4 Reagents and antibodies

DL4-Fc fusion protein was purified from Chinese hamster ovary (CHO) cells stably transfected with pCR3 vector encoding the extra-cellular domain of mouse DL4 and the Fc domain of human IgG₁ using affinity chromatography as previously described [Tussiwand et al. 2011]. Recombinant mouse IL-7 and IL-6 supernatants were purified from IL-7 and IL-6 producing X63 hybridoma cell lines, respectively, generated in house. Recombinant mouse SCF was purified in house from Rosetta pLacI bacteria transfected with pQE-60 vector encoding steel (scf) using metal ion affinity chromatography [von Münchow, 2013]. Mouse monoclonal anti-human IgG₁ Fc antibody was purified from Huf5.4 hybridoma cell line generated in house [Tussiwand et al. 2011]. For flow cytometry, FITC-conjugated anti-CD25; PE-conjugated anti-CD44 (BD Biosciences) and APC-conjugated anti-CD117 (eBioscience) antibodies; anti-BrdU mouse monoclonal antibody (BD Biosciences) and FITC-conjugated anti-mouse IgG (whole molecule) antibody (Sigma-Aldrich) were used. For Western Blotting, anti-53BP1 rabbit polyclonal antibody (Bethyl) anti-phospho-Histone H2A.X (Ser139) mouse monoclonal antibody (Millipore), anti-H2AX rabbit polyclonal antibody, anti-ATM [2C1(1A1)] mouse monoclonal antibody, anti-DNA Ligase IV rabbit polyclonal antibody, anti-Rad51 rabbit polyclonal antibody, anti-p21 rabbit polyclonal antibody, anti-β-Tubulin rabbit
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polyclonal antibody (Abcam), anti-Chk2 rabbit polyclonal antibody, anti-p53 1C12 mouse monoclonal antibody, anti-Bcl-\(_{XL}\) (54H6) rabbit monoclonal antibody, anti-Bcl-2 (D17C4) rabbit monoclonal antibody, anti-Bim (C34C5) rabbit monoclonal antibody, anti-Puma rabbit polyclonal antibody (Cell Signaling Technology), anti-DNA-PKcs Ab-4 mouse monoclonal antibody, Pierce\textsuperscript{®} HRP conjugated rabbit antimouse IgG and ImmunoPure\textsuperscript{®} HRP conjugated goat anti-rabbit IgG antibodies (Thermo Scientific) were used. For Immunofluorescence Staining, anti-phospho-Histone H2A.X (Ser139) mouse monoclonal antibody (Millipore), anti-53BP1 rabbit polyclonal antibody (Bethyl); (FITC)-conjugated AffiniPure F(ab')\(_2\) Fragment goat anti-mouse IgG and Rhodamine Red\textsuperscript{®}-conjugated AffiniPure F(ab')\(_2\) Fragment goat anti-rabbit IgG antibodies (Jackson ImmunoResearch Laboratories Inc.) were used. Antibody preparations used are described in Appendix IV.

4.2.5 Clonogenic survival assay

Cells were irradiated at 0.5-4 Gy, seeded into 6 well plates (Nunc) at a concentration of 500,000 cells/well and harvested when control cultures reached 80% confluency i.e. 3 days (HSC/HSC-Bcl-2/ST4.5) or 5 days (DN2) post irradiation. Cell counts were performed in duplicate in a haemocytometer using Trypan blue exclusion. The percentage survival was determined by normalising the cell concentration in irradiated cultures to that in control cultures.

4.2.6 Flow Cytometry

4.2.6a Cell cycle analysis using BrdU-PI Staining

Cultures incubated with BrdU were centrifuged at 400 g for 10 minutes; washed three times in pre-warmed sterile PBS; re-suspended in the appropriate growth medium (Appendix II) and transferred into 6 well plates. At the indicated time-points post 4 Gy irradiation, cell suspensions were centrifuged at 400 g for 10 minutes; washed in PBS; fixed in ice-cold 70% ethanol and then stained for BrdU incorporation and DNA content using PI as previously described (Section 2.2.7a) [Sugrue et al. 2013a]. The progression of G\(_1\), S and G\(_2\)/M cells through the cell cycle was analyzed by measuring the percentage of cells in each phase until 36 hrs post IR.
4.2.6b Fluorescence Activated Cell Sorting (FACS) of DN thymocytes

Thymi from 6 control or irradiated (8.5 Gy) C57BL/6 mice were provided by Prof. Matthew Griffin (REMEDI, NUI Galway) and by Prof. Noel Caplice, [Centre for Research into Vascular Biology, (University College Cork)]. Thymic lobes were crushed between two pieces of nylon mesh in sterile DMEM (Sigma-Aldrich) to generate thymocyte suspension. CD4+ and CD8+ T lymphocytes were depleted by incubating thymocyte suspension with culture supernatants of rat IgM anti-mouse-CD4 (RL172.4) and rat IgM anti-mouse-CD8 monoclonal antibodies (3.168.8.1) [Ceredig et al. 1983b; Ceredig et al. 1985] and Low-Tox®-M Rabbit Complement (Cedarlane®). DN thymocytes were subsequently separated from the cell suspension using Ficoll-Hypaque density media (Sigma-Aldrich). DN thymocytes were stained with anti-CD25; anti-CD44 and anti-CD117 antibodies at 4°C for 30 minutes in FACS buffer. DN thymocytes were then re-suspended in 300 µl of Sort buffer (Appendix III) following a washing step. Dead cells were excluded using 5 µM Sytox blue dead cell stain (Invitrogen). DN1 were sorted as CD117high, CD25- and CD44high, DN2 as CD117high, CD25+ and CD44high and DN3 as CD117low, CD25+ and CD44low (Figure 4.5A; Figure 1.3) [Ceredig & Rolink, 2002] using BD FACS Aria II® (BD Biosciences) and re-analysis of sorted DN3 cells indicated >97% purity.

All FACS samples were analysed using Diva® (BD Biosciences) and FlowJo® software (TreeStar Inc., OR, USA).

4.2.7 Western Blotting

Whole cell extracts were prepared from harvested control or irradiated cells at the indicated time-points post 4 Gy irradiation as previously described (Section 2.2.5). Samples were separated using 4-15% SDS-PAGE gels and transferred to nitrocellulose membranes. Chemiluminescence was detected using SuperSignal West Pico Chemiluminescent Substrate® (Thermo Scientific) and medical X-ray film (Kodak).
4.2.8 Immunofluorescence staining

Cells were centrifuged onto coverslips at 500 rpm for 10 minutes using a cytospin (Tharmac Cellspin I) at the indicated time-points post 4 Gy irradiation. Following washing in PBS, cells were fixed in 95% methanol (pre-chilled at -20°C) at 4°C for 15 minutes. Following washing in PBS, cells were blocked in 5% FCS/PBS at 37°C for 1 hr; incubated with primary and secondary antibodies and mounted in Vectashield containing Hoechst solution as previously described (Section 2.2.6) [Sugrue et al. 2013a]. All images were captured using 100X magnification on a Zeiss LSM 510 Meta confocal microscope using LSM software (MediaCybernetics, MD, USA). Manual quantification of γ-H2AX and 53BP1 IRIF per nucleus (total of 50 cells per time-point) was performed blind.

4.3 RESULTS

4.3.1 DN2 thymocytes are more radio-resistant than HSCs and DP thymocytes in vitro

To investigate the radio-resistance of DN2 thymocytes (DN2) in vitro, DN2, ST4.5, NH HSCs (HSC), and NH HSC-Bcl-2 (HSC-Bcl-2) were irradiated at 0-4 Gy and cultured for 3 or 5 days before cell counts were performed. HSC-Bcl-2 was the most radio-resistant cell type as expected (Figure 4.1B) [Domen et al. 1998]. The radio-resistance of DN2, ST4.5 and HSC was comparable at IR doses of 0.5-2 Gy (Figure 4.1B). However, at the highest IR dose (4 Gy), DN2 radio-resistance was 10-fold greater than that of HSC and ST4.5 (~18.7% DN2 survival versus ~1.8% HSC and ~1.3% ST4.5 survival) (Figure 4.1B). These results confirm that the radio-resistance of DN2 thymocytes, relative to other immune cell types, can be replicated in vitro.

4.3.2 Irradiated DN2 thymocytes activate a robust DNA Damage Response

To determine whether DN2 and HSC activated the DDR in response to IR-induced DNA DSBs, H2AX Ser139 phosphorylation (γ-H2AX, DNA DSB marker); p53 stabilisation; and p21 and Puma expression were analysed over a 24 hr time-course.
DN2 thymocytes activate a robust DNA Damage Response to IR-induced DNA double-strand breaks (Figure 4.2A). Maximal H2AX phosphorylation was detected in DN2 at 1 hr post IR whereas it was delayed in HSC and accumulated until 4 hrs post IR (Figure 4.2A). p53 was stabilised, and p21 and Puma expression were induced, in irradiated DN2 and HSC, indicating that DDR pathways were intact in these cell types in vitro. However, in contrast to HSC, p53 stabilisation and induction of Puma expression were transient in irradiated DN2 (Figure 4.2A). Also, p21 expression was strongly induced in DN2 at early (1-4 hrs) time-points post IR whereas it was weakly induced in HSC at later time-points (12 & 24 hrs) (Figure 4.2A). Western blot analysis of control DN2 and HSC extracts revealed that DN2 expressed higher levels of ATM and DNA-PKcs than HSC whereas Chk2 levels were comparable (Figure 4.2B). In addition, DN2 expressed higher levels of anti-apoptotic proteins, Bcl-2 and Bcl-XL and of the pro-apoptotic protein, Bim, than HSC (Figure 4.2C).
Figure 4.1 DN2 thymocytes survive γ-irradiation in vitro. (A): Diagram of the ‘plastic thymus’ culture system. DN2 thymocytes were seeded into tissue culture wells coated with DL4-Fc fusion bound to the well surface using with anti-human IgG₁-Fc antibody (HUF) (Section 4.2.1) and were maintained in vitro by supplemental addition of recombinant IL-7 and SCF. (B): Clonogenic survival assay of mouse DN2 thymocytes and NH-HSC, NH-HSC-Bcl-2 and ST4.5 DP thymocytes γ-irradiated at 0.5-4 Gy and cultured for 3 or 5 days (depending on cell type) as previously described. Error bars represent mean ± SD, n=3.
Figure 4.2 DN2 thymocytes activate a robust DNA Damage Response. Western blot analysis of (A): H2AX (Ser139) phosphorylation (γ-H2AX – marker of DNA double-strand breaks); p53 stabilisation; and p21 and Puma expression in DN2 and NH-HSC at 0-24 hrs post 4 Gy irradiation; (B): Total H2AX, ATM, DNA-PKcs and Chk2 and (C): Bcl-2, Bcl-XL and Bim expression levels in un-irradiated (control) DN2 and NH-HSCs. β-Tubulin expression was used as a loading control. All Western blot images are representative of one of three independent experiments.
4.3.3 Irradiated DN2 thymocytes activate DNA damage checkpoints

Differences in (i) the long-term survival (Figure 4.1B) and (ii) the kinetics of p53 stabilisation and downstream induction of p21 and Puma expression (Figure 4.2A) between irradiated DN2 and HSC suggested that DNA damage checkpoints may be differentially activated in these cell types. The ability of DN2 and HSC to activate IR-induced cell cycle arrest was analysed using a flow cytometry-based BrdU incorporation assay as previously described (Section 2.2.7a) [Xu et al. 2002; Sugrue et al. 2013a]. Under normal conditions, ~16% DN2 population were in S phase (BrdU positive), compared with ~56% HSC and ~62% HSC-Bcl-2 (Figure 4.3A, black box in Untreated panels; Figure 4.3B). In contrast, ~78% DN2 population were in G1 phase compared with ~41% HSC and ~36% HSC-Bcl-2 (Figure 4.3B). These results indicate that a greater proportion of HSCs were actively cycling than DN2 under normal conditions in vitro.

Dramatic differences in the cell cycle status between DN2 and HSC were observed post irradiation. BrdU labelled DN2 accumulated as a cohort in late S/G2 until 12 hrs post IR, indicative of the activation of intra-S-phase and G2 checkpoints (Figure 4.3A, * in top 12 hr panel) (Figure 2.3A – 2.3C) [Xu et al. 2002]. In contrast, BrdU labelled HSC rapidly underwent cell death post IR (Figure 4.3A, black arrowheads in centre panels). Interestingly, unlike HSC, irradiated HSC-Bcl-2 did not undergo extensive cell death and activated DNA damage checkpoints in a similar manner to DN2 (Figure 4.3A). Irradiated BrdU labelled HSC-Bcl-2 recovered from cell cycle arrest and re-initiated DNA synthesis (Figure 4.3A, bottom 24 hr panel and 4.3E). However, BrdU labelled HSC and DN2 did not re-initiate DNA synthesis following cell cycle arrest and likely entered cell death (Figure 4.3A, top 24 hr panel; 4.3D and 4.3E). Strikingly, in contrast to irradiated G1 HSCs, G1 phase DN2 cells did not undergo extensive cell death and persisted long-term post IR (Figure 4.3A and 4.3C). Taken together, these results indicate that DN2 that survived long-term post IR primarily originated from G1 phase of the cell cycle.
Figure 4.3 (Overleaf) DN2 thymocytes activate DNA damage checkpoints. (A): Representative cytograms of DN2, HSC and HSC-Bcl-2 harvested at 0-24 hrs post 4 Gy irradiation and stained for BrdU incorporation and DNA content using PI. Black box represents BrdU labelled S phase population; black arrowheads indicate sub-G1 cells and * indicates cohort of BrdU labelled cells accumulated in late S/G2. (B): Quantification of the percentage DN2, HSC and HSC-Bcl-2 cells in each phase of the cell cycle under normal conditions. (C): Quantification of the percentage G1 DN2 and HSC at 0-36 hrs post 4 Gy irradiation. (D): Quantification of the percentage BrdU labelled G1/early S phase DN2 and HSC at 0-36 hrs post 4 Gy irradiation. (E): Quantification of the percentage BrdU labelled G1/early S phase DN2 and HSC-Bcl-2 cells at 0-36 hrs post 4 Gy irradiation. All cytograms are representative of one of three independent experiments. Error bars represent mean ± SD, n=3.
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Figure 4.3 DN2 thymocytes activate DNA damage checkpoints.
4.3.4 DN2 thymocytes can repair IR-induced DNA double-strand breaks

To characterise DNA DSB repair in irradiated DN2 and HSC, the kinetics of γ-H2AX and 53BP1 IRIF formation and resolution were analysed. 53BP1 IRIF was chosen as a marker of DNA DSB repair in this study because (i) preliminary data indicated that Rad51 IRIF were relatively scarce in irradiated DN2 and HSC nuclei in vitro (data not shown) and (ii) BrdU analysis demonstrated that (a) the majority of DN2 were in G1 phase at the time of irradiation and (b) DN2 thymocytes that survived irradiation primarily originated from G1 phase (Figure 4.3) and therefore, would require NHEJ, rather than HR, to repair DNA DSBs in which 53BP1 is implicated [Bothmer et al. 2011; Chapman et al. 2013; Rybanska-Spaeder et al. 2013; Zimmerman et al. 2013].

In correlation with robust H2AX phosphorylation (Figure 4.2A), greater numbers of γ-H2AX IRIF were generated in irradiated DN2 nuclei, and at a faster rate, than in HSC nuclei (Figure 4.4A and 4.4B). An overall maximum of ~17 γ-H2AX IRIF was detected per DN2 nucleus at 1 hr post IR in comparison with ~10 γ-H2AX IRIF per HSC nucleus at 2 hrs post IR (Figure 4.4B). Furthermore, greater numbers of 53BP1 IRIF were also formed in DN2 nuclei, and at a faster rate post IR, than in HSCs (Figure 4.4A and 4.4C). An overall maximum of ~4-5 53BP1 IRIF were detected in DN2 nuclei at 2 hrs post IR in comparison with ~3 53BP1 IRIF in HSCs at 4 hrs post IR (Figure 4.4C). In DN2 nuclei, 53BP1 IRIF began to co-localise with γ-H2AX IRIF at 2 hrs post IR and were highly co-localised at 4 and 8 hrs (Figure 4.4A) whereas co-localisation was weak in HSCs (Figure 4.4A). γ-H2AX IRIF were largely resolved in DN2 by later time-points (8-24 hrs post IR) (Figure 4.4A and 4.4B). Interestingly, ~1-2 53BP1 IRIF persisted in DN2 nuclei at 24 hrs post IR (Figure 4.4A). γ-H2AX and 53BP1 IRIF numbers were also reduced in HSC nuclei at 12 and 24 hrs post IR but this was likely due to cell death rather than DNA DSB repair (Figure 4.4A – 4.4C; Figure 4.3A). In addition, Western blot analysis demonstrated that DN2 expressed higher levels of DNA-PKcs, 53BP1 and DNA ligase IV; and lower levels of Rad51 than HSC under normal conditions (Figure 4.4D).
4.3.5 DN thymocytes activate the DNA Damage Response in vivo

Since irradiated DN2 robustly activated the DDR in vitro (Figure 4.2A; 4.4A and 4.4B), I finally wanted to determine whether DN2 respond to IR-induced DNA DSBs in vivo. In a preliminary experiment carried out between UCC and NUI Galway, viable DN1, DN2 and DN3 thymocyte subsets were sorted from control and irradiated (8.5 Gy) thymi (Figure 4.5A) and then stained for γ-H2AX and Rad51. γ-H2AX and Rad51 IRIF were detected in irradiated DN2 and DN3 nuclei (Figure 4.5B, bottom panels). DN1 thymocytes could not be analysed due to low numbers (<10,000 cells) on coverslips. γ-H2AX foci were also detected in control DN2 and DN3 nuclei (Figure 4.5B, top panels). Surprisingly, Rad51 foci were abundant in control DN2 nuclei whereas they were largely absent in control DN3 nuclei (Figure 4.5B, bottom panels). This preliminary data suggests that irradiated DN thymocytes activated the DDR in vivo.

Figure 4.4 (overleaf) DN2 thymocytes can repair IR-induced DNA double-strand breaks. (A): Representative immunofluorescent images of DN2 and NH-HSC at 0-24 hrs post 4 Gy irradiation and stained for DNA content (DAPI), γ-H2AX (green) and 53BP1 (red) IRIF captured using 100X magnification. Quantification of the average number of (B): γ-H2AX IRIF and (C): 53BP1 IRIF per nucleus (50 cells in total per time-point) at 0-24 hrs post IR. Error bars represent mean ± SD, n=3. (D): Western blot analysis of 53BP1, DNA Ligase IV and β-Tubulin (loading control) expression in control DN2 and NH-HSC whole cell extracts. All images are representative of one of three independent experiments.
Figure 4.4 DN2 thymocytes can repair IR-induced DNA double-strand breaks.
DN2 thymocytes activate a robust DNA Damage Response to IR-induced DNA double-strand breaks

Figure 4.5 DN thymocytes activate the DNA Damage Response \textit{in vivo}. (A): Viable DN thymocytes (Sytox blue negative – Panel 1) were sorted following doublet discrimination (Panel 2) as CD25^-CD117^{high} CD44^{high} (DN1); CD25^+ CD117^{high} CD44^{high} (DN2) and CD25^+ CD117^{low} CD44^{mid} (DN3) (Panels 4 and 5). Re-analysis of the DN3 sorted sample indicated >97% purity (Panel 6). (B): Representative immunofluorescent images of sorted control and irradiated (8.5 Gy, 1 hr time-point) DN2 and DN3 thymocytes stained for DNA content (DAPI), γ-H2AX (green) and Rad51 (red) IRIF captured using 100X magnification. All cytograms and images are representative of one experiment.
4.4 DISCUSSION

DN2 thymocytes are radio-resistant early intra-thymic T lymphocyte precursors exclusively responsible for initiating HD thymic auto-reconstitution post irradiation [Bosco et al. 2010]. Using the novel ‘plastic thymus’ culture system, I have characterised the DDR of irradiated DN2 thymocytes for the first time. Irradiated DN2 executed an efficient DDR characterised by (i) rapid induction of γ-H2AX (Figure 4.2A, 4.4A and 4.4B); (ii) robust activation of DDR effector pathways, indicated by IR-induced p53 stabilisation and downstream p21 and Puma expression (Figure 4.2A); (iii) induction of DNA damage checkpoints, particularly of a radio-protective G1 checkpoint (Figure 4.3A - 4.3C) and (iv) repair of IR-induced DNA DSBs (Figure 4.4A – 4.4C). Finally, detection of γ-H2AX IRIF in DN2 and DN3 nuclei directly sorted from irradiated thymi suggested that DN thymocytes activated the DDR to IR-induced DNA DSBs in vivo (Figure 4.5B). Altogether, these results indicate that the execution of an effective DDR contributes to DN2 radio-resistance.

Deficiency or loss-of-function mutations in multiple DNA DSB response genes including ATM, Nbs1, 53BP1 and DNA ligase IV results in (i) reduced thymic cellularity and (ii) increased preferential susceptibility to thymic lymphomas which frequently harbour defective V(D)J rearrangements and chromosomal aberrations involving TCR loci [Barlow et al. 1996; Xu et al. 1996; Kang et al. 2002; Morales et al. 2003; Nijnik et al. 2009]. It was originally hypothesised that physiological RAG-dependent DNA DSBs bypass the DDR. However, multiple DDR proteins (e.g. ATM, H2AX and 53BP1) are now directly implicated in monitoring correct V(D)J rearrangement [Bredemeyer et al. 2006; Huang et al. 2007; Difilippantonio et al. 2008; Bothmer et al. 2011; Savic et al. 2009; Liu et al. 2012; Bowen et al. 2013]. In addition, γ-H2AX and Nbs1 foci are generated at RAG-induced DNA DSBs within antigen receptor loci [Chen et al. 2000; Savic et al. 2009]. This may partly explain the presence of γ-H2AX foci observed in control DN2 and DN3 nuclei (Figure 4.5B, top panels). Furthermore, deficiencies in particular DDR proteins affect DN thymocyte development at different stages. For example, ATM deficiency blocks the DN3a-DN3b transition [Isoda et al. 2012] whereas 53BP1 deficiency blocks DN development at the DN2 and DN3 stages [Morales et al. 2003].
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DN2 thymocytes expressed higher levels of key DNA DSB response proteins including ATM, DNA-PKcs, 53BP1 and DNA ligase IV, than radio-sensitive HSCs (Figure 4.2B and 4.4D). Since the findings described above collectively suggest that the DDR is involved in (i) maintaining genomic stability at critical checkpoints in thymocyte differentiation and (ii) in regulating DN survival and proliferation, this may partly explain why DN2 thymocytes express high levels of key DDR proteins. Surprisingly, Rad51 foci were abundant in control DN2 nuclei (Figure 4.5). The potential function(s) of Rad51 in DN2 thymocytes and in thymopoiesis are currently unknown. However, DN2 thymocytes are the most rapidly proliferating DN population in vivo, despite on-going rearrangement of TCR-β D and J segments at this stage [Laurent et al. 2004; Masuda et al. 2007]. Therefore, one may speculate whether DNA repair proteins, such as Rad51, are required at this stage to police genomic instability potentially ensued by unresolved gene rearrangements in cycling DN2 thymocytes. Interestingly, DN2b thymocytes, which harbour rearranged TCR-β D and J segments, cycle more rapidly in vivo than DN2a thymocytes in which the TCR-β locus is largely un-rearranged [Masuda et al. 2007]. Therefore, it would be interesting to compare (i) the survival rate; (ii) DDR transcript levels; (iii) DNA DSB repair and (iv) DNA damage checkpoint activation in these DN2 subsets to determine whether they exhibit a similar DDR to IR-induced DNA DSBs.

While an abundance of key DDR proteins may prime DN2 to activate DDR pathways that promote survival rather than cell death, this is not likely to fully account for the unique radio-resistance of DN2 because DDR proteins are implicated throughout thymopoiesis and are expressed in other thymocyte subsets [Morales et al. 2003; Bosco et al. 2010; Isoda et al. 2012]. Therefore, it would be interesting to investigate whether the DDR of DN2 thymocytes could also be influenced by signals from the thymic microenvironment.

Notch1-DL4 signalling is required for DN2 survival, proliferation and T lineage commitment [Radtke et al. 1999; Balciunaite et al. 2005b; Massa et al. 2006; Koch et al. 2008]. Identification of Notch target genes in thymocytes is on-going but has been aided by continuing studies on T-acute lymphoblastic leukaemia (T-ALL) in which
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Notch1 acts as an oncogene with ~50% of T-ALL cases harbouring Notch1 gain-of-function mutations [Weng et al. 2004; Graux et al. 2006 Aster et al. 2008]. Interestingly, Notch1 over-activation in T-ALL results in de-regulated expression of numerous genes involved in G1 cell cycle control. Activated intracellular Notch1 (ICN1) can (i) suppress expression of key G1 CKIs including p21, p27 and p16INK4a and p19ARF; (ii) suppress p53 function and (iii) induce expression of potent promoters of G1-S progression including Cdk4, Cdk6, cyclin D3 and c-Myc [Sicinska et al. 2003; Massagué, 2004; Beverly et al. 2005; Sarmento et al. 2005; Joshi et al. 2008; Rao et al. 2009]. Furthermore, inhibition of Notch signalling using γ-secretase inhibitors (preventing generation of ICN1), represses Notch-dependent effects on G1 cell cycle machinery, leading to G0/G1 arrest in T-ALL cells [Joshi et al. 2008; Rao et al. 2009].

ATM and p53 are required for maintaining thymocytes with unresolved DNA DSBs in G0/G1 [Yan et al. 2002; Dujka et al. 2010]. Interestingly, 30% of T-ALL tumours derived from H2AX−/− and H2AX−/−/Tp53−/− mice harbour Notch1 gain-of-function mutations [O’Neil et al. 2006; Aster et al. 2008]. In addition, there is a high frequency of Notch1 rearrangements in T-ALL cells derived from control and irradiated Atm−/− and scid−/− mice [Tsuji et al. 2003 & 2009]. Altogether, these findings suggest that (i) Notch-mediated suppression of G1 checkpoint control contributes to T lymphocyte transformation and thus, (ii) DDR modulation of Notch1 signalling may be important for maintaining genomic stability in thymocytes. Irradiated DN2 thymocytes activated a robust G1/S checkpoint characterised by (i) impaired entry of irradiated G1 cells into S phase post IR (Figure 4.3C); (ii) p53 stabilisation (Figure 4.2A) and (iii) downstream induction of p21 expression (Figure 4.2A). In addition, DN2 that survived long-term post IR primarily originated from G1 phase (Figure 4.3A). Therefore, one may speculate whether DDR activation in irradiated DN2 may suppress Notch signalling to prevent DN2 thymocytes with unresolved DNA DSBs from entering DNA synthesis. To investigate the potential interplay between the DDR and Notch signalling, the expression levels of ICN1 and of Notch1 target genes in DN2 could be analysed over a time-course post IR. In addition, DN2 could be treated with γ-secretase inhibitor(s) to determine whether inhibition of Notch signalling affects DN2 radio-sensitivity and cell cycle status. Furthermore, DN2 could be
isolated from an ICN1-expressing transgenic mouse (e.g. NotchIC transgenic mouse strain [Robey et al. 1996]) to determine whether constitutive Notch1 activity affects the DDR of DN2 thymocytes.

Thymopoiesis is arrested at the DN stage in SCID- and RAG-deficient mice due to impaired V(D)J recombination at TCR loci. However, surprisingly, irradiation rescues TCR loci rearrangement in these mice, leading to the spontaneous generation of DP thymocytes [Danska et al. 1994; Bogue et al. 1996; Zhu et al. 1996]. As previously discussed, evidence is mounting that the DDR is important for co-ordinating V(D)J recombination. In addition, activation of G1 arrest is essential for V(D)J recombination and differentiation in lymphocytes [Lin & Desiderio, 1994]. Since DN2 are exclusively responsible for thymic auto-reconstitution, these findings may suggest that irradiation could stimulate DN2 to differentiate via induction of a G1 checkpoint in which the repair IR-induced DNA DSBs within TCR loci by the DDR machinery results in TCR rearrangements that lead to the spontaneous generation of DP thymocytes. Interestingly, 1-2 53BP1 IRIF persisted in most DN2 nuclei in the absence of γ-H2AX IRIF at 24 hrs post IR (Figure 4.4A). Furthermore, 53BP1 can co-localise with TCR loci [Bowen et al. 2013]. Therefore, it would be interesting to (i) determine whether irradiation stimulates DN2 differentiation in vitro; (ii) analyse TCR loci rearrangements in irradiated DN2 thymocytes and (iii) determine whether persistent 53BP1 foci in irradiated DN2 nuclei co-localise with TCR loci or are potentially associated with other chromosomal rearrangements.

IL-7 signalling is also indispensable for normal T lymphopoiesis [Murray et al. 1989; Peschon et al. 1994; von Freeden-Jeffry et al. 1995; Maraskovsky et al. 1997]. In early thymopoiesis, IL-7 plays key roles in (i) promoting DN1-DN3 survival and proliferation; (ii) influencing αβ versus γδ lineage commitment and (iii) regulating the differentiation of β-selected DN3 thymocytes [Maraskovsky et al. 1997; von Freeden-Jeffry et al. 1997; Kang et al., 2001; Balcuiunaitė et al., 2005c; Ceredig & Rolink, 2012]. Notch1 signalling up-regulates IL-7Ra (CD127) expression on DN2 thymocytes, leading to IL-7 dependent bcl-2 transcription [González-García et al. 2009]. As a result, DN2 express very high levels of Bcl-2 that promotes their survival in vivo by blocking apoptosis [Maraskovsky et al. 1997; Bosco et al. 2010]. Similarly,
I found that DN2 expressed higher levels of Bcl-2 and Bcl-XL than HSCs \textit{in vitro} (Figure 4.2C), likely due to the combined presence of recombinant DL4 and IL-7 in the ‘‘plastic thymus’’ culture system.

Bcl-2 over-expression increases the radio-resistance of haematopoietic cells, including DN2, improving host-derived BM and thymic auto-reconstitution following TBI [Domen \textit{et al.} 1998; Ogilvy \textit{et al.} 1999; Erlacher \textit{et al.} 2005; Bosco \textit{et al.} 2010]. Similarly, HSC-Bcl-2 were more radio-resistant than HSC and DN2 \textit{in vitro} (Figure 4.1B). Interestingly, unlike irradiated HSC, irradiated HSC-Bcl-2 activated DNA damage checkpoints in a similar manner to irradiated DN2 (Figure 4.3A and 4.3C). Overall, these results suggest that Bcl-2 expression may promote DN2 radio-resistance by enabling robust activation of cell cycle arrest and DNA repair through the suppression of apoptosis. To determine this, one could compare the DDR of irradiated WT DN2; DN2 isolated from H-2K.BCL-2 Tg mice (DN2-Bcl-2) and Bcl-2 depleted WT DN2 using RNA interference. In addition, it would be interesting to analyse CD127 and Bcl-2 expression levels in DN2 post IR and post treatment with γ-secretase inhibitor(s) to determine whether irradiation and/or Notch inhibition affects IL-7 signalling in DN2 in the ‘‘plastic thymus’’ culture system.

DN2 thymocytes also express high levels of c-kit (CD117), the receptor for the cytokine SCF, and is required for DN2 self-renewal, T lineage commitment and \textit{in vitro} expansion [Godfrey \textit{et al.} 1992; Rodewald \textit{et al.} 1995; Massa \textit{et al.} 2006]. Since DN2 largely remain in G1 following irradiation \textit{in vitro}, it would be interesting to determine whether irradiation affects CD117 expression on DN2 thymocytes. In addition, by treating DN2 with the c-kit inhibitor, Gleevec [Massa \textit{et al.} 2006], one could determine whether inhibition of c-kit signalling affects DN2 radio-sensitivity \textit{in vitro}. Since the SCF-c-kit signalling pathway is also required for driving HSC self-renewal and expansion [Broudy \textit{et al.} 1997], it would also be interesting to compare the effects of inhibiting c-kit signalling on HSC and DN2 radiobiology.

The experiments described herein will enable us to determine the potential roles of Notch, IL-7 and c-kit signalling pathways in mediating DN2 radio-resistance \textit{in vitro}. However, there are currently a few limitations to the \textit{in vitro} study of DN2 radio-
DN2 thymocytes activate a robust DNA Damage Response to IR-induced DNA double-strand breaks

biology. Firstly, DN2 cycle slowly in vitro (Figure 4.3A and 4.3B) whereas they are rapidly cycling in vivo [Laurent et al. 2004; Masuda et al. 2007]. In addition, Rad51 foci were scarce in control or irradiated DN2 in vitro whereas they were present in DN2 nuclei in vivo (Figure 4.5B). Therefore, while ‘‘the plastic thymus’’ culture system is a powerful tool for analysing DN2 radio-resistance at the molecular level, their DDR in vitro may not be truly reflective of that in vivo. Furthermore, DN1-DN3 survival is Notch- and IL-7 dependent and DN1-DN2 survival is c-kit dependent (Balciunaite et al. 2005b; Massa et al. 2006). Therefore, while these signalling pathways are likely to affect DN2 radio-resistance, how this compares with other DN thymocyte subsets also needs to be addressed.

Given more time, one could address these limitations by analysing (i) γ-H2AX; (ii) 53BP1 and (iii) Rad51 IRIF kinetics in DN1-DN4 thymocytes directly sorted from control and irradiated thymi over a defined time-course. This would enable one to determine whether (i) DDR activation; (ii) Focal recruitment of DNA repair proteins to DNA DSBs and (iii) DNA DSB repair are differentially regulated in individual DN thymocyte subsets. Following this, (i) PI staining and (ii) BrdU incorporation analysis of sorted control and irradiated DN thymocyte subsets (labelled with BrdU in vivo) could be performed to (i) characterise DNA damage checkpoints activated by irradiated DN subsets and (ii) determine whether the proliferation rates of DN subsets are affected by irradiation. In addition, subsequent analysis of DN subsets isolated from irradiated mice treated with a γ-secretase inhibitor, Gleevec or soluble IL-7Rα (to block IL-7 signalling) could enable one to investigate whether the DDR of DN subsets is affected by Notch, c-kit and IL-7 signalling in vivo, respectively. In conclusion, I have determined that the activation of an efficient DDR contributes to the radio-resistance of DN2 thymocytes in vitro. These findings will enable us to further investigate the aspects of the DDR that contribute to irradiated DN2 survival in vivo and to determine the potential roles of the thymic microenvironment in driving DN2 radio-resistance.
DN2 thymocytes activate a robust DNA Damage Response to IR-induced DNA double-strand breaks
5.1 MOUSE MSC AND DN2 RADIO-RESISTANCE: WHAT HAVE WE LEARNED?

Herein, I have performed the first investigation into the role of the DDR in mediating mouse MSC resistance to IR-induced DNA DSBs. Detailed characterisation of MSC radio-biology has thus far been impaired due to the widespread use of heterogeneous bulk MSC cultures. Therefore, the use of cloned MSC lines in this study has enabled us to perform the first in depth analysis of the molecular pathways governing MSC radio-resistance. I have demonstrated that the activation of the DDR is fundamental for mouse MSC survival post irradiation. Multiple DDR signalling pathways were found to synergistically promote MSC survival. Specifically, mouse MSCs (i) rapidly activated the DDR (Figure 2.3 and 3.6); (ii) induced robust intra-S and G2/M DNA damage checkpoints (Figure 2.4 and 3.3) and (iii) could repair DNA DSBs (Figure 2.3A; 2.7; 3.6 and 3.8). Moreover, despite possessing a functional intrinsic apoptotic pathway, apoptosis was weakly activated by MSCs in response to γ-irradiation (Figure 2.6A – 2.6C; Figure 3.5B). This was likely due to the activation of protective DNA damage checkpoints and of efficient DNA DSB repair (summarised in Figure 5.1). In addition, I found that irradiated mouse MSCs continued to differentiate, indicating that their multi-potency remains intact following genotoxic insult (Figure 2.9).

Hypoxia is emerging as an important modulator of MSC proliferation, differentiation potential and function. I have shown, for the first time, that hypoxia alters the DDR of MSCs to IR-induced DNA DSBs, enhancing MSC radio-resistance (Figure 3.1; 3.3; 3.6 and 3.9). Furthermore, I found that HIF-1α stabilisation contributed to the up-regulation of DNA DSB repair in hypoxic MSCs via (i) increased DNA-PKcs and DNA ligase IV expression and (ii) accelerated Rad51 IRIF formation in response to DNA DSBs (Figure 3.10 and 3.11). This consequently (i) accelerated DNA DSB resolution; (ii) enhanced recovery from IR-induced cell cycle arrest and thereby, (iii) improved the long-term survival of MSCs post irradiation (summarised in Figure 5.2). This is the first study to identify HIF-1α as a mediator of enhanced DNA DSB repair in hypoxic mouse MSCs. In addition, these findings provide the first evidence that mouse MSC radio-resistance is likely to be influenced by environmental factors within the BM and tumour microenvironments.
Figure 5.1 Overview of the DNA Damage Response in irradiated mouse MSCs and CD4<sup>+</sup> CD8<sup>+</sup> DP thymocytes. (A): Irradiated MSCs rapidly phosphorylate H2AX and form γ-H2AX IRIF in response to DNA DSBs which may be promoted by high expression levels of ATM and DNA-PK<sub>cs</sub>. These attributes likely promote (i) rapid initiation of DNA DSB repair and (ii) activation of protective DNA damage checkpoints that suppress apoptosis and promote DNA DSB repair. Efficient DNA DSB repair, aided by high expression levels of DNA-PK<sub>cs</sub>, DNA ligase IV and Rad51, may enable MSCs to recover from cell cycle arrest and to resume proliferation. MSC survival could also be promoted by (i) transient p53 stabilisation (due to efficient DNA DSB repair) and (ii) biased expression of pro-survival (Bcl-2 and Bcl<sub>-XL</sub>) versus pro-apoptotic proteins (Bim and Puma). (B): Irradiated ST4.5 thymocytes exhibit delayed H2AX phosphorylation and γ-H2AX IRIF formation, which may be correlated with low endogenous levels of ATM and DNA-PK<sub>cs</sub>. Inefficient activation of protective DNA damage checkpoints may be correlated with low expression levels of ATM and Chk2 in this cell type and likely contributes to the rapid activation of IR-induced apoptosis. Delayed γ-H2AX formation and inefficient checkpoint activation, combined with low endogenous levels of DNA DSB repair proteins (DNA-PK<sub>cs</sub>, DNA ligase IV and Rad51, are likely to impair efficient DNA DSB repair in ST4.5 thymocytes. These attributes, together with high expression levels of potent pro-apoptotic proteins, Bim and Puma, likely stimulate ST4.5 thymocytes to undergo extensive apoptosis post irradiation. **Abbreviations:** DSB (Double-strand break).
Figure 5.1 Overview of the DNA Damage Response in irradiated mouse MSCs and CD4^+ CD8^+ DP ST4.5 thymocytes.
**Figure 5.2** HIF-1α contributes to the enhanced DNA DSB repair capacity of hypoxic mouse MSCs. In normoxia (left panel) and hypoxia (right panel), irradiated MSCs activate DNA damage checkpoints and DNA DSB repair (NHEJ and HR) to resolve genotoxic DNA DSBs. Resolution of DNA DSBs likely enables irradiated MSCs to recover from cell cycle arrest and promotes MSC survival. In hypoxia (right panel), HIF-1α stabilisation resulted in (i) increased endogenous expression of the two key NHEJ enzymes, DNA-PKcs and DNA ligase IV, and (ii) accelerated recruitment of Rad51 to DNA DSBs, an essential component of repair by HR. Consequently, the DNA DSB repair capacity of MSCs is increased, accelerating recovery from cell cycle arrest and improving long-term survival.

**Abbreviations:** DSB (Double-strand break); HIF-1α (Hypoxia inducible factor-1α); HR (Homologous recombination); IRIF (IR-induced foci); NHEJ (Non-homologous end-joining).
In this study, I have also characterised the DDR of DN2 thymocytes to IR-induced DNA DSBs for the first time. I have uniquely demonstrated that the novel ‘plastic thymus’ culture system can, with certain limitations, be used to study signalling pathways in DN2 thymocytes in vitro. In addition, by using the NH-HSC cell line as a radio-sensitive control, I have also performed the first characterisation of the DDR of clonal bona-fide HSCs in vitro. The radio-resistance of DN2 thymocytes, relative to other immune cell types (i.e. HSCs and DP thymocytes), could be replicated in the ‘plastic thymus’ culture system (Figure 4.1). In addition, in contrast to radio-sensitive HSCs and DP thymocytes (ST4.5), irradiated DN2 (i) did not undergo rapid cell death; (ii) activated DNA damage checkpoints, particularly of a radio-protective G1 checkpoint and (iii) could repair DNA DSBs, likely using NHEJ. These results indicate that the execution of DDR signalling pathways contributes to DN2 radio-resistance and provide a foundation on which to investigate the DDR of DN2 thymocytes in vivo in the future.

Overall, I have compared the DDR of irradiated MSCs versus DP thymocytes (ST4.5), and of DN2 thymocytes versus HSCs. By comparing these cell types, I have clearly shown that the DDR is differentially executed by individual cell types, irrespective of (i) being located within the same organ (MSC/HSC in BM; DN2/DP in the thymus); (ii) deriving from the same cell lineage (HSC/DN2/DP) or (iii) being at a stem/progenitor (MSC/HSC/DN2) or a more differentiated state (DP). To my knowledge, this is the first study to directly compare the effects of irradiation on the DDR of haematopoietic cells and their supportive stromal cells and of different cell types within the haematopoietic lineage. Therefore, this study has added significant knowledge to our understanding of how the DDR is modulated in different cell types involved in the immune system. The DDR of mouse MSCs has been extensively characterised in this study. Therefore, I would now like to discuss several aspects of MSC radio-biology and its potential implications in more detail.

5.2 THE DNA DAMAGE RESPONSE OF MSCs: PROGRAMMED FOR SURVIVAL?

My findings collectively suggest that the response of MSCs to IR-induced DNA damage is to activate survival mechanisms, such as DNA damage checkpoint activation and DNA DSB repair, which likely inhibit cell death and enable MSCs to continue to proliferate and to differentiate along mesenchymal-derived lineages. Therefore, the question remains why
irradiated MSCs preferentially execute pro-survival DDR mechanisms, rather than those that lead to their demise? An interesting observation made during this study was that irradiated MSCs induced maximal H2AX phosphorylation and γ-H2AX IRIF formation at a faster rate than radio-sensitive DP thymocytes (ST4.5) (Figure 2.3A; 2.7A; 3.5E and 3.6A). In addition, MSCs expressed higher levels of the key DNA DSB response proteins, ATM and DNA-PKcs, than DP thymocytes (Figure 2.3B). ATM and DNA-PKcs function redundantly to phosphorylate H2AX and inhibition of their kinase activity delays H2AX phosphorylation [Stiff et al. 2004; Wang et al. 2005]. Furthermore, ATM- and DNA-PKcs-dependent γ-H2AX induction is required for efficient DNA DSB repair by acting as a docking site for the assembly of DDR mediators and DNA DSB repair proteins at damaged sites [Downs et al. 2000; Paull et al. 2000; Bassing et al. 2002; Celeste et al. 2002 & 2003; Xie et al. 2004; Franco et al. 2006]. Therefore, these findings may suggest that high endogenous levels of ATM and DNA-PKcs may promote DNA DSB repair in MSCs by rapidly initiating γ-H2AX induction and thereby, facilitating rapid recruitment of DNA DSB repair proteins to damaged sites (summarised in Figure 5.1). Given more time, it would be interesting to either (i) knock-down ATM and/or DNA-PKcs in MSCs or (ii) over-express these DDR proteins in ST4.5 thymocytes to determine whether their endogenous expression levels influence the rate of IR-induced γ-H2AX formation in these cell types.

It has been recently shown that the majority of IR-induced DNA DSBs are repaired at early time-points post IR [Beucher et al. 2009]. Similarly, DNA DSBs were largely repaired in MSCs by 4-8 hrs post IR (Figure 3.6D and 3.9D). This suggests that DNA DSB repair must be initiated quickly following damage to promote cell survival. MSCs (i) expressed higher levels of DNA DSB repair proteins, e.g. ATM, DNA-PKcs and DNA ligase IV and (ii) had an increased NHEJ capacity in comparison with DP thymocytes (Figure 2.3B; 2.7F - 2.7I). In addition, hypoxic MSCs (i) expressed higher levels of DNA-PKcs and DNA ligase IV and (ii) were more efficient at DNA DSB repair in comparison with normoxic MSCs (Figure 3.6 and 3.8). Taken together, these findings suggest that high expression levels of DSB repair proteins may also provide MSCs with a survival advantage by facilitating rapid initiation of DNA DSB repair post IR, thereby suppressing the activation of cell death pathways. This hypothesis may be supported by the finding that (i) maintained p53 stabilisation and (ii) induction of Puma expression, indicative of apoptosis activation, occurred in MSCs in the presence of persisting un-repaired DNA DSBs induced by etoposide (Figure 2.5B).
MSCs also expressed high levels of the key pro-survival proteins, Bcl-2 and Bcl-XL, and low levels of the potent pro-apoptotic proteins, Bim and Puma (Figure 2.6D and 2.6E). The intrinsic expression levels of various apoptotic proteins are known to influence the therapeutic sensitivity of various cell types, including HSCs, BSCs, KSCs and lymphocytes [Domen et al. 1998; Ogilvy et al. 1999; Erlacher et al. 2005; Sotiropoulou et al. 2010]. Similarly, in this study, HSC-Bcl-2 exhibited (i) increased resistance to cell death and (ii) improved long-term survival in comparison with WT HSCs. Therefore, it is possible that high expression levels of pro-survival proteins may also contribute to the ability of irradiated MSCs to suppress apoptosis. Overall, my findings may suggest that the endogenous expression levels of proteins involved in pro-survival and pro-death DDR signalling pathways may influence the radio-sensitivity of a given cell type. It seems that MSCs express high levels of proteins involved in multiple pro-survival DDR signalling pathways which together, are likely to enable MSCs (i) to rapidly respond to DNA damage; (ii) to repair IR-induced DNA DSBs and (iii) to suppress cell death mechanisms, thereby contributing to their enhanced ability to recover from high dose irradiation in comparison with radio-sensitive cell types.

Why MSCs appear to be intrinsically primed for survival is unknown. However, mounting evidence indicates that the DDR plays fundamental roles in stem cell biology. The DDR functions in stem cells to monitor endogenous DNA damage caused by environmental factors, e.g. ROS production, and by telomere shortening, preventing stem cell dysfunction and exhaustion in vivo [Ito et al. 2004; Chambers et al. 2007; Kenyon & Gerson, 2007; Rübe et al. 2011; Sperka et al. 2011 & 2012]. In addition, several key DDR proteins (e.g. ATM, p53 and p21) and DNA DSB repair proteins are also required in stem cells for (i) maintaining self-renewal; (ii) inhibiting cellular senescence and (iii) regulating their differentiation potential [Luo et al. 1999; Cheng et al. 2000; Nijnik et al. 2007; Rossi et al. 2007; Qing & Gerson, 2007; Inomata et al. 2009; Milyavsky et al. 2010; Sperka et al. 2011; Wang J et al. 2012]. Interestingly, proteins involved in genome stability and cell cycle control have also been directly implicated in MSC biology. For example, loss of (i) the cell cycle regulator, Rb; (ii) the microRNA processor, Dicer, and (iii) telomerase (required for telomere maintenance and stability); impairs the ability of MSCs to regulate haematopoiesis, resulting in myeloproliferation and leukaemia development [Ju et al. 2007; Walkley et al. 2007; Raajimakers et al. 2010]. In addition, deletion or loss-of-function mutations in several DDR genes, including
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ATM, ATR and DNA ligase IV, can result in growth delay, skeletal abnormalities and osteoporosis [Hishiya et al. 2005; O’Driscoll & Jeggo, 2006; Rasheed et al. 2006; Ruzankina et al. 2007]. Overall, these findings suggest that MSC radio-resistance may perhaps be a consequence of the importance (i) of the maintenance of genomic stability for stem cell homeostasis and (ii) of DDR components in regulating MSC self-renewal, differentiation and function in the BM. Given more time, it would be interesting to (i) knock down the expression of DDR genes (e.g. ATM, Chk2, Ligase IV) and/or (ii) to isolate MSC populations from DDR gene-deficient mice to investigate the potential roles of the DDR in regulating MSC self-renewal, immuno-modulatory properties and differentiation potential in more detail.

5.3 RADIO-RESISTANT MSCs: FOR BETTER OR FOR WORSE?

There are several important implications of MSC radio-resistance. The ability of MSCs to survive high dose irradiation is highly significant in BMT. Over 25,000 allogeneic bone marrow transplantations are performed each year to treat a variety of conditions including haematological and lymphoid cancers and various genetic diseases of the immune system [Copelan, 2006]. TBI is often used in allogeneic BMT because functioning BM is distributed throughout various regions of the body, with ~60% of total body BM located in the pelvis and the vertebrae [Mauch et al. 1995]. It is also able to target cancer cells that are unaffected by chemotherapy [Copelan, 2006]. As previously discussed, MSCs are essential for supporting haematopoiesis in the BM (Section 1.1). Moreover, co-administration of MSCs with donor HSCs substantially improves haematopoietic engraftment and reconstitution following allogeneic BMT [Anklesaria et al. 1987; Koç et al. 2000; Mugurama et al. 2006; Hu et al. 2010; Shim et al. 2013]. Taken together, these findings indicate that the ability of host-derived MSCs to survive irradiation is likely to be important for supporting haematopoietic recovery following myeloablative radiotherapy. Interestingly, it was recently reported that IR-induced damage to the BM vasculature impedes MSC recovery in mouse femurs [Cao et al. 2011]. Therefore, advancing our understanding of the impact of radiation on MSCs and the HSC niche in vivo could have important implications on the modification of pre-conditioning regimens for BMT. These preparative regimens should potentially aim to adequately deplete haematopoietic cells while minimising harmful effects to the BM microenvironment in order to improve the success rates of allogeneic BMT.
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As previously described, MSCs are potent immuno-modulators that home to inflammatory and damaged sites where they function to reduce inflammation and to promote tissue repair (Section 1.2). As well as having detrimental effects on the immune system, acute radiation exposure also causes extensive damage to other tissues including the gut, the skin and the central nervous system [Hall & Giaccia, 2006]. Interestingly, studies have demonstrated that transplanted MSCs can home and engraft into radiation-injured non-haematopoietic tissues [François et al. 2006; Mouiseddine et al. 2007]. Therefore, the radio-resistance of host-derived MSCs may also have important implications for the suppression of inflammation and for tissue regeneration following acute radiation injury.

The abilities of MSCs to home to sites of injury and inflammation, to repair damaged tissues and to modulate immune responses are likely to be beneficial for systemic recovery following irradiation, particularly of the immune system. However, these characteristics could prove to be detrimental in the context of cancer therapy. As previously described, MSCs are an integral component of the tumour stroma [Hanahan & Coussens, 2012; Kidd et al. 2012]. Tumour formation causes changes in the tissue environment which mimic the formation of a wound site, resulting in tissue injury and inflammation which consequently stimulate MSC recruitment [Bergfeld & DeClerck, 2010; Schichor et al. 2010; Marigo & Dazzi, 2011]. Here, MSCs are transformed into CAFs which promote tumourgenesis by (i) inhibiting apoptosis of cancer cells; (ii) secreting growth factors that stimulate cancer cell growth; (iii) secreting angiogenic factors and differentiating into pericytes, contributing to the tumour vasculature and (iv) suppressing anti-tumour immune cells [Bergfeld & DeClerk, 2010; Kraman et al. 2010; Marigo & Dazzi, 2011; Hanahan & Coussens, 2012]. Since MSCs are radio-resistant, MSC-derived CAFs within the tumour microenvironment may not be ablated by conventional radiotherapy. Consequently, these CAFs may continue to support the growth of remaining therapeutically resistant cancer cells and cancer stem cells, preventing tumour elimination and promoting the development of a more aggressive tumour.

Solid tumours often contain an abnormal, disorganised vascular network resulting in heterogeneously distributed hypoxic regions within the tumour mass. Intra-tumoural hypoxia is an adverse prognostic factor in cancer associated with increased therapeutic resistance, malignancy and metastasis. The increased radio-resistance of hypoxic cancer cells is thought to be mediated by two factors: (i) A reduction in IR-induced ROS production which causes
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chemical alterations in DNA that contribute to the generation of DNA damage and (ii) The activation of signalling cascades, mainly regulated by HIF-1α and HIF-2α, which lead to biological alterations in hypoxic cancer cells that promote therapeutic resistance and genomic instability [Hall & Giaccia, 2006; Bristow & Hill, 2008]. In this study, I found that hypoxia enhanced MSC radio-resistance, particularly by up-regulating DNA DSB repair in a HIF-1α-dependent manner. Therefore, my findings suggest that hypoxia may also contribute to tumourigenesis by altering the radio-resistance of the tumour stroma, including MSC-derived CAFs. Thus, it will likely be important to consider the effects of hypoxia on the tumour microenvironment, in addition to cancer cells, in order to develop successful strategies for combating hypoxia-enhanced therapeutic resistance in the future.

MSCs within the BM niche itself are highly implicated in the development and therapeutic resistance of various haematological malignancies, particularly of leukaemias. The production of various cell-cell adhesion molecules (e.g. VCAM-1 and fibronectin) and paracrine factors (e.g. CXCL12 and SCF) by MSCs stimulate homing and engraftment of leukaemic cells in the BM [Michigami et al. 2000; Alsayed et al. 2007; Ishikawa et al. 2007; Ayala et al. 2009]. These interactions enable leukaemic cells to establish malignant niches within the BM where leukaemic stem cells (LSCs) enter a quiescent state in which they are highly resistant to anti-cancer therapies [Ishikawa et al. 2007; Ninomiya et al. 2007; Colmone et al. 2008; Nwajei & Konopleva, 2013]. MSCs also contribute to the therapeutic resistance of leukaemic cells by suppressing apoptosis. For example, interaction of MSC-derived CXCL12 with CXCR4 expressed on leukaemic cells can (i) suppress chemotherapy-induced caspase-3 activation and (ii) induce increased expression of anti-apoptotic proteins, e.g. Bcl-2, Bcl-XL and Mcl-1, in leukaemic cells [Balakrishnan et al. 2009 & 2010; Vianello et al. 2010]. Interestingly, hypoxia has recently been shown to (i) improve the ability of MSCs to support leukaemic cells and (ii) promote LSC survival and therapeutic resistance [Benito et al. 2011; Fecteau et al. 2013]. Therefore, the resistance of MSCs to DNA damaging agents, including IR, may also contribute to the survival of LSCs in the BM, leading to disease relapse following radio- and/or chemotherapy.

Overall, it is evident that MSC radio-resistance has important implications for haematopoietic re-constitution, immune system recovery, tissue regeneration and for tumour eradication following radiotherapy. Therefore, whether MSC radio-resistance is therapeutically beneficial
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or unfavourable is highly dependent on the disease being treated and thus, should be taken into account when designing therapeutic regimes to combat the disease in question.

5.4 MSC RADIO-RESISTANCE: FUTURE DIRECTIONS

I have shown that MSC lines generated from un-irradiated (ST2) and irradiated (MS5) BM of two independent mouse strains, executed a similar DDR to IR-induced DNA DSBs in both normoxia and hypoxia. In addition, p53 was found to be functional in these MSC lines and unpublished data has demonstrated that they are non-tumourigenic in vivo. These findings suggest that cloned MSC lines provide a reliable means for modelling MSC behaviour in response to genotoxic stress and for dissecting the molecular pathways involved. However, there are some limitations of this in vitro study that should be considered before translating these findings in vivo. Firstly, MSCs reside and function in a dynamic microenvironment within the BM niche in vivo (Section 1.1). Here, I demonstrated that hypoxia, a key feature of the BM microenvironment, can alter the DDR of mouse MSCs. In addition, under the current method of culturing MSCs on plastic and in normoxia, primary MSCs alter their (i) self-renewal capacity, (ii) differentiation potential and (iii) surface marker expression and (iv) frequently become senescent in vitro, indicating that these culture conditions are not optimal for MSCs. Therefore, micro-environmental factors, including not only hypoxia, but also ECM production and interactions with various other cell types, are likely to influence MSC radio-biology in vivo which cannot be mimicked in vitro.

Secondly, the MSC compartment consists of multiple sub-populations of varying function, proliferative capacity and differentiation potential in vivo (Section 1.1). Whether MS5 and ST2 MSC lines derive from the same, or different, MSC sub-populations in vivo is unknown. Recently, mouse MSCs isolated from flushed BM aspirates (denoted BM-SC) were reported to be more radio-sensitive than MSCs isolated from collagenase digested bone (denoted OB-SC) [Carbonneau et al. 2012]. Interestingly, p16^{INK4A} tumour suppressor protein was expressed at higher levels in irradiated OB-SCs than in BM-SCs whereas p19^{ARF} expression levels were only increased in irradiated BM-SCs. Furthermore, BM-SC numbers were significantly reduced in irradiated mouse femurs and this reduction was rescued in ink4a/arf-deficient mice whereas OB-SC numbers were unaffected by irradiation. In addition, human MSCs derived from maxilla and mandibular trabecular bones (i.e. orofacial MSCs or OF-
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MSCs) were reported to be more radio-resistant than MSCs derived from iliac crest (i.e. IC-MSCs) [Damek-Propawa et al. 2010]. OF-MSCs induced higher p21 expression and harboured less DNA damage than IC-MSCs post IR treatment. Furthermore, injection of irradiated MSCs into nude mice demonstrated that bone formation by IC-MSCs in vivo was delayed in comparison with OF-MSCs [Damek-Propawa et al. 2010]. Taken together, these findings suggest that MSC sub-populations are likely to differentially execute the DDR following genotoxic insult in vivo.

As previously mentioned, the methods used to isolate and culture primary mouse MSCs currently hinder our ability to characterise the radiation response of distinct, purified mouse MSC sub-populations in vitro. Also, although the human MSC phenotype is maintained in vitro, extensive characterisation of human MSC populations directly isolated from irradiated BM is technically difficult. However, Cre-Rosa26-GFP knock-in reporter mouse strains used to identify and characterise specific MSC sub-populations in the BM, e.g. Nestin+ MSCs [Méndez-Ferrer et al. 2010], could provide a powerful tool for investigating MSC radio-biology in vivo. In these transgenic mice, normal ubiquitous expression of the Rosa26 locus, genetically modified to encode a fluorescent protein (e.g. GFP), is inactivated due to the presence of loxP-flanked stop sequence. Following tamoxifen treatment, activated Cre recombinase, whose expression is under the control of a cell type-specific promoter, deletes the loxP-flanked stop sequence, resulting in GFP expression in the targeted cell type. Therefore, this transgenic system enables one to identify, monitor and sort rare cell populations in vivo. It would be interesting to isolate MSC sub-populations from these transgenic mice at various time-points post irradiation to determine (i) whether IR affects MSC proportions over time; (ii) whether individual MSC sub-populations differ in radiosensitivity in vivo (iii) whether MSCs remain in the BM or transit to other tissues post IR.

To analyse the DDR of MSCs in vivo in more detail, one could sort MSC populations directly from irradiated BM of these transgenic mice and (i) analyse DNA DSB repair kinetics via γ-H2AX resolution using IF staining or flow cytometry; (ii) analyse cell cycle checkpoint kinetics using PI staining and (iii) analyse apoptosis using Annexin-V/PI staining. Furthermore, one could perform quantitative RT-PCR of DDR mRNA transcripts expressed in sorted control and irradiated MSCs and HSCs in vivo to determine (i) whether MSCs express high levels of pro-survival DDR genes in vivo and (ii) whether the DDR gene
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expression levels differ between MSC sub-populations. Interestingly, it was recently shown that irradiation stimulates the expansion of the osteoblast compartment in mouse BM which was suggested to be due to the proliferation of a currently un-identified osteogenic MSC population *in vivo* [Dominici *et al.* 2009]. Therefore, one could also label MSCs in transgenic mice with a proliferation marker, such as BrdU, to determine whether irradiation differentially affects the cycling status of MSC sub-populations *in vivo*. In addition, one could perform immuno-staining of bone, cartilage and adipose tissue of these mice to determine whether irradiated MSCs continue to differentiate into osteocytes, adipocytes and/or chondrocytes *in vivo*.

*In vivo* analysis of MSCs would also provide important insight into their contribution to tumourigenesis. Whether specific MSC sub-populations are recruited to and contribute to the tumour microenvironment is currently unknown. To address this, one could potentially engraft transgenic MSC sub-population(s) into lethally irradiated mice in which tumour initiation can be controlled, e.g. B16 melanoma or Lewis lung carcinoma mouse models. In this way, one could potentially monitor the influx of specific MSC sub-populations during tumour development and analyse their distribution and interactions with other cell types within the tumour microenvironment. Following this, similar to work performed on FAP*+* stromal cells [Kraman *et al.* 2010], one could engraft MSC sub-populations, genetically engineered to express the diphtheria toxin receptor (DTR), in the BM of a tumourigenic mouse model and subsequently analyse the effects of conditional deletion of these populations on tumourigenesis via diphtheria toxin administration. Furthermore, it was recently demonstrated that tumour irradiation stimulates the recruitment of transplanted MSCs into the tumour microenvironment [Klopp *et al.* 2007]. Therefore, using a mouse model in which endogenous MSCs can be tracked *in vivo*, it would also be interesting to determine whether tumour irradiation increases intra-tumoural recruitment of endogenous MSCs.

Genetic alterations in genome stability and cell cycle regulators (e.g. p53 and Rb) in MSCs can affect their ability to regulate haematopoiesis [Epperly *et al.* 1999; Walkley *et al.* 2007; Raajimakers *et al.* 2010]. In addition, irradiation stimulates MSCs to produce a variety of cytokines, chemokines, growth factors and ECM proteins including TNF-α, TGF-β, GM-CSF, IL-6, CXCL12, collagen and fibronectin, many of which are implicated in
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carcinogenesis and leukaemogenesis [Greenberger et al. 1996a & 1996b; Dominici et al. 2009]. Irradiation of MSCs also causes increased intracellular ROS levels in haematopoietic cells [Greenberger et al. 1996a]. Therefore, in addition to identifying radio-resistant MSCs in the BM and tumour microenvironments and characterising their DDR, it will also be important to determine (i) whether radio-resistant MSCs are genetically stable and (ii) whether their immuno-modulatory properties are altered post IR.

5.5 CONCLUSIONS

This study has demonstrated that irradiated mouse MSCs activate a robust DDR to IR-induced DNA DSBs in which multiple mechanisms synergistically promote MSC survival: (i) rapid DDR initiation; (ii) activation of DNA damage checkpoints; (iii) efficient DNA DSB repair and (iv) suppression of apoptosis. Alterations in this multi-faceted DDR, particularly in DNA DSB repair, enhances the radio-resistance of mouse MSCs in hypoxia and are mediated, at least in part, by HIF-1α. This detailed investigation into MSC radio-biology has important implications for therapeutic regimens used in allogeneic BMT and for our advancing our understanding of the tumour microenvironment. Continued investigation into MSC radio-resistance will have significant impact on our understanding of how MSCs (i) restore haematopoiesis; (ii) modulate the immune system and (iii) sustain cancer development in response to genotoxic stress.
Overall Discussion
# APPENDIX I: CELL LINES

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Cell type</th>
<th>Mouse strain</th>
<th>Source</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS5</td>
<td>MSC line</td>
<td>C3H/HeNSlc</td>
<td>Prof. Antonius Rolink, DBM, Basel</td>
<td>Adherent cell line isolated from γ-irradiated mouse bone marrow [Itoh et al., 1989] CD45^-CD44^+CD29^+</td>
</tr>
<tr>
<td>ST2</td>
<td>MSC line</td>
<td>BALB/c</td>
<td>Prof. Antonius Rolink, DBM, Basel</td>
<td>Adherent cell line isolated from non-irradiated foetal liver [Hardy et al. 1987] CD45^-CD44^+CD29^+</td>
</tr>
<tr>
<td>ST4.5</td>
<td>CD4^+ CD8^+ thymocyte line</td>
<td>BALB/c</td>
<td>Dr. Anne Wilson, Ludwig Institute of Cancer Research, Lausanne</td>
<td>Suspension thymoma cell line isolated from S-MuLV-infected mouse [Kemp et al. 1980] CD45^-CD4^-CD8^+, CD29^-CD44^-</td>
</tr>
<tr>
<td>J774A.1</td>
<td>Monocyte / macrophage cell line</td>
<td>BALB/cN</td>
<td>Prof. Benjamin Bradley, Dept. of Orthopaedics, University of Bristol</td>
<td>Adherent cell line isolated from murine reticulum cell sarcoma [Ralph et al. 1976] CD45^-FCGR1^-CD11b^-</td>
</tr>
<tr>
<td>NH HSC</td>
<td>HSC line</td>
<td>C57BL/6 CD3e^-</td>
<td>Prof. Antonius Rolink, DBM Basel</td>
<td>Suspension cell line isolated from bone marrow and genetically engineered to over-express NUP98-HoxB4 fusion protein [Ruedl et al., 2008; von Münchow, 2013] CD45^- c-kit^- Sca-1^+, Lin^-</td>
</tr>
<tr>
<td>NH HSC-Bel-2</td>
<td>HSC line</td>
<td>H-2K.BCL-2 transgenic</td>
<td>Prof. Antonius Rolink, DBM Basel</td>
<td>Suspension cell line isolated from bone marrow [Domen et al. 1998] and genetically engineered to over-express NUP98-HoxB4 fusion protein CD45^- c-kit^- Sca-1^+, Lin^-</td>
</tr>
</tbody>
</table>
## APPENDIX II: MEDIA

### GROWTH MEDIA

<table>
<thead>
<tr>
<th>Bulk MSC Growth Medium</th>
<th>Cell Line Growth Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMDM</td>
<td>High Glucose DMEM</td>
</tr>
<tr>
<td>FCS (10%)</td>
<td>FCS (10 %)</td>
</tr>
<tr>
<td>Equine serum (10%)</td>
<td>Penicillin/Streptomycin (1%)</td>
</tr>
<tr>
<td>Penicillin/Streptomycin (1%)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>DN2 Growth Medium</th>
<th>HSC Growth Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF-IMDM</td>
<td>SF-IMDM</td>
</tr>
<tr>
<td>FCS (5%)</td>
<td>FCS (5 %)</td>
</tr>
<tr>
<td>Ciproxin (0.2%)</td>
<td>Ciproxin (0.2%)</td>
</tr>
<tr>
<td>IL-7 (10%)</td>
<td>IL-6 (5%)</td>
</tr>
<tr>
<td>SCF (0.1 µg/ml)</td>
<td>SCF (0.1 µg/ml)</td>
</tr>
</tbody>
</table>
## APPENDIX II: MEDIA

### DIFFERENTIATION MEDIA

<table>
<thead>
<tr>
<th>Osteogenic Differentiation Medium</th>
<th>Adipogenic Induction Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-MEM</td>
<td>High glucose DMEM</td>
</tr>
<tr>
<td>Dexamethasone (100 nM)</td>
<td>Dexamethasone (1 μM)</td>
</tr>
<tr>
<td>Ascorbic acid-2-phosphate (50 μM)</td>
<td>Insulin (10 μg/ml)</td>
</tr>
<tr>
<td>β-glycerophosphate (20 mM)</td>
<td>Indomethacin (200 μM)</td>
</tr>
<tr>
<td>L-thyroxine (50 ng/ml)</td>
<td>3-Isobutyl-1-Methyl-Xanthine (500 μM)</td>
</tr>
<tr>
<td>FCS (10%)</td>
<td>FCS (10%)</td>
</tr>
<tr>
<td>Penicillin/Streptomycin (1%)</td>
<td>Penicillin/Streptomycin (1%)</td>
</tr>
</tbody>
</table>

### Adipogenic Maintenance Medium

- High glucose DMEM
- Insulin (10 μg/ml)
- FCS (10%)
- Penicillin/Streptomycin (1%)
**APPENDIX III: BUFFERS**

**BUFFERS**

<table>
<thead>
<tr>
<th><strong>10X ACK Buffer</strong></th>
<th><strong>Annexin-V binding buffer</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>NH$_4$Cl (1.5 M)</td>
<td>HEPES (10 mM), pH 7.4</td>
</tr>
<tr>
<td>NaHCO$_3$ (100 mM)</td>
<td>NaCl (1.5 M)</td>
</tr>
<tr>
<td>EDTA (10 mM)</td>
<td>KCl (5 mM)</td>
</tr>
<tr>
<td>H$_2$O</td>
<td>MgCl$_2$ (1 mM)</td>
</tr>
<tr>
<td>Adjust to pH 7.4</td>
<td>CaCl$_2$ (1.8 mM)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>DNA Loading Buffer</strong></th>
<th><strong>FACS Buffer</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl (10 mM), pH 7.6</td>
<td>PBS</td>
</tr>
<tr>
<td>Bromophenol blue (0.03%)</td>
<td>FCS (2%)</td>
</tr>
<tr>
<td>Xylene cyanol (0.03%)</td>
<td>Sodium azide (0.05%)</td>
</tr>
<tr>
<td>Glycerol (60%)</td>
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</tr>
<tr>
<td>EDTA (60 mM)</td>
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</table>

<table>
<thead>
<tr>
<th><strong>NHEJ Sample Buffer</strong></th>
<th><strong>PBST</strong></th>
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</thead>
<tbody>
<tr>
<td>dNTPs (0.25 mM)</td>
<td>PBS</td>
</tr>
<tr>
<td>Tris-acetate (25 mM), pH 7.5</td>
<td>Tween-20® (1%)</td>
</tr>
<tr>
<td>Potassium acetate (100 mM)</td>
<td></td>
</tr>
<tr>
<td>Magnesium acetate (10 mM)</td>
<td></td>
</tr>
<tr>
<td>DTT (1 mM)</td>
<td></td>
</tr>
<tr>
<td>ATP (1 mM)</td>
<td></td>
</tr>
</tbody>
</table>
APPENDIX III: BUFFERS

BUFFERS

10X Running Buffer
Tris (250 mM)
Glycine (1.92 mM)
SDS (2% w/v)

1X Running Buffer
10X Running Buffer (1 part)
H₂O (9 parts)

4X SDS Loading Buffer
SDS (12% w/v)
Tris pH 6.8 (300 mM)
EDTA (20 mM)
Glycerol (60% v/v)
Bromophenol blue (0.05% w/v)

1X SDS Loading Buffer
4X SDS Loading Buffer (6 parts)
β-mercaptoethanol (1 part)
H₂O (3 parts)

Sort Buffer
Ca²⁺/Mg²⁺-free PBS
FCS (1%)
HEPES (25 mM)
EDTA (2 mM)
H₂O
## APPENDIX III: BUFFERS

### BUFFERS

<table>
<thead>
<tr>
<th>10X TBS</th>
<th>TBST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl, pH 7.5 (200 mM)</td>
<td>1X TBS</td>
</tr>
<tr>
<td>NaCL (150 mM)</td>
<td>Tween-20® (1%)</td>
</tr>
<tr>
<td>KCl (16 mM)</td>
<td>H₂O</td>
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<table>
<thead>
<tr>
<th>10X Transfer Buffer</th>
<th>1X Transfer Buffer</th>
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</thead>
<tbody>
<tr>
<td>Tris (500 mM)</td>
<td>10X Transfer Buffer (1 part)</td>
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<tr>
<td>Glycine (400 mM)</td>
<td>Methanol (2 parts)</td>
</tr>
<tr>
<td>H₂O</td>
<td>H₂O (7 parts)</td>
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## APPENDIX IV: ANTIBODY PREPARATIONS

<table>
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<tr>
<th>ANTIBODY</th>
<th>SOURCE</th>
<th>Conditions used</th>
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<tr>
<td><strong>WESTERN BLOTTING</strong></td>
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<tr>
<td>anti-53BP1 rabbit polyclonal</td>
<td>Bethyl Laboratories</td>
<td>5% milk PBST 1:5,000</td>
</tr>
<tr>
<td>anti-ATM [2C1(1A1)] mouse monoclonal</td>
<td>Abcam</td>
<td>5% milk PBST 1:1,000</td>
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<tr>
<td>Anti-ATR (N-19) goat polyclonal</td>
<td>Santa Cruz Biotechnology</td>
<td>5% milk PBST 1:1,000</td>
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<tr>
<td>anti-β-Actin rabbit polyclonal</td>
<td>Sigma-Aldrich</td>
<td>5% milk PBST 1:10,000</td>
</tr>
<tr>
<td>anti-β-Tubulin rabbit polyclonal</td>
<td>Abcam</td>
<td>5% milk PBST 1:10,000</td>
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<td>anti-Bcl-2 (D17C4) rabbit monoclonal</td>
<td>Cell Signaling Technology</td>
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<tr>
<td>anti-BclXL (54H6) rabbit monoclonal</td>
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<tr>
<td>anti-Bim (C34C5) rabbit monoclonal</td>
<td>Cell Signaling Technology</td>
<td>5% BSA TBST 1:1,000</td>
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<tr>
<td>anti-Caspase-3 rabbit polyclonal</td>
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<td>5% BSA TBST 1:5,000</td>
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<td>anti-Chk2 rabbit monoclonal</td>
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<tr>
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<td>Abcam</td>
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<td>anti-phospho-Histone H2A.X (Ser139)</td>
<td>Millipore</td>
<td>5% BSA TBST 1:5,000</td>
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<td>anti-HIF-1 alpha rabbit polyclonal</td>
<td>Novus Biologicals</td>
<td>5% milk, TBST 1:4,000</td>
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<tr>
<td>ANTIBODY</td>
<td>SOURCE</td>
<td>Conditions used</td>
</tr>
<tr>
<td>-----------------------------------------</td>
<td>-------------------------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>anti-HIF-2 alpha rabbit polyclonal</td>
<td>Novus Biologicals</td>
<td>5% milk, TBST 1:1,000</td>
</tr>
<tr>
<td>anti-Ku70 [N3H10] mouse monoclonal</td>
<td>Abcam</td>
<td>5% milk PBST 1:1,000</td>
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<tr>
<td>anti-Mcl-1 rabbit antibody</td>
<td>Cell Signaling Technology</td>
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<td>anti-p21 rabbit polyclonal</td>
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<td>anti-Rad51 rabbit polyclonal</td>
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<td>Thermo Scientific</td>
<td>Antibody dependent</td>
</tr>
<tr>
<td>ImmunoPure® HRP-conjugated goat anti-rabbit IgG antibody</td>
<td>Thermo Scientific</td>
<td>Antibody dependent</td>
</tr>
<tr>
<td>IMMUNOFLUORESCENCE STAINING</td>
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<td>anti-phospho-Histone H2A.X (Ser139)</td>
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<tr>
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<td>Abcam</td>
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</tr>
<tr>
<td>(FITC)-conjugated AffiniPure F(ab’)2 Fragment goat anti-mouse IgG</td>
<td>Jackson Immunoresearch</td>
<td>1:180 5% FCS/PBS</td>
</tr>
<tr>
<td>Rhodamine Red®-conjugated AffiniPure F(ab’)2 Fragment goat anti-rabbit IgG antibody</td>
<td>Jackson Immunoresearch</td>
<td>1:180 5% FCS/PBS</td>
</tr>
<tr>
<td>ANTIBODY</td>
<td>SOURCE</td>
<td>Conditions used</td>
</tr>
<tr>
<td>----------------------------------------------------</td>
<td>------------------</td>
<td>-----------------------</td>
</tr>
<tr>
<td><strong>FLOW CYTOMETRY</strong></td>
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</tr>
<tr>
<td>Anti-BrdU mouse monoclonal antibody</td>
<td>BD Biosciences</td>
<td>1:20 PBS / 0.5% BSA / 0.1%</td>
</tr>
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<td>BD Biosciences</td>
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</tr>
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<td>PE-conjugated anti-mouse CD8a</td>
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<td>FITC-conjugated anti-CD25</td>
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<td>eBioscience</td>
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<td>BD Biosciences</td>
<td>1:50 FACS Buffer</td>
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<td>PE-conjugated anti-mouse CD44</td>
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<td>1:100 FACS Buffer</td>
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<td>Merck Millipore</td>
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<tr>
<td>FITC-conjugated anti-mouse IgG (whole molecule) antibody</td>
<td>Sigma-Aldrich</td>
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<td>FITC-conjugated AffiniPure F(ab')2 Fragment goat anti-rabbit IgG</td>
<td>Sigma-Aldrich</td>
<td>1:50 PBS / 1% BSA</td>
</tr>
</tbody>
</table>
APPENDIX V: PUBLICATIONS, PRESENTATIONS AND ACHIEVEMENTS

PUBLICATIONS

**Sugrue T, Lowndes NF & Ceredig Rh.** (2013) Hypoxia enhances the radio-resistance of mouse mesenchymal stromal cells. *In revision.*


**Sugrue T, Brown JAL, Lowndes NF & Ceredig Rh.** (2013) Multiple facets of the DNA damage response contribute to the radio-resistance of mouse mesenchymal stromal cell lines. *Stem Cells* 31: 137-145.


PRESENTATIONS

**Hypoxia enhances the radio-resistance of mouse mesenchymal stromal cells (Poster)** – International Society for Hematology and Stem Cells annual meeting, August 2013, Vienna, Austria.

**Hypoxia alters the DNA Damage Response of mouse mesenchymal stromal cells to γ-radiation treatment (Poster)** – Iberian Society of Cytometry meeting, June 2013, Porto, Portugal.

**DNA damage checkpoint activation contributes to the radio-resistance of mouse mesenchymal stromal cells (Oral)** - Irish Cytometry Society conference November 2012, Galway, Ireland.
The DNA Damage Response contributes to the radio-resistance of mouse mesenchymal stromal cells (Poster) - The Tumour Microenvironment EACR-IACR joint conference September 2012, Dublin, Ireland.

The DNA Damage Response contributes to the radio-resistance of mouse mesenchymal stromal cells (Poster) - Royal Academy of Medicine Ireland (RAMI) Biomedical Sciences conference June 2012, Galway, Ireland.

Characterisation of the DNA Damage Response of mouse bone marrow stromal and haematopoietic cell lines to ionising radiation (Oral & Poster) – Irish Society for Immunology conference September 2011, Galway, Ireland.

ACHIEVEMENTS

Thomas Crawford Hayes bursary awarded by the National University of Ireland (NUI) Galway (2013).

EMBO short-term fellowship for collaborative project between Regenerative Medicine Institute (Prof. Rhodri Ceredig), NUI Galway, Ireland & Department of Biomedicine (Prof. Antonius Rolink), Basel, Switzerland (2012).

Best Student Poster Prize at the Royal Academy of Medicine Ireland (RAMI) Biomedical Sciences conference, NUI Galway (2012).

Irish Research Council Government of Ireland Embark Postgraduate Scholarship in Science, Engineering and Technology (Grant No. RS20102702) (2010).
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Tong J, Kishi H, Matsuda T & Muraguchi A. (1999) A bone marrow-derived stroma cell line, ST2, can support the differentiation of fetal thymocytes from the CD4-CD8- double negative to the CD4+CD8+ double positive differentiation stage in vitro. Immunology 97: 672-678.


von Münchow AL. (2013) CD19 plays a crucial role in the selection of mature B cells. M.Sc. thesis: University of Basel (Switzerland) & University of Freiburg (Germany).


