NOVEL EFFECTS OF BISPHOSPHONATES ON STEM CELLS AND TISSUE REGENERATION

THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY BY

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विद्या ददाति विनयम् विनयाद्याति पात्रताम् । पात्रत्वा धनमाप्नोति धनात्धर्मम् ततः सुखम् ।।
(Vidya dadati vinayam, Vinaya dadati paatrataam, Paatratva dhanamaapnoti, Dhanaatdharmam tatah sukham)

From knowledge one attains discipline and humbleness, from humbleness one achieves worthiness. Worthiness brings fortune that helps one do a good deed and this culminates into ultimate happiness and joy. (-Hithopadesh)

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Publications

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Juhi Misra, F. Hal Ebetino, R. Graham G. Russell, Penelope D Ottewell and Ilaria Bellantuono. ***'Zoledronate protects from radiation induced mucositis'.***
(Manuscript in preparation)

Oral presentations

J Misra, ST Mohanty, S Madan, JA Fernandes, FH Ebetino, P Ottewell, RGG Russell and I Bellantuono '*Zoledronate delays stem cell ageing and protects them from radiation induced damage via mTOR signalling*' at Healthy Ageing: From Molecules to Organisms conference 2015 held at the Wellcome Trust Conference Centre, Hinxton Cambridge UK **(Bursary Award)**

J Misra, ST Mohanty, S Madan, JA Fernandes, FH Ebetino, P Ottewell, RGG Russell and I Bellantuono '*Non Skeletal effects of Zoledronate: Protection from radiation induced mucositis*' at the 5th Mellanby Centre for Bone Research Annual meeting 2014 held at University of Sheffield, UK

J Misra, ST Mohanty, S Madan, JA Fernandes, FH Ebetino, P Ottewell, RGG Russell and I Bellantuono '*Non Skeletal effects of Zoledronate: Protection from radiation induced mucositis*' at 6th International Workshop on Advances in the Molecular Pharmacology and Therapeutics of Bone and other Musculoskeletal Diseases 2014, held at University of Oxford, UK

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Poster presentation

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J Misra, ST Mohanty, S Madan, JA Fernandes, FH Ebetino, H Roehl, RGG Russell and I Bellantuono *'Can Bisphosphonates extend Life Span? Effects on Stem Cell survival, DNA Repair and Tissue Regeneration'* at the CIMA meeting 2013 held at University of Sheffield, UK

J Misra, ST Mohanty, S Madan, JA Fernandes, FH Ebetino, H Roehl, RGG Russell and I Bellantuono *'Can Bisphosphonates extend Life Span? Effects on Stem Cell survival, DNA Repair and Tissue Regeneration'* at the ASBMR meeting 2013 held at Baltimore, USA

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Summary

The integrity of most tissues is highly dependent on resident self-renewing stem cells, which are vulnerable to DNA damage due to physical and chemical insults thus compromising their repopulation capacity and maintenance of tissue integrity. Examples include accumulation of DNA damage with age or following radiotherapy and chemotherapy in cancer patients. Interventions that can reduce accumulation of DNA damage in normal stem cells and maintain tissue integrity may improve patient survival and quality of life. Reports on a robust but unexplained reduction in mortality in osteoporotic patients treated with zoledronate pointed to the question whether this effect was associated with an extension in stem cells' lifespan due to reduced accumulation of DNA damage. To test this hypothesis, human mesenchymal stem cells (hMSC, progenitors of osteoblast) that are known to undergo cellular ageing were used as a model.

Indeed, zoledronate extended hMSC lifespan and this was associated with reduced incidence of DNA double strand breaks. Pre-treatment with zoledronate followed by ionizing radiation also resulted in enhanced DNA repair with extended lifespan of hMSC compared to both irradiated and non-irradiated hMSC used as controls. It also significantly increased their clonogenic and differentiation ability compared to untreated controls. Moreover, administration of zoledronate in C57Bl/6 mice exposed to irradiation significantly reduced the loss of surviving MSC following whole body irradiation. In a model of zebrafish, zoledronate enhanced tail regeneration following irradiation and amputation. All these effects were mediated by inhibition of the mevalonate pathway and of the mammalian target of rapamycin (mTOR) signalling.

These effects were not restricted to hMSC. Zoledronate had similar effects in other stem cell types, such as the murine intestinal stem cells (ISC). Irradiation resulted in a significant reduction in the number of leucine-rich G-protein coupled receptor-5 (LGR5) ISC; however, this was rescued by administration of zoledronate prior to irradiation. Moreover, a significant reduction in accumulation of DNA damage foci was observed in LGR5 cells in mice treated with zoledronate prior to irradiation compared to irradiated controls and this resulted in preserved tissue integrity,

preventing radiation induced mucositis, an important side effect of radiotherapy in cancer patients.

Interestingly, zoledronate had differential action in cancer cells. Following irradiation, zoledronate was unable to enhance repair of DNA damage in myeloma, breast and prostate cancer lines and when combined with irradiation, zoledronate treatment resulted in significant decline in their clonogenic ability.

In conclusion zoledronate protected normal stem/progenitor cells (both mesenchymal and intestinal) from accumulation of DNA damage due to ageing and irradiation via mTOR signalling and had differential action in cancer cells. Given the good safety profile of zoledronate, this drug offers new therapeutic opportunities in cancer and ageing which may be readily exploited.

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Chapter 1: Introduction

1.1 Stem cells: embryonic and adult stem cells

Stem cells are defined as cells that have the ability to self-renew and can undergo differentiation process to form mature cells of finite life span that perform specialized functions, and that can reconstitute *in vivo* following transplant (Lakshmipathy and Verfaillie 2005). Such properties allow these cells to facilitate repair in case of injuries, thus making them much coveted in regenerative medicine. Stem cells can be isolated from different tissues and in principle be culture expanded and administered to patients for repairing injured tissues; however it is not possible to establish this in many cases. The challenges include the paucity of unique identification markers for isolation and lack of well-established culture conditions to facilitate expansion to meet sufficient numbers for clinical usage without loss of their stem cell abilities to self-renew and differentiate following expansion *in vitro* (Colman and Kind 2000, Stocum 2001). Therefore interventions that can facilitate long term expansion of these cells while retaining their stem cell abilities are much sought after to facilitate their usage in tissue repair.

Stem cells from vertebrates can be classified as **a) embryonic stem cells (ESC)** that are obtained from embryos, are pluripotent and can self-renew and differentiate to the 3 germ layers ectoderm, mesoderm and endoderm (Thomson, Itskovitz-Eldor et al. 1998, Wobus and Boheler 2005); **b) adult stem cells (ASC)** that are tissue specific, multipotent and can self-renew and differentiate into mature cells with specialised functions (Jiang, Jahagirdar et al. 2002). Both these cells are useful in regenerative medicine as they can carry out function of tissue repair following injury however there are several reasons for choosing adult stem cells over embryonic stem cells. The

primary advantage of ASC is that they are tissue specific and can be therefore obtained from patient's own tissue as opposed to ESC. This is important from the point of view of immune rejection which is a major problem with ESC following transplants as they may come from different donors (Fairchild, Robertson et al. 2005, Gardner 2007). Moreover ESC can give rise to teratomas if injected in an undifferentiated state (Amit, Carpenter et al. 2000, Reubinoff, Pera et al. 2000, Shih, Forman et al. 2007). In fact use of ESC also raises ethical concerns as opposed to ASC that are comparatively less controversial (Rippon and Bishop 2004).

Adult stem cells are tissue specific and they reside in specific microenvironment known as the 'niche' (first proposed by Schofield) which is a spatially defined anatomic structure responsible for regulating stem cell number, motility and commitment (Schofield 1978). These cells have been reported to exist in all possible tissues including the skin (Watt 2000), liver (Alison, Poulsom et al. 2000), brain (Gage 2000), fat tissue (Hawke and Garry 2001), kidneys (Rookmaaker, Verhaar et al. 2004), pancreas (Choi, Ta et al. 2004), bone marrow (Weissman 2000) and intestines (Whitehead, Demmler et al. 1999, Potten, Booth et al. 2003, Barker and Clevers 2010, Barker and Clevers 2010).

1.1.1 Stem cells and tissue homeostasis

In the tissue which is composed of mature cells with specialised function; tissue-specific stem cells play an important role in maintenance, repair and tissue regeneration. Essentially the stem cells replace cells that were lost to the homeostatic turnover or due to injury (caused by various extrinsic and intrinsic factors) or in a disease state (Weissman 2000). Tissue homeostasis is the maintenance of the above status quo. There are two proposed cellular mechanism by which stem cells maintain

homeostasis (Watt and Hogan 2000). A stem cell population may behave such that on average, 50% of their progeny are replicas of these stem cells and the remaining 50% are differentiating cells, a process defined as 'population asymmetry'(Klein and Simons 2011). This may be a cell autonomous property or may be controlled by external regulators such as the restricted size of niche which allows a stem cell to divide only when a differentiated cell leaves the niche. Alternatively a single stem cell may undergo 'division asymmetry' giving rise to one daughter cell and one differentiated cell that may further divide (Klein and Simons 2011). However lineage tracing and live imaging reveal a higher dominance of population asymmetry in tissues that have high rates of turnover (Fig. 1.1A)(Alcolea and Jones 2013, Rompolas and Greco 2014). Examples of tissue systems with high turnover rate include the skin, intestinal tissues and blood wherein throughout the life span of individual, adult stem cells have to produce large number of mature cells to maintain tissue functions.

Squamous epithelia of skin, mouth and oesophagus consist of layers of keratinocytes (Fig. 1.1B). Proliferation is confined to the deepest basal layer that forms the stem cell niche (Alcolea and Jones 2014). Lineage tracing experiments reveals that a single population of keratinocyte stem cells contribute to homeostasis by equal divisions resulting in equal number of stem cells and differentiating cells (Fig.1.1B) (Clayton, Doupe et al. 2007, Doupe, Klein et al. 2010, Doupe and Jones 2012, Lim, Tan et al. 2013). This is an example of 'population asymmetry' however whether this is governed by cells intrinsic property or a consequence of external regulations is not known (Watt and Hogan 2000, Clayton, Doupe et al. 2007, Jones and Simons 2008).

Intestinal epithelium also has a rapid turnover (Fig. 1.1C). Homeostasis achieved in this case is by linking differentiation to self-duplicating stem cells that divide within a

niche of restricted size. The differentiated cells are shed from the villus tips quite rapidly and replacement is achieved by stem cells and progenitor cells residing in the base of the crypts (Clevers and Bevins 2013).

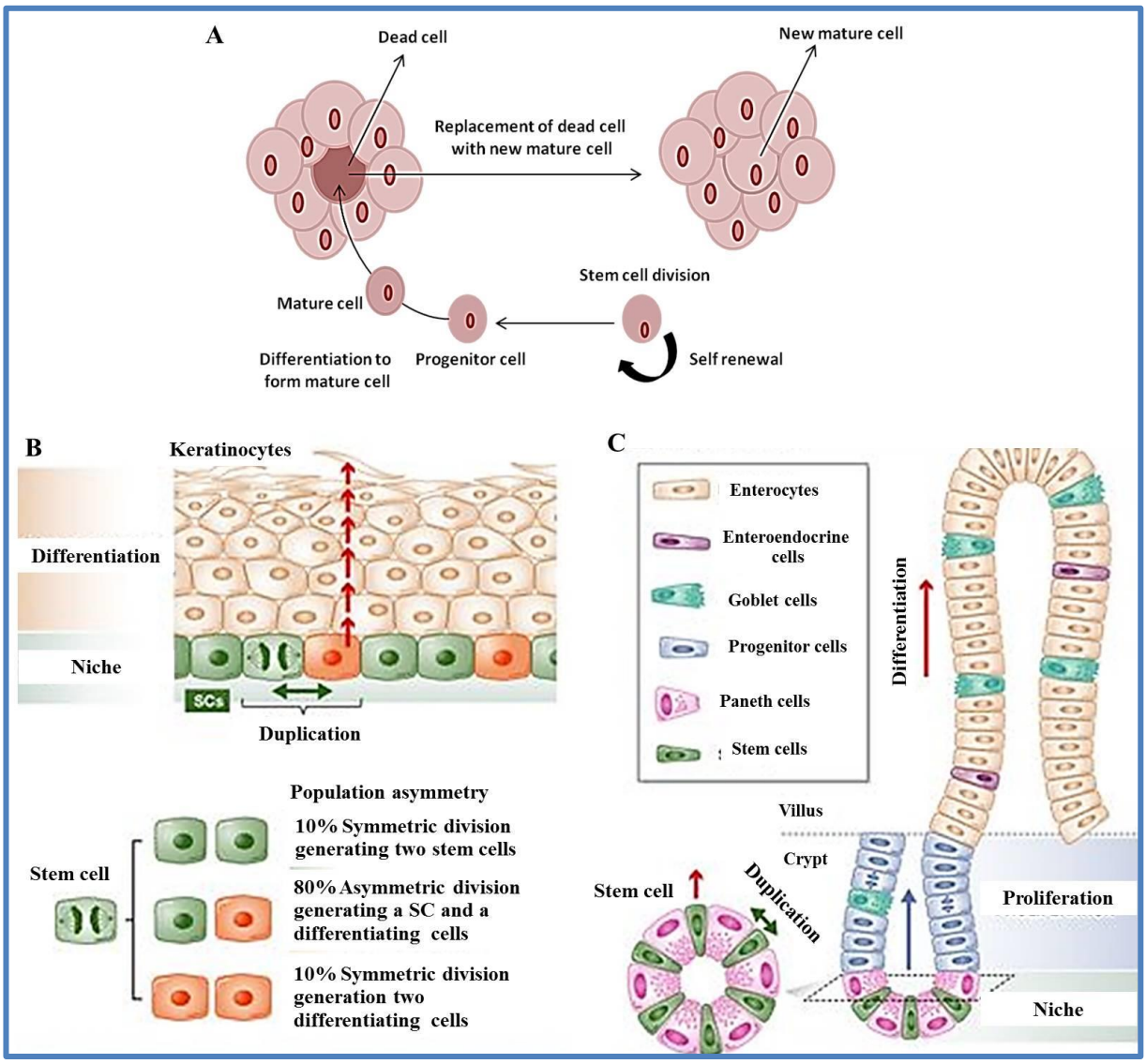


Figure 1.1 Tissue homeostasis

A) Cells (red) at end of life span gets eliminated and replaced by healthy cell which arises as a result of stem cell (SC) proliferation and differentiation to mature cells. B) Squamous epithelia consist of layers of keratinocytes, SCs (green) reside in the basal cell layer, along with post-mitotic cells waiting to stratify (red). When a differentiating cell leaves the niche (arrow), a nearby SC divides with one of the three division outcomes shown to maintain constant cell density in the niche. The probabilities of each outcome (expressed as per cent, oesophageal epithelium shown) are balanced, so equal numbers of SCs and differentiated cells are produced across the population. C) Intestinal epithelium contains four lineages sustained by SCs (green) that lie between Paneth cells in the crypt base. Differentiating cells migrate through a progenitor compartment in the upper crypt from which post-mitotic cells populate the villus, from which they are shed. Inset shows a simplified top-down view of the niche. As a differentiating SC exits the niche, it is replaced by the self-duplicating division of an immediately adjacent SC. (Wabik and Jones 2015).

These cells express leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5) and maintain four lineages of differentiated cells via the lineage committed transit amplifying progenitor cells (Barker, van Es et al. 2007). Lineage tracing studies reveal that as a stem cells differentiate, they migrate upwards to form the transit amplifying cells, at this point the neighbouring stem cell self-duplicates and is maintained by adjoining Paneth cells (Fig. 1C) (Lopez-Garcia, Klein et al. 2010, Snippert, van der Flier et al. 2010, Sato, van Es et al. 2011).

In case of maintenance of adult haematopoiesis, nearly $2X10^{11}$ erythrocytes and 10^{10} white blood cells are replaced each day and this constant renewal of the blood through the production of new blood cells is carried out by haematopoietic stem cells (HSC) (Harrison's Principles of Internal Medicine 17e, 2009). These haematopoietic stem cells that reside in the bone marrow share this niche along with another adult stem cell called the mesenchymal stem cells (MSC) which are known to differentiate to cells of bone, fat and cartilage and help maintain stroma to support haematopoiesis and maintain tissue homeostasis. In this thesis the primary focus is on these mesenchymal stem cells that hold great potential in therapy of bone regeneration and maintenance of HSC.

1.2 Mesenchymal Stem Cells (MSC)

Mesenchymal stem cells are adult stem cells that primarily are known to reside in the bone marrow but are easy to isolate from many other tissues such as adipose tissues (Gronthos, Franklin et al. 2001), peripheral blood (Zvaifler, Marinova-Mutafchieva et al. 2000), fetal liver (Campagnoli, Roberts et al. 2001), umbilical cord (Kim, Kim et al. 2004), amniotic fluid and chorionic villi of placenta (Igura, Zhang et al. 2004), lungs(in 't Anker, Noort et al. 2003) and teeth (Miura, Gronthos et al. 2003). In 1976, studies

conducted by Friedenstein et al (1976) led to identification and characterization of this population of adult multipotent stem cells. These cells have the ability to self-renew or proliferate and differentiate (Friedenstein, Chailakhjan et al. 1970, Friedenstein, Gorskaja et al. 1976). The universal criteria proposed by 'The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy' for identifying MSC (Dominici, Le Blanc et al. 2006) were established as follows:

a) Plastic Adherent Cells: MSC are phenotypically identified as spindle shaped cells that adhere to plastic and are capable of forming fibroblast colonies (Colony forming unit-fibroblasts or CFU-F) (Friedenstein, Chailakhjan et al. 1970, Friedenstein, Gorskaja et al. 1976).

b) Cluster of Differentiation (CD) Markers: Cluster of differentiation (CD) are identifiers and distinguishers of cell types based on cell surface molecules such as receptors or ligands ('+' is presence of antigen, '-' is absence of antigen).The absence of cell membrane protein CD45 (marker of haematopoietic cells) and CD31 (marker of endothelial cells) is employed to identify MSCs. Cells identified as MSC must express CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR (class II) surface molecules (Dominici, Le Blanc et al. 2006).

c) Differentiation ability: MSC have the ability to differentiate to multiple lineages including osteoblasts, chondrocytes and adipocytes that give rise to bone, cartilage and fat respectively as has been demonstrated through several studies (Jaiswal, Haynesworth et al. 1997, Pittenger, Mackay et al. 1999, Barry, Boynton et al. 2001).

Experimental evidence suggests a stepwise model of MSC differentiation in which the stem cells commit to either osteochondro or osteoadipo progenitors before

undertaking differentiation to osteoblasts, adipocytes and chondrocytes (Fig 1.2). This is based on in vitro studies by Muraglia et al (2000) who suggested that MSC cultures

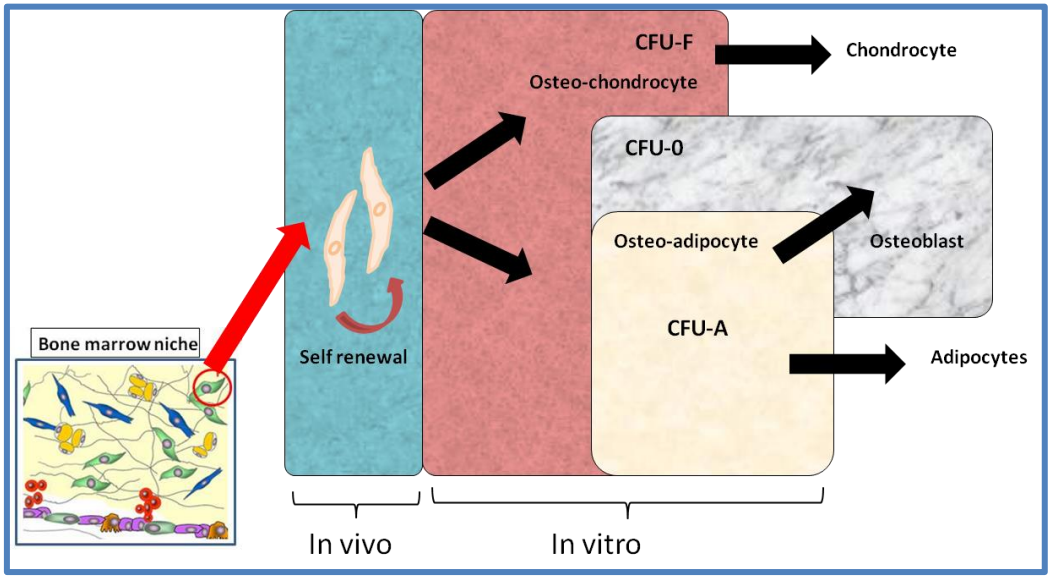


Figure 1.2 Proposed model of MSC differentiation and assays

MSC derived from the bone marrow undergo differentiation to osteo-adipo and osteo-chondro lineage that give rise to the mature cells osteoblasts, adipocytes and chondrocytes. The colony forming unit-fibroblast (CFU-F) assay reflects the clonogenecity of bone marrow stromal cells. Assays such as the colony forming unit-osteoblast (CFU-O) and colony forming unit-adipocyte (CFU-A) are employed to assess the number or progenitors with osteogenic and adipogeinc potential.

are heterogenous. Clonal analysis showed that one third of the MSC clones have the potential to differentiate into all the three lineages while about a third of clones was able to differentiate to either in osteogenic and chondrogenic but not adipogenic (osteo-chondro) or osteogenic and adipogenic but not chondrogenic (osteo-adipo) lineage. Moreover a third was able to differentiate only in one lineage. This suggests that not all cells have equal potential and at least upon long term culture, some clones with tri-potent ability lose their adipo- and chondro- differentiation capacity (Muraglia, Cancedda et al. 2000).

It is important to mention that set of criteria defined initially for identification of MSC have been severely criticized by Bianco et al (2013). This is because of the very limited

number of assays that retrospectively enumerate and characterize properties of stem cells may indeed not be reflective of true stem cells and may be the progenitor cells (Bianco, Cao et al. 2013). Moreover limitations in identifying markers detected in vitro and the potential to differentiate towards the osteoblast, adipocyte and chondrocyte may not necessarily facilitate a striking distinction between the progenitor cells from true stem cells. This is because even the progenitor cells exhibit the potential to proliferate and differentiate in vitro. Therefore for true identification of stem cells, these cells should have the ability to regenerate tissue *in vivo*. This ability helps define and distinguish a stem cell from progenitors that are incapable of long term tissue regeneration.

1.2.1 Assays to assess MSC

The most common assay to assess the number of MSC is based on clonogenicity. This is assessed by the ability of these cells to generate fibroblast like cell colonies from a single cell and is called colony forming unit fibroblast (CFU-F) assay. The colony forming unit-osteoblast (CFU-O) and colony forming unit-adipocyte (CFU-A) assays are employed to assess the number of progenitors with osteogenic and adipogenic potential (Fig 1.2). Although the populations detected by these assays overlap they are not the same. For example in Gamberdella et al (2011) the response to a small molecule activating the Wnt pathway is different for CFU-F and CFU-O (Gambardella, Nagaraju et al. 2011). Although clonogenic assays are used routinely, such assays do not necessarily distinguish between the number of progenitors and the number of primitive stem cells capable of tissue regeneration in response to injury or transplantation (Gronthos, Brahim et al. 2002, Bianco, Kuznetsov et al. 2006). More over cells *in vitro* may lose their stemness. Indeed it was suggested that

approximately only 10% of CFU-F were able to form a myelo-supportive stroma, bone and adipose tissue following *in vivo* ectopic transplantation in immunocompromised mice (Bianco, Kuznetsov et al. 2006).

As mentioned earlier *in vivo* transplantation is the best way to assess stem cell properties. The best assay available for *in vivo* purposes is the ectopic bone formation assay. In ectopic bone formation assay MSC are implanted under the skin of mouse in a porous scaffold and after 5 to 6 weeks ossicles are removed and assessed for areas of bone formation including host hematopoiesis. This allows one to determine whether cells have the ability to undergo differentiation and form hematopoiesis stroma (Daga, Muraglia et al. 2002). Alternatively MSC that are labelled with enhanced green fluorescent protein (eGFP) can be transplanted *in vivo* directly into the bone marrow, which is their site of origin by intra bone transplantation method (Muguruma, Yahata et al. 2006). Ideally to test long term repopulating capacity of MSC, a secondary transplant is necessary which involves re-isolating cells from primary transplant and re-injecting them to a secondary host.

Evidence of the existence of this stem/progenitor cell population of MSC comes from two important studies by Sacchetti et al (2007) and Mendez-Ferrer et al (2010). In the first study, MSC characterized by expression of melanoma associated adhesion protein (MCAM) and CD146 (population of cells lying close to sinusoids and HSC) were transplanted in immune compromised mice. These MCAM/CD146+ cells were able to generate heterotropic ossicles and on re-isolation gave rise to CFU-Fs suggesting the capability of these cells to function as MSC *in vivo* (Sacchetti, Funari et al. 2007). In the second study, Mendez–Ferrer et al. identified a population of murine stromal cells that expressed nestin and these cells were able to give rise to secondary

haematopoietic ossicles 8 months after transplant of nestin+ isolates from primary recipient mice, thus illustrating self-renewal potential of these cells. These cells were also capable of multi-lineage differentiation (Mendez-Ferrer, Michurina et al. 2010). These studies emerge as evidences in support of the existence of MSC that can self-renew and function as true stem cells.

Several surface markers of MSC have been defined and proposed to facilitate in identification of MSC and validating them through functional assays. These are indeed a panel of markers that are specific to other cell types but not specific to MSC and therefore help in negative selection of MSC. Cultured MSC are negative for CD45 (haematopoietic marker) and CD31 (endothelial marker) (Pittenger, Mackay et al. 1999, Jones, Kinsey et al. 2002). They are positive for CD105 (endoglin, also expressed by endothelial cells), CD29, CD44, SH2 (also expressed by fibroblasts), SH3 and STRO-1 (Pittenger, Mackay et al. 1999, Deans and Moseley 2000, Jones, Kinsey et al. 2002, Zhou, Huang et al. 2003). Despite the availability of MSCs with these specific markers, no stringent evaluations have been carried out to assess the regeneration potential of these cells *in vivo* (Bellantuono, Aldahmash et al. 2009). Table 1.1 shows the general phenotypic surface markers of MSC.

MSC cell surface marker	Status	Reference
CD45,CD34, CD14, CD11, Lin	Negative	(Pittenger, Mackay et al. 1999, Deans and Moseley 2000, Zhou, Huang et al. 2003, Horwitz, Le Blanc et al. 2005, Kassis, Zangi et al. 2006, Tormin, Li et al. 2011)
CD146 (MCAM)	Positive	(Sacchetti, Funari et al. 2007)
CD44	Positive	(Pittenger, Mackay et al. 1999, Zhou, Huang et al. 2003)
CD10, CD13	Positive	(Jones, Kinsey et al. 2002)
CD73 (SH3/SH4)	Positive	(Martinez, Hofmann et al. 2007)
CD90	Positive	(Tormin, Li et al. 2011)
CD105	Positive	(Jones, Kinsey et al. 2002, Tormin, Li et al. 2011)
CD271	Positive	(Tormin, Li et al. 2011)
NG2	Positive	(Kozanoglu, Boga et al. 2009)
STRO-1	Positive	(Simmons and Torok-Storb 1991, Jones, Kinsey et al. 2002, Tormin, Li et al. 2011)
LNGFR	Positive	(Jones, Kinsey et al. 2002, Quirici, Soligo et al. 2002)
HLA-DR	Positive	(Jones, Kinsey et al. 2002)
D7-FIB	Positive	(Martinez, Hofmann et al. 2007)
CD49-a	Positive	(Deschaseaux, Gindraux et al. 2003, Tormin, Li et al. 2011)
CD140b(PDGFβ)	Positive	(Buhring, Treml et al. 2009, Tormin, Li et al. 2011)
MHC Class-1	Positive	(Young, Steele et al. 1999)

Table 1.1 Surface markers of MSC

1.3 Therapeutic applications of MSC

The multi-lineage differentiation potential of MSC and the ease of their isolation from multiple tissues make them a very attractive candidate for therapeutic applications. Potential application of MSC has been reviewed in a wide variety of disorders. These include the haematopoietic system (Pontikoglou, Deschaseaux et al. 2011, Bernardo, Pagliara et al. 2012); bone (Kagami, Agata et al. 2011); neurological disorders(Joyce, Annett et al. 2010, Uccelli, Laroni et al. 2011); kidney (Wise and Ricardo 2012); cardiovascular system (Bernstein and Srivastava 2012); autoimmune disorders (Yi and Song 2012) and cancers (Dwyer, Khan et al. 2010, Shah 2012). The two major applications focused on are the use of MSC in correction of bone and cartilage defects and their role in supporting the haematopoiesis.

1.3.1 Role of MSC in correction of bone and cartilage defects

Mesenchymal stem cells differentiate to cells of mesenchymal lineage such as osteoblasts and chondrocytes thus they have great importance as candidates in therapies to regenerate bone and cartilage tissues. Culture expanded autologous MSC have been successfully loaded on bio-ceramic scaffolds and implanted in sites of fractures in patients with large bone fractures. Results from the follow up for 7 years showed integration of the implants within the host bones accompanied with new bone formation. In fact regenerated bones were highly durable, despite the occurrence of cracks in the implants (bio-ceramic disintegration) and successful functional recovery of the limbs was achieved (Quarto, Mastrogiacomo et al. 2001, Marcacci, Kon et al. 2007).

Genetic modifications of MSC to enhance fracture healing have been studied as well with promising outcomes especially in genetically engineered MSC that over express bone morphogenetic protein (BMP2). These modified MSC were shown to heal bone defects in a rat model although the mechanism of repair is unclear (Peterson, Iglesias et al. 2005). Other studies have suggested that MSC have both autocrine and paracrine mechanisms that mediate repair of tibial fracture especially when MSC overexpressing insulin like growth factor-1 was transplanted (Granero-Molto, Myers et al. 2011).

One of the newer strategies using MSC to induce osteogenic differentiation at bone surface was demonstrated by using ligand LLP2A to the bisphosphonate alendronate (Ale) to target Alpha-4 Beta-1 (α4β1) integrin expressed on MSC undergoing osteoblast differentiation(Guan, Yao et al. 2012). Alendronate affinity to bone directed the hMSC to bone surface and resulted in increase in endosteal, trabecular

and periosteal bone formation. These strategies hold promise in potential treatment of conditions like osteoporosis and repair of fractures.

MSC have also been used in correcting inherited disorders such as the osteogensis imperfecta (OI) characterized by osteopenia, bone deformities, multiple fractures and short stature due to defects in collagen type I. MSC transplants have shown to reduce fractures and increase strength of bones *in vivo* (Pereira, O'Hara et al. 1998, Le Blanc, Gotherstrom et al. 2005, Wang, Li et al. 2006, Guillot, Abass et al. 2008, Panaroni, Gioia et al. 2009). Treatment of pediatric OI cases with allogenic bone marrow cells or MSC conducted by Howritz et al (1999, 2001, 2002) showed improvements in bone structure, mass and mineral content including reduced rates of fractures (Horwitz, Prockop et al. 1999, Horwitz, Prockop et al. 2001, Horwitz, Gordon et al. 2002). However these beneficial effects were only short lived and did not sustain long enough (Horwitz, Prockop et al. 2001). In further follow up studies, the effect of MSC therapy were short lived and lasted for 3-6months (Horwitz, Gordon et al. 2002). These short lived effects could be attributed to the clinical benefits of the cytokines that MSC secrete as opposed to the proliferation and differentiation of these transplanted MSC themselves (Horwitz and Dominici 2008, Meirelles Lda, Fontes et al. 2009, Otsuru, Rasini et al. 2011, Otsuru, Gordon et al. 2012). These studies highlight the need for higher engraftment of MSC with their proper functioning and survival especially in target tissues for therapeutic success.

MSC have also been investigated in treatment of femoral head necrosis where autologous MSC that were eGFP labelled demonstrated better survival, proliferation, increased differentiation to osteoblasts with significant increase in trabeculae bones in the surgically induced necrotic femoral heads in dogs (Yan, Hang et al. 2009). Other

animal studies also successfully demonstrated use of MSC in the treatment of full thickness articular cartilage defects(Wakitani, Goto et al. 1994, Yan and Yu 2007) including a few clinical reports in treating defects in human patellae (Wakitani, Mitsuoka et al. 2004, Wakitani, Nawata et al. 2007).

Despite the promising results from clinical trials , it is still unknown what factors govern the survival and proliferation of MSC and their ability to differentiate after systemic delivery and more importantly how can higher level engraftment be achieved to meet specific clinical needs.

1.3.2 Role of MSC in supporting haematopoiesis

Both MSC and HSC reside in the bone marrow. MSC are known to provide the specialized environment (supporting stroma) for controlling and maintaining the process of hematopoiesis by maintaining the HSC growth and differentiation. Osteoblasts have also shown to be involved in supporting the survival and proliferation of long term repopulating stem cells and also in regulating hematopoietic stem cell niche. These osteoblasts are differentiated from MSC thus highlighting the importance of these cells in HSC maintenance (Calvi, Adams et al. 2003, Zhang, Niu et al. 2003).

Mendes-Ferrer et al (2010) through their studies indicate the existence of a unique MSC-HSC pairing niche demonstrated that Nestin+ MSC serve as niche component for HSC and co-localize with HSC in bone marrow. In their study they also found that administration of parathormone increased the osteoblast differentiation of Nestin+ cells along with their doubling. Further depletion in Nestin+ cells by inter-crossing the Nes-Cre^{ERT2} mice with Cre-recombinase-inducible diphtheria toxin receptor line (iDTR)

found to rapidly reduce HSC activity thus suggesting that Nestin+ cells are required for HSC maintenance in bone marrow(Mendez-Ferrer, Michurina et al. 2010).

Survival, proliferation and differentiation of HSC requires several cytokines such as the interleukin (IL)-6, IL-11, macrophage colony stimulating factor (M-CSF) and leukemia inhibitory factor (LIF). Most of these have been identified to be secreted by MSC (Majumdar, Thiede et al. 2000, Wang, Liu et al. 2000, Li and Wu 2011). In fact certain MSC also express certain antigens (CD166, CD54, CD106), chemokines(SDF-1/CXCL12) and integrins (CD49d/α4 integrin,CD29/β1 integrin, CD11/β2 integrin) that are of great relevance in the proliferation, homing, migration and adhesion of HSC (Pittenger, Mackay et al. 1999, Deans and Moseley 2000, Sugiyama, Kohara et al. 2006) (Majumdar, Keane-Moore et al. 2003). These studies evidence the important of MSC in the maintenance and support of the HSC niche. Hence use of MSC in hematological malignancies treatment, especially to enhance the post transplantation recovery of HSC has been investigated with great interest.

MSC differentiate to osteoblasts that have been shown to support hematopoiesis (Zhang, Niu et al. 2003). Studies by Lymperi et al (2008) have demonstrated that a certain subset of osteoblasts (N Cadherin+) was necessary to support the expansion of HSC *in vivo* and when these N cadherin + osteoblasts were blocked by using strontium relanate in mice, the expansion of HSC and their functioning was hampered (Lymperi, Horwood et al. 2008). Even in studies by Dominici et al (2009), an increase in N cadherin + osteoblasts was observed following irradiation during the restoration phase of the bone marrow. In fact following transplantation in these irradiated mice, the GFP labelled bone marrow cells were detected at the sites of N Cadherin + cells suggesting the roles of osteoblasts in HSC maintenance(Dominici, Rasini et al. 2009).

Irrespective of the cell type that may support HSC, *in vivo* studies have revealed that co-transplantation of MSC with HSC facilitate HSC engraftment (Almeida-Porada, Flake et al. 1999, Muguruma, Yahata et al. 2006). Co-transplant of HSC with MSC has also shown improved efficiency of HSC transplant in cases when HSC numbers are either low (using cord blood) or there is damage to the microenvironment (due to radiation/chemotherapy). Treatment of various diseases requires the use of detrimental doses of radiation or chemotherapy that results in intensive damage to the bone marrow stroma ultimately leading to defects in the osteogenic development and the hematopoiesis (Banfi, Bianchi et al. 2001, Banfi, Podesta et al. 2001). In vitro studies using etoposide treatment to cause damage to the stromal layer was rescued by transplanted MSC (Carrancio, Blanco et al. 2011). In fact the *in vivo* studies by Carrancio et al (2011) demonstrated that following etoposide treatment that severely damaged the bone marrow stroma, co-transplant of hMSC with HSC resulted not only in engraftment of MSC and their differentiation but also enhanced donor derived hematopoietic cell proliferation, possibly due restoration of stroma. Studies by Muguruma et al (2006) have also shown that co-transplant of eGFP MSC with HSC in to NOD/SCID mice resulted in a two fold increase in CD45+ cells (Muguruma, Yahata et al. 2006). In studies by Maitra et al (2004), 8 out of 10 mice showed improved engraftment when MSC were co-transplanted with umbilical cord blood cells(Maitra, Szekely et al. 2004).

In a Phase I clinical trial of 15 patients with hematological malignancies, the successful use of intravenously infused MSC derived from bone marrow have demonstrated the safety of these cells in use for therapeutic purposes (Lazarus, Haynesworth et al. 1995). In fact culture expanded autologous MSC have also demonstrated to be

therapeutically beneficial in breast cancer patients to enhance their hematopoietic recovery following treatment with high dose chemotherapy and autologous hematopoietic stem cells transplant (Koc, Gerson et al. 2000). Although an enhanced recovery was observed in these patients without any toxicity, the claims made for reconstitution of hematopoietic compartment was made in comparison to controls from older cases, hence more robust randomized controlled trials are required to establish the true therapeutic benefits of MSC on recovery of the HSC compartment. However this study has been able to establish the feasibility in using the *ex vivo* expanded MSC in aiding improvement in outcomes of hematopoietic stem cells transplantations. This has also paved the way to other trials. Meuleman et al (2009) have shown an improved hematopoietic recovery in 2 out of 6 patients with MSC transplantations alone (Meuleman, Tondreau et al. 2009). In another study as well, a patient with severe idiopathic aplastic anemia had modest improvements in hematopoiesis upon allogenic co-transplants with MSC (Stute, Fehse et al. 2002). Although all the data from published literature are very encouraging, no reports from phase III clinical trials have been generated and therefore long term follow up studies with appropriate controls are much warranted. Moreover the use of MSC in therapy also faces some challenges that are highlighted in the next section.

1.3.3 Challenges in MSC therapeutic applications

One of the major challenges of MSC therapy is the inability to acquire high levels of engraftment when delivered systemically (Horwitz, Prockop et al. 2001, Horwitz, Gordon et al. 2002). Because of low level of engraftment, the positive effect of this therapy disappears and is short lived. It is therefore imperative to understand the mechanisms and molecular cues that are associated with survival and functioning of

these cells in the host. Moreover, investigations are needed to determine whether the resilience of the host MSC can be boosted in situ.

Poor engraftment can also be attributed to inefficient migration and homing to target tissue due to lack/low levels of expression of cell surface adhesion molecules such as the receptor C-X-C chemokine receptor type 4 (CXCR4) that is used by stem cells for their migration (Brenner, Whiting-Theobald et al. 2004, Kahn, Byk et al. 2004) and whose expression is lost following in vitro expansion (Ploemacher and Brons 1989, Ploemacher 1997, Rombouts and Ploemacher 2003, Wynn, Hart et al. 2004).

MSC are also known to undergo cellular ageing following culture expansion that result in reduced proliferative capacity as the cells age (Banfi, Bianchi et al. 2002, Ksiazek 2009). With increasing passage numbers there is a decline the heterotropic bone formation capacity of MSC indicating the poor quality of the cells with age (Agata, Asahina et al. 2010). Moreover, complications with systemic delivery of MSC such as entrapment in the lungs or even non-specific in other organs have been reported (Allers, Sierralta et al. 2004, Mouiseddine, Francois et al. 2007).

In vitro cultured MSC lose their stemness and previous studies on hMSC have shown that following expansion, hMSC undergo cellular ageing (described in detail in the next sections) that has limiting effects on their proliferation and differentiation abilities (Baxter, Wynn et al. 2004, Mohanty, Cairney et al. 2012). Since expansion of these cells is important for generating sufficient numbers for use in therapy, therefore understanding underlying mechanisms and interventions to prevent the loss of stemness are much needed.

1.4 Cellular ageing leading to loss of tissue homeostasis

Stem cells are important in maintenance of tissue homeostasis. Despite the potential of stem cells to maintain tissue integrity and function, ageing is inexorable and characterised by loss of tissues homeostasis with gradual impairment of normal biological functions, decreased ability to respond to stress/injury, increased susceptibility to disease and tissue dysfunction and increased rates of mortality (Kirkwood 2005, Hayflick 2007, Kirkwood and Shanley 2010). It has been identified in a number of tissues including blood, skeletal muscle, skin and brain among others that ageing results in imbalanced homeostatic and regenerative capacities that are maintained by resident adult stem cells, therefore age related changes are clear reflections of a declining ability of stem cell function (Table 1.2)(Bell and Van Zant 2004, Dorshkind, Montecino-Rodriguez et al. 2009, Dorshkind and Swain 2009, Jones and Rando 2011).

Cellular ageing can be characterised by accumulation of damaged macromolecules such as the proteins and DNA (Deoxyribose nucleic acid). Protein damage resulting in 'proteotoxicity' may also attribute to normal cellular ageing and age related diseases (e.g. neurodegenerative diseases) wherein replacement of mis-folded or damaged protein is much slower resulting in aggregate formations that are eventually not degraded by the cell (Douglas and Dillin 2010, Koga, Kaushik et al. 2011)

Population of Stem Cells	Frequency of Stem Cells	Proliferation Ability	Differentiation Ability	Other Defects	References
Haematopoietic	Increase in long lived mouse strains Decrease in short lived mouse strains	Increase in cycling, Decrease in self-renewal	Decrease in lymphoid, Increases in myeloid	Decrease in homing, mobilization and engraftment	(Morrison, Wandycz et al. 1996, de Haan, Nijhof et al. 1997, Chen, Astle et al. 2000)
Neural	Decrease in lateral ventricle SVZ Decrease in dentate gyrus subgranular layer	Decrease in cycling, Decrease in self-renewal	Decrease in neurogenesis Increase in gliogenesis		(Kuhn, Dickinson-Anson et al. 1996, Maslov, Barone et al. 2004, Molofsky, Slutsky et al. 2006)
Muscle	Decrease in satellite cells associated with muscle fibers	Decreases in proliferation	Decrease in Myogenic, Increase in fibrogenic and adipogenic		(Conboy, Conboy et al. 2003, Conboy, Conboy et al. 2005)
Melanocyte stem cells in hair bulge	Decrease in melanocyte stem cells		Increase in terminal differentiation of melanocytes		(Nishimura, Granter et al. 2005)

Table 1.2 Age related changes in various mammalian stem cell populations Adapted from (Signer and Morrison 2013)

Moreover abnormal modifications of proteins by a process of glycation resulting in 'advanced glycation end products' also result in proteotoxicity and are known to contribute to many age associated pathologies such as diabetes, atherosclerosis (Ahmed 2005). Apart from protein, DNA can be modified due to the accumulated free radicals and reactive molecules that are usually produced by normal cellular metabolism and function but due to age are increasingly accumulated (Haigis and Yankner 2010). Apart from free radicals, damage to chromosome ends (telomeres) can also occur due to continuous cell division, ultimately leading to a critical length beyond which cells trigger a DNA damage response (DDR). Upon sensing damage, cells respond by activating checkpoints to arrest cell cycle progression, thereby

allowing time for repair to occur before the damage is passed on to daughter cells (Zhou and Elledge 2000). If the damage is not easily repaired then it accumulates in the cell and when this reaches a critical threshold, cells undergo apoptosis (programmed cell death) or cellular senescence (isolated cells demonstrating a limited and irreversible inability to divide in culture). The consequence of such damage is an imbalance in homeostasis. To curtail such damage, the cells follow pathways to trigger repair, but some damages just evade such repair systems and accumulate with age (Hamilton, Van Remmen et al. 2001).

1.5 DNA damage response: cell cycle arrest, apoptosis or senescence

Irrespective of the type of damage and repair mechanism the activation of DDR results in a trigger of signals to the DNA-damage- sensing protein kinases such as ataxia-telangiectasia mutated (ATM), ataxia-telangiectasia Rad3 related (ATR) and DNA-damage sensing protein kinases (DNA-PK). These proteins through a series of phosphorylation steps in response to the DDR, converge upon p53 (tumour suppressor protein) phosphorylation and stabilization (d'Adda di Fagagna, Reaper et al. 2003, Blanpain, Mohrin et al. 2011). Activation of p53 and transactivation of genes by p53 then determine the fate of cells (Fig 1.3).

The cells may undergo transient **cell cycle arrest** due to induction of cyclin dependent kinase inhibitor (CDKI) p21 whose transactivation is achieved by p53 (Itahana, Dimri et al. 2001) (Fig. 1.3). Cell cycle arrest by p53-p21 activation can be reversed by inactivating p53, and cells can resume cell division, suggesting this fork of DDR acts as a time gaining mechanism wherein damaged cells have an opportunity to engage in

repair mechanisms when levels of damage are low (Beausejour, Krtolica et al. 2003, d'Adda di Fagagna, Reaper et al. 2003).

Apoptosis may result when levels of damage are severe (Fig. 1.3). Activation of p53 may cause apoptosis or programmed cell death by inducing activation of pro-apoptotic bcl2 gene family members such as the Bcl2 associated X (BAX), p53 upregulated modulator of apoptosis (PUMA) also known as Bcl-2-binding component3 (BBC3), and noxa (Miyashita, Krajewski et al. 1994, Selvakumaran, Lin et al. 1994, Miyashita and Reed 1995, Pietsch, Sykes et al. 2008).

Following severe damage cells may also become **senescent** (metabolically active and arrested in G1 phase of cell cycle) through induction of the CDKI: p16/Ink4a and tumour suppressor gene p19/alternate reading frame (ARF)(Campisi and d'Adda di Fagagna 2007). Senescent cells exhibit enlarged and flatted morphology and express the senescence associated β-galactosidase, higher levels of tumour suppressors and hypo-phosphorylated retinoblastoma protein (Dimri, Lee et al. 1995, Campisi 2001, Campisi, Kim et al. 2001, Stolzing, Jones et al. 2008). Senescent cells don't divide but they secrete several factors such as pro-inflammatory cytokines and chemokines, termed as senescence associated secretory phenotype (SASP) and these may affect the behaviour of neighbouring cells (Coppe et al. 2010).

Upon DNA damage, there are sensors (eg Ku70/80) that identify and amplify signal with the help of transducers (eg DNA-PK; ATM, ATR) and mediators (eg 53BP1, Brca1) to finally trigger effector (p53; PUMA; p16, p21) molecules that determine the cellular outcome (cell cycle arrest/apoptosis/senescence).

Factors that trigger DNA damage response have been discussed in the next sections with evidences highlighting their implication on stem cells and tissue homeostasis.

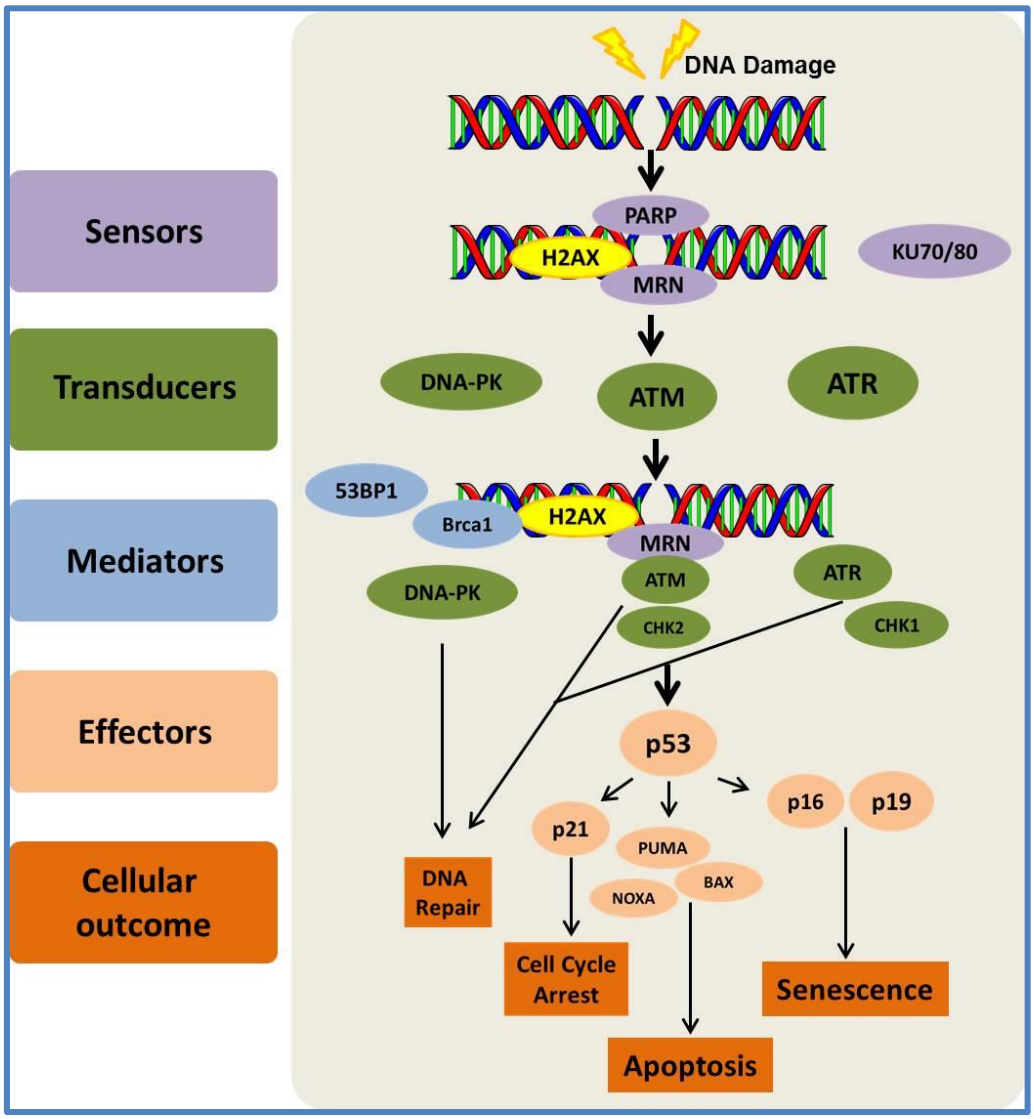


Figure 1.3 DNA-damage response pathways

Upon DNA damage, distinct factors detect, transmit, and amplify the DNA-damage signal. This DNA-damage response converges upon p53 which, depending on the target genes activated, regulates different cellular outcomes.

1.5.1 Telomere Shortening and telomerase activity

Telomeres are nucleoprotein complex structures at the ends of chromosomes and consist of non-coding TTAGGG double strand repeats, 3' overhang and telomere binding proteins. Telomere protects the chromosome ends from fusion, damage, degradation and instability (Blackburn 2001, Wright and Shay 2002). Cellular senescence can be induced due to persistent double strand breaks (stress induced premature senescence) or shortening of telomere length (uncapping of the telomere leading to replicative senescence). Many cells may senescence in response to mitogenic signals delivered by oncogenes (Rodier and Campisi, 2011).

At each cell division there is telomere shortening due to 'end replication problem'. This occurs till they become critically very short. Critically short telomeres become dysfunctional (d'Adda di Fagagna, Reaper et al. 2003, Takai, Smogorzewska et al. 2003). As DDR is triggered, a state of cell cycle arrest termed replicative senescence is achieved after a finite number of divisions defined as Hayflick limit (Harley, Futcher et al. 1990, de Lange 2002, Wright and Shay 2002, Allsopp, Morin et al. 2003, Allsopp, Morin et al. 2003). This replicative senescence is telomere dependent senescence.

Cellular senescence can also be induced by telomere independent mechanisms such as oncogene induced oxidative stress (Chandeck and Mooi, 2010) and mitochondrial dysfunction (Correia-Melo and Passos 2015) including other exogenous factors.

Telomere shortening majorly contributes to uncapping of the telomere and can be largely caused by breaks in the telomere specific DNA. One of the factors causing such damage leading to telomere attrition is oxidative stress (von Zglinicki, 2002). This causes various types of DNA damage triggering DNA damage response, which if remain persistent, can activate senescence via tumour suppressors' p53 and p21 or

p16 retinoblastoma protein (pRB). Oxidative stress at low intensity but chronic exposure generate single strand breaks, that maybe acquired by the telomeres (Petersen, Saretzki and Zglinicki. 1998). This may be less efficiently repaired resulting in DNA replication of the telomeres containing high frequency of damage thus shortening its length (von Zglinicki, Saretzki et al. 1995; Richter, Saretzki et al. 2007). Oxidative stress may also affect the maintenance of telomeres via its effect on telomerase activity. Telomere loss can be attenuated by an enzyme telomerase. Telomerase is an enzyme structurally composed of two components, telomerase reverse transcriptase (TERT) and a telomerase RNA template (TR or TERC) and functionally it adds DNA sequence repeats to the telomere regions (Cohen, Graham et al. 2007). It is expressed in a few cell types including germ cells, adult stem cells and T cells, where it attenuates telomerase loss (Kim, Piatyszek et al. 1994, Liu, Snow et al. 2000, Blackburn 2001, Wright and Shay 2005).

However even in cells where telomerase is expressed, this does not seem to be sufficient to maintain telomere length. For example, studies by Weng et al (1995) and Rufer et al (1999) examining telomere lengths of T cells from aged donors demonstrated telomere length attrition occurred in both naive and memory T cells with increasing age of donor (Weng, Levine et al. 1995, Rufer, Brummendorf et al. 1999, Rufer, Brummendorf et al. 1999). Son et al (2000) demonstrated that similar to T cells, B cells also exhibit telomere shortening with age but at a slower rate than in T cells (Son, Murray et al. 2000). Although oxidative stress has resulted in accelerated telomere attrition in endothelial cells, vascular smooth muscle cells or leukemic cancer cell lines due to diminished telomerase activity (Haendeler, Hoffman et al. 2004; Kurz, Decary et al. 2004; Matthews, Gorenne et al. 2006; Pizzimenti, Briatore et al. 2006).

Mitochondrial dysfunction may also contribute to cellular senescence as mitochondria is major source of oxygen free radicals accumulation of these reactive oxygen species (ROS) have shown to occur in senescent cells (Sitte, Merker et al. 2000; Sitte, Merker et al. 2001). Depolarisation of mitochondria resulted in increased ROS levels, telomere loss and fusion of chromosomes (Liu, Trimarchi et al. 2002). Moreover patients with mitochondrial dysfunction also show shortened telomeres in white blood cells (Oxele and Zwirner, 1997). Thus senescence could be determined by processed associating mitochondrial dysfunction, oxidative stress and telomere shortening (Passos, Saretzki et al. 2007; Passos and von Zglinicki, 2005; Sozou and Kirkwood, 2001).

1.5.2 DNA damage independent of telomere shortening

DNA damage response can be triggered independent of telomere shortening. Endogenous and exogenous sources play a major role in damaging the DNA. A rough estimate of the DNA lesions experienced by cells could sum up to 10^5 lesions per day (Table 1.3) (Hoeijmakers 2009). This can be caused by insults that are either physical or chemical in nature. For example ionizing radiation and ultraviolet radiation including radiation therapy or metabolic activities in the cells are physical genotoxic agents that cause production of reactive oxygen species (ROS) and resulting in damage to the DNA; whereas chemical agents include chemotherapy drugs, alkylating agents such as methyl-methanosulfonate, temozolomide, crosslinking agents such as mitomycin C, cisplatin, topoisomerase inhibitors such as camptothecin and etopside. Lesions may also occur due to DNA replication errors. Specific DNA lesions such as O6-methylguanine, base N-alkylations, bulky DNA adducts, DNA cross-links and DNA double-strand breaks (DSBs), single strand breaks and point mutations among others

(Roos and Kaina 2006) cause apoptotic attrition, senescence or cellular dysfunction (Fig. 1.4). The consequence of such damage is an imbalance in homeostasis. In response to damage cells trigger evolutionary conserved signalling mechanisms trigger cell cycle arrest and DNA repair (Lombard, Chua et al. 2005, Blanpain, Mohrin et al. 2011). The different mechanisms of DNA repair are described in the following sections.

Endogenous DNA Damage	DNA Lesions Generated	Number Lesions/Cell/ Day	References
Depurination	AP site	10000	(Lindahl and Barnes 2000)
Cytosine deamination	Base transition	100--500	(Lindahl and Barnes 2000)
SAM-induced methylation	3meA	600	(Lindahl and Barnes 2000)
	7meG	4000	(Lindahl and Barnes 2000)
	O6meG	10--30	(Rydberg and Lindahl 1982)
Oxidation	8oxoG	400--1500	(Klungland, Hoss et al. 1999)

Exogenous DNA Damage	Dose Exposure (mSv)	DNA Lesions Generated	Estimated Number Lesions/Cell	References
Peak hr sunlight	Not measurable	Pyrimidine dimers, (6-4) photoproducts	100,000/day	(Hoeijmakers 2009)
Cigarette smoke	Not measurable	aromatic DNA adducts	45-1029	(Phillips, Hewer et al. 1988)
Chest X-rays	0.02	DSBs	0.0008	Hall and Giaccia, 2006; Elkind and Redpath 1977
Dental X-rays	0.005	DSBs	0.0002	Hall and Giaccia, 2006; Elkind and Redpath 1977
Mammography	0.4	DSBs	0.016	Hall and Giaccia, 2006; Elkind and Redpath 1977
Body CT	7	DSBs	0.28	Elkind and Redpath 1977
Head CT	2	DSBs	0.08	Elkind and Redpath 1977
Coronary angioplasty	22	DSBs	0.88	Hall and Giaccia, 2006; Elkind and Redpath 1977
Tumor PET scan (18F)	10	DSBs	0.4	Hall and Giaccia, 2006; Elkind and Redpath 1977
131I treatment	70-150	DSBs	2.8-6	Hall and Giaccia, 2006; Elkind and Redpath 1977
External beam therapy	1800-2000	DSBs	72-80	Elkind and Redpath 1977
Airline travel	0.005/hr	DSBs	0.0002/hr	Elkind and Redpath 1977
Space mission (60 days)	50	DSBs	2	(Ciccia and Elledge 2010)
Chernobyl accident	300	DSBs	12	(Ciccia and Elledge 2010)
Hiroshima and Nagasaki atomic bombs	5-4000	DSBs	0.2-160	(Ciccia and Elledge 2010)

Table 1.3 DNA lesions caused by exogenous and endogenous agents *(Adapted from (Ciccia and Elledge 2010))*

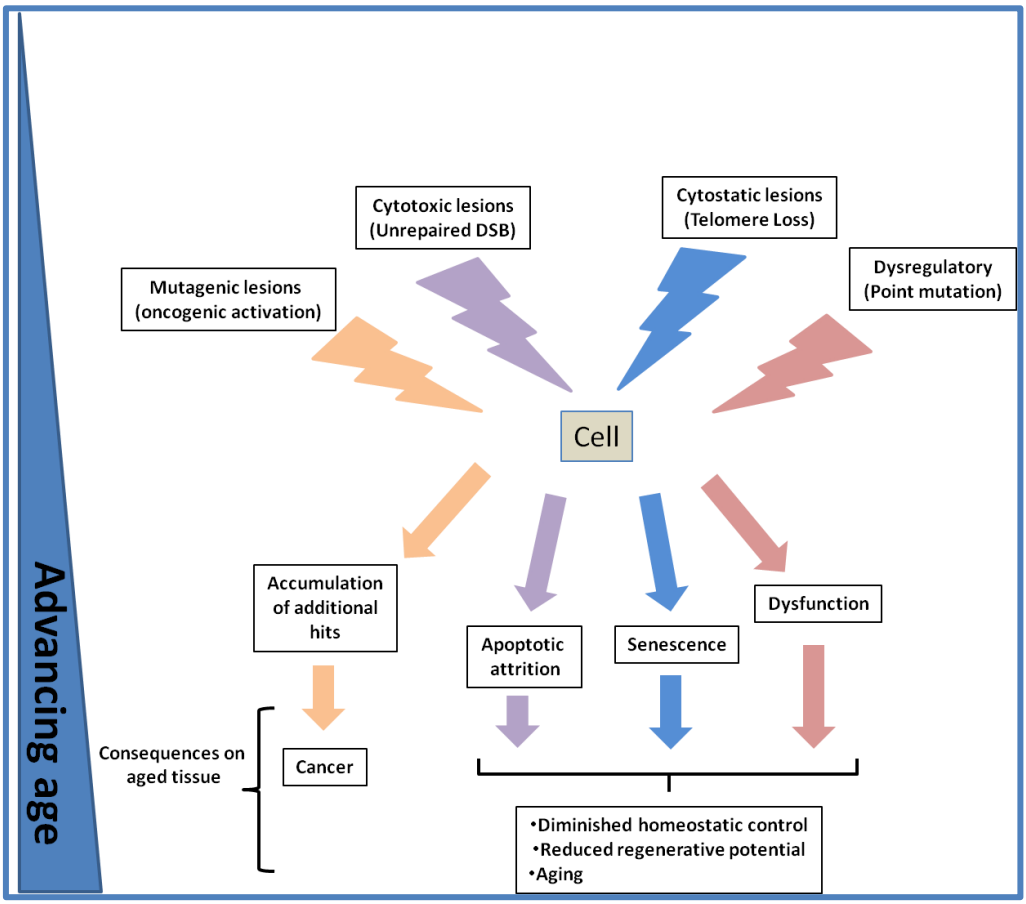


Figure 1.4 Cellular responses to damage

With advancing age the response of the cells is to accumulate damage which contributes to the diminished homeostasis control and regenerative potential.

Base modifications are caused by oxidation, ultraviolet (UV) or cigarette smoke more commonly by reactive oxygen species (ROS). Such DNA lesions are repaired by **base excision repair** (BER). BER involves repair of two types: a short patch repair (repairing lesion with single nucleotide) (Matsumoto and Kim 1995) and long patch repair (repairing lesion with approximately 2-10 nucleotides)(Matsumoto, Kim et al. 1994). Base excision repair reduce the modified (oxidized/reduced /alkylated/deaminated/mismatched) bases and AP sites from the DNA (Wilson and Bohr 2007)

Nucleotide modifications induced by UV light are also repaired by **nucleotide excision repair** (NER) pathway. Nucleotide excision repair (transcriptional controlled

or global genome) repair transcribed and non-transcribed genome using repair enzymes (Hanawalt 2001, Hanawalt 2002). They involve a series of steps from 1) recognition of DNA damage, 2) local unwinding of DNA 3) DNA strand dual incision and finally 4) DNA repair synthesis and strand ligation (Batty and Wood 2000).

Replication errors due to template slippage causing insertion/deletion loops and DNA polymerase mis-incorporation of nucleotides causing base-base mismatch are corrected by the **Mismatch repair** (MMR) pathway (Gradia, Subramanian et al. 1999, Clark, Valle et al. 2000, Marti, Kunz et al. 2002).

Double strand breaks are caused by a variety of sources such as ionizing radiations, genotoxic chemicals, ROS and they differ from other types of DNA lesions in that they affect both strands of DNA. Cells have evolved two repair pathways that operate during different phases of cell cycle (Harper and Elledge 2007, Lieber and Wilson 2010). During the G0/G1 phase, the **non-homologous end-joining** (NHEJ) pathway is involved in repairing the DSB, while during the S-G2M phase; lesions are repaired by the **homologous recombination** (HR) pathway (Fig 1.5).

HR directed repair occurs in S-G2 phase of cell cycle when an undamaged sister chromatid is available for use as template to repair DSB in an error free manner (Mahaney, Meek et al. 2009). The damage is identified by proteins that generate 3' single strand DNA overhangs bound with protein RAD52. The damaged strand protein complex then searches for undamaged DNA on sister chromatid and invades the duplex followed by extension and subsequent ligation resulting in a hetero-duplexed DNA structure (Sonoda, Takata et al. 2001, Jackson, Dhar et al. 2002).

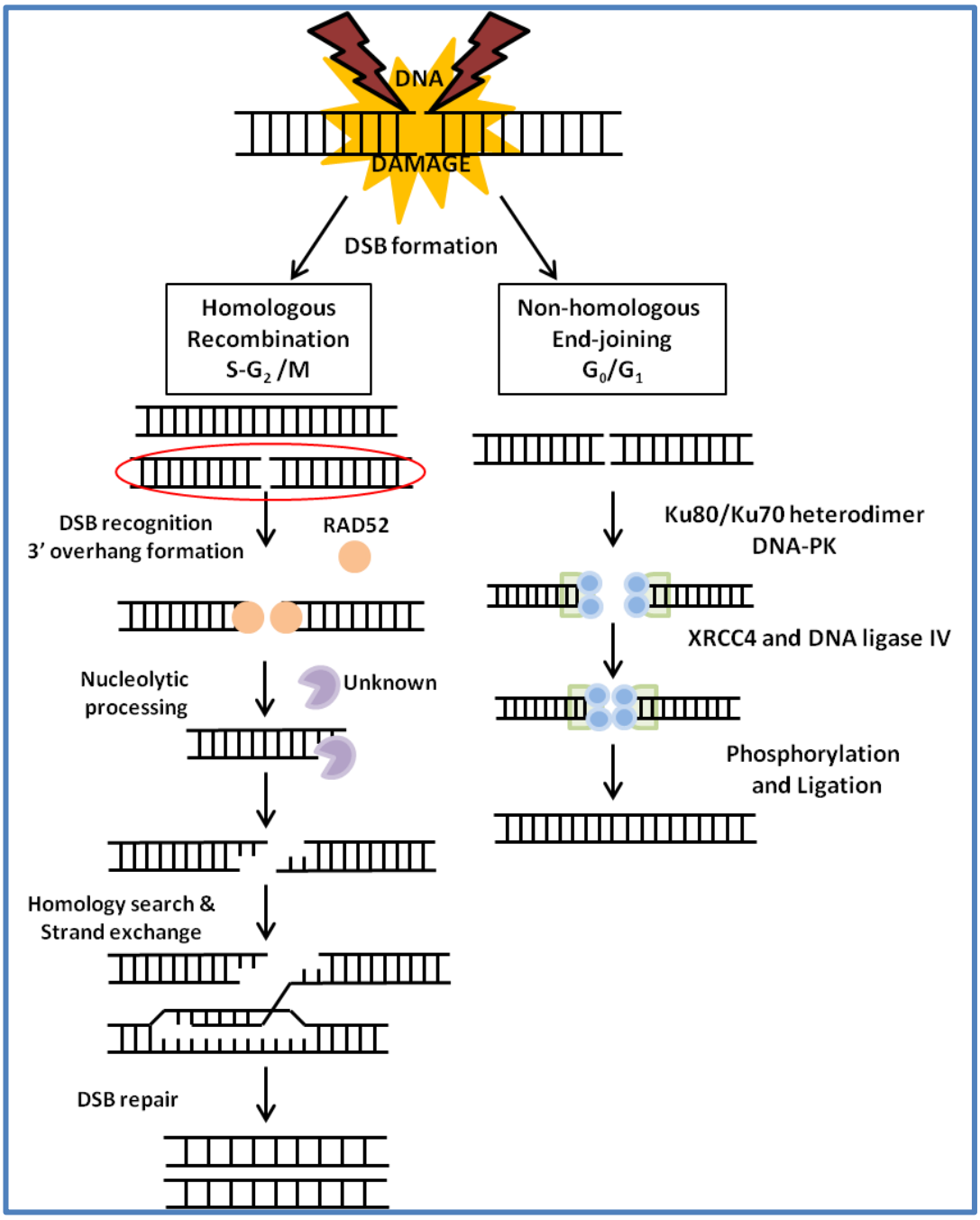


Figure 1.5 DNA double strand break (DSB) repair pathways

Homologous recombination (HR) and Non-Homologous End joining (NHEJ) pathways are involved in repair of DSB. HR pathway occurs in S-G2 phase of cell cycle and uses an undamaged sister chromatid as a template to repair DSB. NHEJ pathway occurs in the G0/G1 phase of cell cycle and repair is initiated by heterodimer Ku80/Ku70 that in the presence of DNA-PK recruits XRCC4 and DNA ligase IV to repair DSB.

Repair by **NHEJ**, occurs mainly when cells are in G0/G1 phase where an intact template is absent. The KU70/Ku80 hetero-dimeric protein initiates NHEJ by binding to the free DNA end and recruits other factors such as the DNA dependent protein kinase (DNA-PK), XRCC4 and DNA ligase IV to the site of injury. This is an error prone repair mechanism (Collis, DeWeese et al. 2005). Small deletions, insertions, nucleotide changes or chromosomal translocations are associated with this mode of DNA repair.

If the damage is unresolved/incorrectly repaired by these repairs mechanism and/or the damaged cells are not eliminated then mutations accumulate and these have been reported to occur with age. Point mutations (GC→AT transitions, GC→TA / GC→CG / T→CG transversions, deletions, expanded simple tandem repeats or ESTR) and large genomic rearrangements (deletions and translocations) accumulate with age in murine small intestines (mucosa and serosa), brain (except ESTR), sperm, bone marrow and heart (Dolle, Giese et al. 1997, Dolle, Snyder et al. 2000, Busuttil, Bahar et al. 2007, Hardwick, Tretyakov et al. 2009). These age accumulated mutations can also be attributed to the declining function of DNA repair pathways whose activity have been shown to weaken with age or even have an age-specific repair-pathway usage bias to avoid excessive energy and time consumption (Preston, Flores et al. 2006, Engels, Johnson-Schlitz et al. 2007, Gorbunova, Seluanov et al. 2007).

1.6 Evidence of stem cell ageing

1.6.1 Ageing due to telomere shortening and telomerase activity

Most studies of telomere shortening are in hematopoietic stem cells to date due to the availability of better assays to assess the self-renewal and differentiation ability. Despite telomerase being expressed in HSC, shortening of telomeres has been

observed with donor age. This has also been shown in situation of stress following bone marrow transplantation (Lee, Kook et al. 1999, Allsopp, Cheshier et al. 2001, Brummendorf, Rufer et al. 2001, Ito, Hirao et al. 2006, Rocci, Ricca et al. 2007). Vaziri et al 1994 showed that telomere length in purified human stem cells of $CD34^{+}CD38^{low}$ phenotype are shorter than cells from fetal liver or umbilical cord blood. This suggests an age related decline in telomere length (Vaziri, Dragowska et al. 1994).

Terc-/- mice devoid of telomerase activity and with signs of telomere dysfunction have demonstrated in the studies by Saeed et al, a decreased bone mass, MSC number and ability to differentiate, an intrinsic osteoblastic defect and signs of increased senescence following in vitro culture suggesting that in absence of telomerase which is known to protect the telomere caps there is ageing phenotype with loss of stem cell functions (Saeed, Abdallah et al. 2011).

Moreover*, in vivo* studies involving telomerase knockout mice demonstrate that telomeres undergo accelerated shortening and this has functional consequences (Allsopp, Morin et al. 2003). They demonstrated telomerase deficient HSCs could be serially transplanted for only two rounds where as wild type HSCs could be serially transplanted for at least four rounds (Allsopp, Morin et al. 2003) suggesting that short telomere affects stem cell regeneration capacity.

Jaskelioff M et al., (2011) engineered a 4-hydroxytamoxifen (4-OHT)-inducible telomerase reverse transcriptase-estrogen receptor (Tert-ER) knock-out mice which in their 4th generation exhibited short dysfunctional telomeres along with a depletion in stem cell and signs of tissue atrophy. Interestingly when telomerase was reactivated for only 3 weeks there was a marked increase in proliferation of early

neural progenitor cells and the Dcx+ (Doublecortin, neuronal marker) neurons and Oligo2+ oligodendrocyte populations (Jaskelioff, Muller et al. 2011). There was also a rescue in the tissue function. The sense of smell that was lost with age was regained following reactivation of telomerase similar to that of younger mice. These evidences thus suggest the important association that exists between tissue impairment in ageing and repair by stem cell functions that can be enhanced by chemical interventions to allow amplification and rejuvenation of these cells to maintain tissue function.

1.6.2 Ageing due to DNA damage

Impact of DNA damage accumulation in stem cells leading to age related pathologies has been evidenced (Nijnik, Woodbine et al. 2007, Rossi, Bryder et al. 2007, Rossi, Seita et al. 2007, Charville and Rando 2011, Mandal, Blanpain et al. 2011). Replication independent DNA damage such as abasic mutations and strand breaks may accumulate with advancing age(Charville and Rando 2011). Frequency of DNA double strand breaks in CD34+ and CD34- stem/progenitor cells were higher in older population (>50 years) compared to younger groups (<50 years age) and following exposure to radiation this difference increased with donor age (Rube, Fricke et al. 2011). In fact Yahata et al (2011) showed restriction of self-renewal capacity of hematopoietic stem cells that had accumulation oxidative damage with age of donors (Yahata, Takanashi et al. 2011). Even in wild type mice an age related decline in functional capacity was reported in hematopoietic (Rossi, Bryder et al. 2007, Rossi, Seita et al. 2007)and muscle (Lavasani, Robinson et al. 2012)derived stem/progenitor cells. Moreover defects in DNA repair mechanism have resulted in profound effects on stem cell function and viability thus causing imbalance of homeostasis.

Ruzankina Y et al., (2007) showed that conditional depletion of ATR gene (a DNA-damage sensing gene) in mice led to defects in tissue homeostasis resulting in tissue atrophy due to loss of thymus and hair follicle stem and progenitor cells pool. Mice were characterized by an ageing phenotype (Ruzankina and Brown 2007, Ruzankina, Pinzon-Guzman et al. 2007). They had hair loss and graying, reduced thymopoiesis, kyphosis, osteoporosis, fibrosis of heart and kidney, compared to wild type (Ruzankina, Pinzon-Guzman et al. 2007).

Mice deficient in ATM (DNA-damage sensing gene) present age related pathologies including neurological abnormalities, premature hair growth, loss of T cells and are also extremely sensitive to radiation exposures (Barlow, Hirotsune et al. 1996). In fact mutations in ATM in patients have shown accelerated ageing pathologies associated with neural degeneration, high incidence of cancer, growth retardation, telangiectasia and other age related clinical pathologies (Lavin 2008).

Similarly accumulation of DNA damage was found in murine HSC with age as shown by accumulation of gamma H2AX phosphorylation of double stranded breaks in DNA in older mice compared to young mice (Rossi, Bryder et al. 2007, Rossi, Seita et al. 2007). Moreover, mice deficient for genes involved in repair of DSB following the NHEJ repair pathway, including DNA ligase 4 (LIG4) and Ku80, showed impaired repopulating potential (Nijnik, Woodbine et al. 2007, Rossi, Bryder et al. 2007), suggesting that increased accumulation of DNA damage can lead to functional impairment.

Decline in functional capacity in muscle derived stem/progenitor cells has also been reported (Lavasani, Robinson et al. 2012). Interestingly, evidence of an extension in

life span and health span of mice with progeroid phenotype following transplant with stem cells from a young donor presents an important role of the stem cell pool in tissue regeneration and tissue homeostasis maintenance (Lavasani, Robinson et al. 2012).

1.6.3 Ageing due to environment

Not all stem cells may behave similarly and an age-related decline in function may also result from a change in local or systemic environment which effects stem cells ability to produce mature cells. This has been seen in case of muscle stem cells/satellite cells and HSC. By setting up parabiotic pairings which shared the circulatory system between a young and an old mouse (heterochronic) as test and two old mice (isochronic) as controls Conboy et al established that post muscle injury, muscles in old mice (isochronic) regenerated poorly as a result of reduced numbers or ability to differentiate in shared circulation in comparison to the heterochronic pairing between young and old mouse (Conboy, Conboy et al. 2005) . The regenerative capacity of ageing muscle satellite cells of old mice was restored substantially by exposure of these older satellite cells to the serum of younger mice in vitro cultures , whereas an inhibitory effect of old mouse serum on young satellite cells was observed (Conboy, Conboy et al. 2005). These data suggest that factors contained in the systemic circulation affected the ability of stem cells to repair a damaged tissue with age.

Similarly in case of HSC, alteration in the local and systemic environment was found to limit the stem and progenitor cell function. In studies by Ju et al (2007) aged telomerase knockout mice (Terc-/-) demonstrated impaired B lymphopoiesis and accelerated myelopoiesis in both endogenous and donor derived wild type cells post

transplantation of bone marrow cells (Ju, Jiang et al. 2007). Rescue of impaired B cell development and normalization of myelopoiesis in aged telomerase knockout donor derived cells after transplant of bone marrow from these knockout mice into young wild type mice suggested role of environment. Further investigating the effect of environment on wild type HSC transplantation in aged telomerase knockout mice showed no engraftment of HSC in Terc-/- mice. Such impairment in engraftment may be due to minimal intrinsic defects in HSC and was more due the defective mesenchymal progenitor cells (critically short telomere lengths) in the niche that had reduced ability to support the HSC (Jiang, Ju et al. 2007, Ju, Jiang et al. 2007). These data suggest that environmental defects generated by extremely short telomeres can impair the function and engraftment of hematopoietic stem and progenitor cells.

The above examples thus suggest that cellular ageing affects stem cells in their function and this leads to loss of tissue homeostasis. This can be attributed to intrinsic defects or environmental effects or a combination of both. Although stem cells have repair mechanisms to minimize damage, they still accumulate damage. Once the threshold is reached cells can either undergo senescence or apoptosis, thus attenuating stem cell function and loss of homeostasis (Mandal, Blanpain et al. 2011).

To date majority of data associated with stem cell ageing has been investigated and established with HSC. Very limited data are available in other stem cells therefore in this thesis I have focused primarily on mesenchymal stem cells as a model of cellular ageing.

1.7 Age related changes in MSC

Evidences of cellular ageing in MSC emanate from important changes occurring with the number and proliferation ability. These age related changes in MSC progenitor numbers are assessed by employing the CFU-F and CFU-O assays. While studies in mice assessed the number of CFU-F with age (Brockbank, Ploemacher et al. 1983, Xu, Hendry et al. 1983)showed controversial results, those in rats demonstrated a decrease in MSC number (Tsuji, Hughes et al. 1990, Egrise, Martin et al. 1992, Quarto, Thomas et al. 1995) . Such discrepancies could be due to the experimental method utilized, the way CFU-F frequencies were calculated or the strain of mice. For example the number of CFU-F decreased with age in Balb/c but not C57Bl/6 strain. This was similar to what was previously described in HSC repopulation studies (Ertl, Chen et al. 2008).

In human studies inconsistencies were also observed in the number of CFU-F or CFU-O and these could possibly be attributed to the method and source of bone marrow samples. The bone marrow were obtained either from the head of the femur of patients undergoing routine hip replacement or corrective surgery (Oreffo, Bennett et al. 1998, Oreffo, Bord et al. 1998) or from the posterior iliac crest of hematologically healthy donors (Bruder, Jaiswal et al. 1997, Stenderup, Justesen et al. 2001). As more primitive MSC have been shown to be present in the Iliac crest rather than in the periosteum or trabecular bone this may explain some of the differences (Sacchetti, Funari et al. 2007). Moreover the definition of CFU-F differed among investigators with some defining CFU-F as colonies more than 50 cells (Stolzing, Jones et al. 2008) and other show colonies with 16 cells (Stenderup, Justesen et al. 2001). It is of interest that investigators using a cut off of 50 cells find a significant decrease with

age (Stolzing, Jones et al. 2008). In contrast no difference has been found in studies by Stenderup et al (2001) where a cut off of 16 cells was considered. As stem cells are considered highly proliferative cells it is possible those two different populations with varying potentials were considered and those colonies with 50 cells or more is more representative of a stem cell population. Another explanation is that the age of donor cohorts considered as 'young' differs. For example in the study by Stolzing et al (2008) donor aged 7-18 years were considered 'young' in contrast to Stenderup et al (2001) donor aged 20-40 years was considered 'young'. As no difference was found in studies by Stenderup et al (Stenderup, Justesen et al. 2001) as opposed to the study by Stolzing et al (2008) this may suggest that majority of changes occur in early days when indices of bone formation are maximal (Szulc, Kaufman et al. 2007) and such changes in the skeletal dynamics may be responsible for the observed decline in CFU-F observed by Stolzing et al (Stolzing, Jones et al. 2008).

The effect of donor age on MSC proliferation has been investigated in vitro and studies showed a decrease in the total number of population doublings obtained with time in culture with an average of 30-40 population doublings in culture derived from BM of younger donors in comparison to approximately 20 population doublings in cultures from older donors suggesting an age related decline in MSC ability to proliferate (Stenderup, Justesen et al. 2003, Baxter, Wynn et al. 2004).

The effect of donor age on MSC differentiation potential was assessed by using MSC isolated by selective culture condition and expanded in vitro and exposed to differentiation supplements. Evidence of decreased differentiation to the osteoblast and adipocyte lineage is contrasting. Studies by Stolzing et al (2008) investigated effects of age on differentiation potential of human MSC derived from donors of

varying age. They found that culturing cells in vitro in osteogenic medium showed a decline in ALP (alkaline phosphatase) activity, a marker of early osteoblast differentiation in older donors in comparison to younger donor (Stolzing, Jones et al. 2008). In contrast studies by (Stenderup, Justesen et al. 2001) and Justesen et al (Justesen, Stenderup et al. 2002) involving early passage cultures from young, old and osteoporotic patients demonstrated a maintained osteoblast differentiation potential.

Similarly assessing bone forming capacity using *in vivo* bone formation assays (subcutaneous implants of MSC mixed with hydroxyapetite/tricalcium phosphate HA/TCP in syngeneic animals) showed a decreased bone formation capacity of MSC derived from aged rats in comparison to young donors (Inoue, Ohgushi et al. 1997). In contrast Stenderup et al in 2004 assessed the bone forming capacity of MSC obtained from early passage and demonstrated that in both young and old human donors no difference in bone formation expressed as a percentage of bone volume over total volume was observed in the implants (Stenderup, Rosada et al. 2004). This discrepancy in results could be attributed to the heterogeneity of MSC when cultured in vitro and to the passage number at which differentiation is induced with no difference in ability at early passage but loss of osteoblast differentiation at late passage. Consistent with this, studies by Muraglia et al demonstrated that with time in culture the tripotent clones lost their adipogenic potential but at early passage had no change (Muraglia, Cancedda et al. 2000).

Cellular ageing in vitro cultured MSC is manifested by changes in morphological appearance from spindle shaped to large flat cells. This is accompanied by telomere shortening and expression of senescence markers (Stenderup, Justesen et al. 2003,

Baxter, Wynn et al. 2004, Stolzing, Jones et al. 2008) . Studies by Banfi et al (2000), Baxter et al (2004), Bonab et al (2006) and Bianchi et al (2003) have consistently demonstrated a significant decrease in mean telomere restriction fragment (mTRF) in MSC from young donors between primary passage and end of culture (Banfi, Muraglia et al. 2000, Bianchi, Banfi et al. 2003, Baxter, Wynn et al. 2004, Bonab, Alimoghaddam et al. 2006). Baxter et al (2004) showed that MSC from young donors had longer telomere lengths in comparison to MSC from old donors when cultured for equal number of population doublings suggesting that the difference was due to loss of mTRF *in vivo* with age of donor (Stenderup, Justesen et al. 2003, Baxter, Wynn et al. 2004). Moreover, MSC obtained from fetal liver, cord blood compared to adult bone marrow showed a significantly greater telomere length, thus supporting that there is loss of telomere length with age (Guillot, Gotherstrom et al. 2007).

In vitro, MSC lack telomerase activity (Zimmermann, Voss et al. 2003) and hTERT expression (Simonsen, Rosada et al. 2002) thus resulting in telomere shortening with serial passaging. Moreover studies by Saeed et al (2011) have shown that Terc-/- mice devoid of telomerase activity and with signs of telomere dysfunction, demonstrated a decreased bone mass. MSC counted as CFU-F were decreased in number and ability to differentiate to osteoblasts and showed signs of increased senescence following in vitro culture (Saeed, Abdallah et al. 2011). These data suggest that MSC may express telomerase but this may be switched off during in vitro culture. Moreover it is not sufficient to maintain telomere length with age. It is possible that similar to HSC only very primitive MSC express telomerase *in vivo* that delay telomere shortening with age. Indeed a study by Isenmann et al (2007) demonstrated that when hMSC were expanded in culture there were high levels of expression of the osteoblast lineage

marker core binding factor alpha (Cbfa) which resulted in inhibition of telomerase activity (Isenmann, Cakouros et al. 2007).

In studies by Alves et al (2010) hMSC had a decrease in their multipotency with time in culture due to accumulation of DNA damage. Accumulated DNA damage was associated with activated NER DNA damage response pathway. Moreover they showed that exposure of hMSC to DNA damaging agents like hydrogen peroxide led to a decrease in their differential potential (Alves, Munoz-Najar et al. 2010). Although in their study they showed that MSC maintained effective NER activity, there was still progressive accumulation of DNA damage that contributed to loss of differential potential. Levels of oxidative stress markers were elevated in this study in late passage MSC, although no associated with telomere associated DNA damage accumulation with age in culture was established (Alves, Munoz-Najar et al. 2010).

In another study in mice mutated for Ercc2 (a DNA repair gene), resulted in accumulation of DNA damage associated with features of accelerated ageing, decreased bone strength and lack of periosteal apposition. These trichothiodystrophy (TDD) mice also showed lack of body fat with age (Nicolaije, Diderich et al. 2012). Thus results obtained were suggestive of impairment in MSC number and differentiation ability however more careful studies with knockout mice models demonstrating the ageing associated loss of MSC function need to be carried out.

MSC function declines in older individuals and this MSC dysfunction influences the effects of autologous MSC transplantation in older individuals (Zhang, Fazel et al. 2005, Kretlow, Jin et al. 2008) Moreover aged cell-extrinsic environment has shown to affect MSC function following xenogeneic MSCs transplantation in older individuals

(Kyriakou, Rabin et al. 2008). Moreover the effects of an aged environment on MSC function have been established in rat MSC where culture of these cells in old rate serum resulted in MSC senescence, loss of cellular proliferation and survival. Moreover, the expression of γ-H2A.X, a molecular marker of DNA damage response, p16INK4a, p53, and p21 was increased in senescent cells. These effects on aged microenvironment on MSC ageing were shown to be modulated by Wnt/β-catenin signaling (Zhang, Wang et al. 2011). These studies suggest that aged environment may also trigger ageing in MSC.

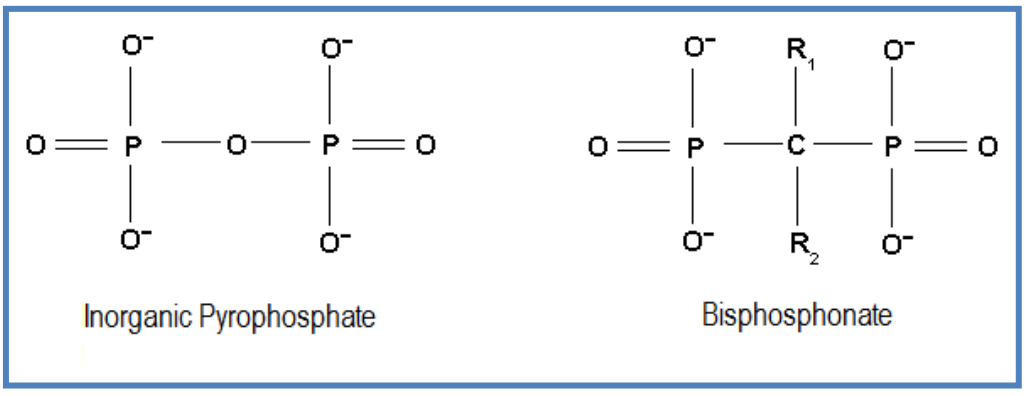
As MSC undergo a process of ageing characterized by decreased self-renewal and differentiation ability with the age of the donor along with time in culture, it is very important to understand the molecular mechanisms underlying the process of ageing to find target molecules which would facilitate their long-term self-renewal and differentiation ability *in vitro* and *in vivo.* One possible pharmacological intervention is the bisphosphonates that is discussed in details in the following sections.

1.8 Bisphosphonates

Bisphosphonates (BP) are class of chemical compounds that are widely used as a treatment choice in excessive bone loss disorders where bone resorption rate by osteoclasts is very high, and these drugs essentially inhibit osteoclast activity (Russell, Watts et al. 2008). They are synthetic analogues of the inorganic pyrophosphates where the central oxygen atom (P-O-P) is replaced by carbon atom (P-C-P). Chemically they form a P-C-P structure where each carbon atom is attached to two phosphate groups and this carbon atom confers resistance to enzymatic or acid hydrolysis (Fig 1.6). The substitution of the central oxygen moiety to the backbone of the naturally occurring inorganic pyrophosphate has made BPs a more stable,

resistant to degradation, biologically active and water soluble compound (Green 2005). Based on their mechanism of action this class of drugs is classified into the non-nitrogen containing BP; alkyl-amino group containing BP and the heterocyclic nitrogen containing BP (Fig 1.6).

The **non-nitrogen containing bisphosphonates** which are structurally closer to inorganic pyrophosphate (PPi), for e.g. clodronate, etidronate and tiludronate act by getting incorporated into adenosine triphosphate molecules (ATP) and inhibit ATP driven cellular reactions which eventually lead to apoptosis of osteoclasts(Russell, Watts et al. 2008). The **alkyl-amino BPs** like the Pamidronate, Alendronate and Ibandronate, act by interfering with the enzyme farneysl pyrophosphate synthase (FPPS) that results in inhibition of the mevalonate pathway (Russell, Watts et al. 2008). The **heterocyclic nitrogen containing BPs** like risedronate and zoledronate, also inhibit the mevalonate pathway by inhibiting FPPS. These compounds also possess additional ability to stabilize conformational changes that magnify their inhibitory potency (Rogers, Frith et al. 1999, Russell and Rogers 1999, Russell, Watts et al. 2008).



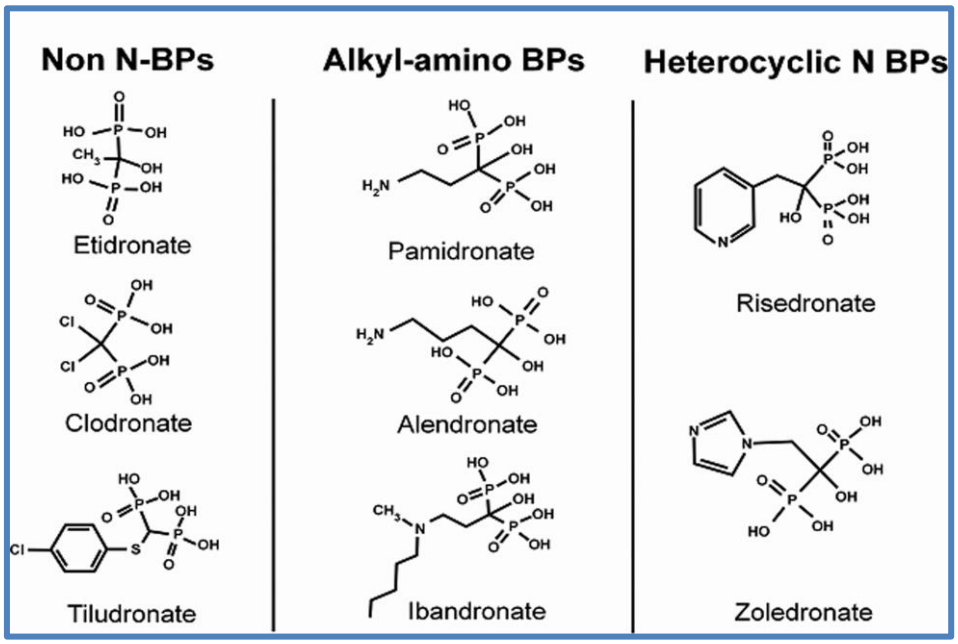


Figure 1.6 Comparison between pyrophosphate and bisphosphonate compounds

The central oxygen atom (P-O-P) in pyrophosphate is replaced by Carbon atom (P-C-P) in bisphosphonate. The classification of BPs (adapted from(Russell 2011))

The molecular mechanisms of BPs have been widely studied in osteoclast where different BPs as described above, inhibit the osteoclasts (Fig 1.7). Among the BPs, the N-BPs have been found to be more potent that the non-nitrogen containing bisphosphonates ((Russell, Watts et al. 2008). The most potent among this N-BPs was found to be zoledronate (Rosen, Gordon et al. 2003).

1.8.1 Zoledronate

Zoledronate (Zol) is a nitrogen containing bisphosphonate (NBP) widely used over the last decade in the treatment of bone loss diseases such as Paget's disease and osteoporosis. It also finds application in malignancy associated osteolysis and hypercalcemia and is currently under evaluation for being used as anti-tumor/cancer drug (Morgan and Lipton 2010, Morgan, Davies et al. 2010). It is by far the most potent commercially available drug with highest inhibitory effect on osteoclasts (Green 2005). The structure of zoledronate incorporates a heterocyclic imidazole ring with a hydroxyl (-OH) group at its R1 and two nitrogen atoms at its R2 moiety (Fig 1.6).

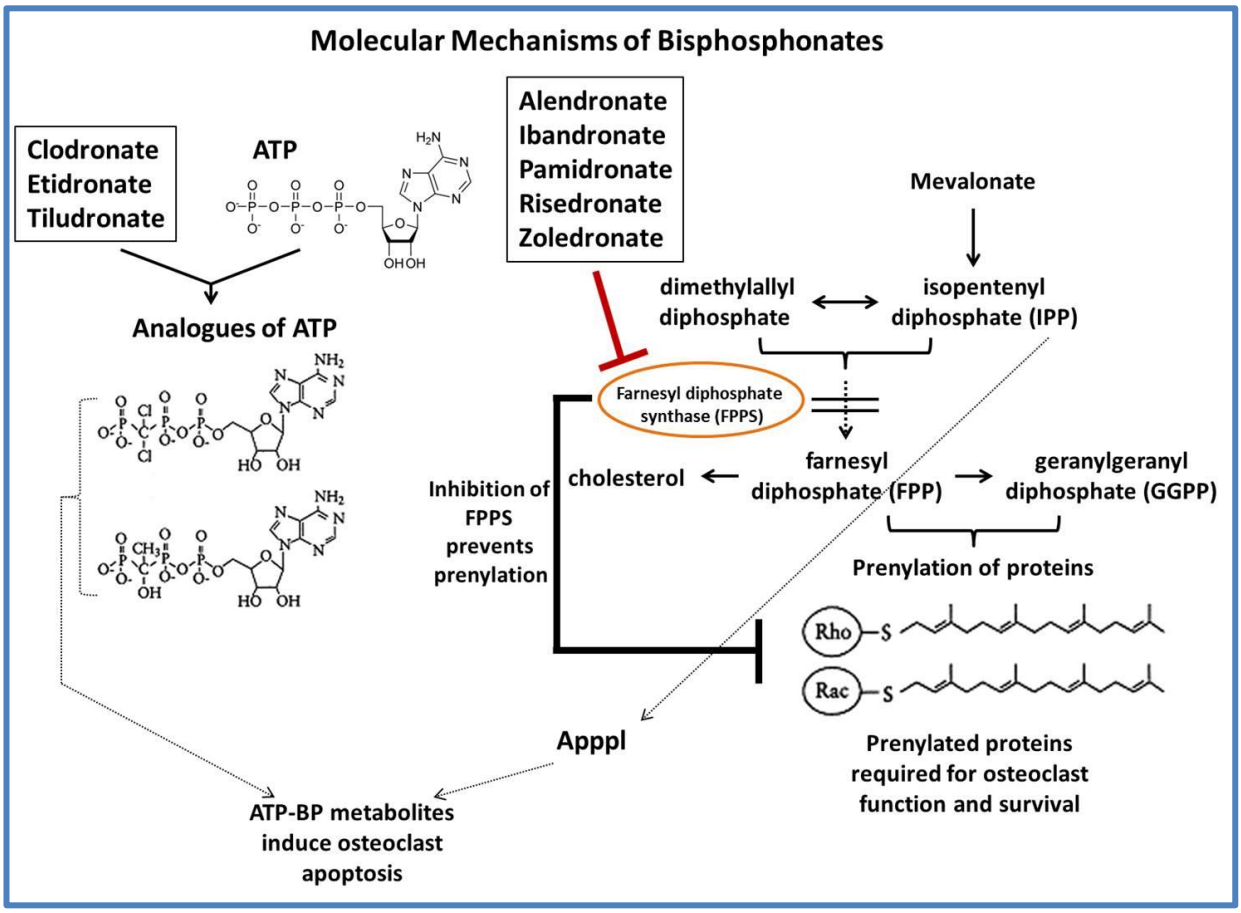


Figure 1.7 Mechanism of action of bisphosphonates on osteoclast

BPs like clodronate, etidronate after internalization into osteoclasts by endocytosis metabolize to form ATP-BP metabolites (ATP analogues) that induce apoptosis in osteoclasts whereas nitrogen containing BPs inhibit FPPS and this results in inhibition of protein prenylation, thus affecting function and survival of osteoclasts. This FPPS inhibition also results in accumulation of IPP which is incorporated into analog of ATP namely Apppl (Triphosphoric acid 1-adenosin-5'–yl ester 3-(3-methyl-but-3-enyl) ester), that results apoptosis of osteoclasts (adapted from(Russell 2011))

1.8.2 Zoledronate – Mechanism of Action

Bone resorption is prevented by zoledronate via inhibition of osteoclast activity. This is achieved by inhibition of geranyl diphosphate and farnesyl diphosphate synthase enzyme in the mevalonate pathway (Fig 1.8) (Amin, Cornell et al. 1992, Luckman, Hughes et al. 1998). This is a biosynthetic pathway whose end products are cholesterol, other sterols, isoprenoid lipid, farnesyl diphosphate (FPP) and geranyl-

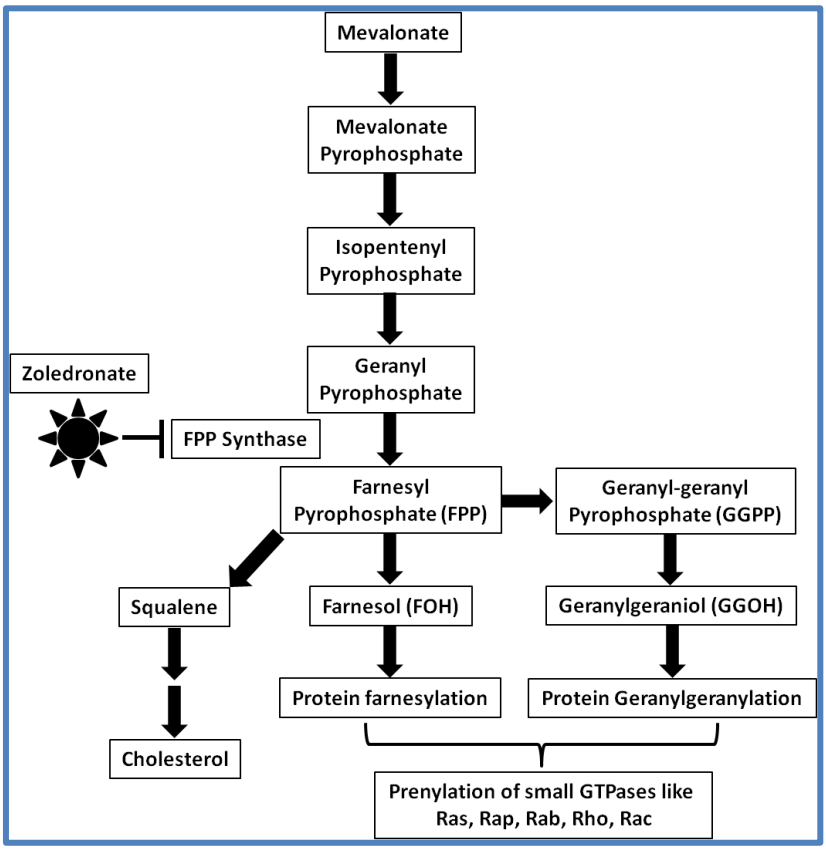


Figure 1.8 The Mevalonate pathway

This figure depicts the point at which zoledronate intervenes in the mevalonate pathway. zoledronate inhibits the enzyme farnesyl diphosphate synthase (FPP synthase) and therefore the formation of farnesyl diphosphate and all its downstream products.

-geranyl diphosphate (GGPP) (Russell, Watts et al. 2008). The FPP and GGPP molecules are involved in the post translational modification or prenylation of small GTPases like Ras, Rab, Rap, Rho and Rac (signaling proteins). Prenylation or isoprenylation is a post-translational modification process in which cysteine residues close to the C-terminal regions of some eukaryotic proteins are biosynthetically modified with an isoprenoid lipid: the 15-carbon farnesyl group or the 20-carbon geranylgeranyl group. These small GTPases are signalling molecules that play an important part in the regulation of cellular processes involved in osteoclast function, morphology and apoptosis (Russell, Watts et al. 2008). In cells that readily take up

Zol, there is inhibition of geranyl diphosphate and farnesyl diphosphate synthase (FPPS) which plays a critical role in the conversion of isopentenyl diphosphate into farnesyl diphosphate (FPP) of the mevalonate pathway (van Beek, Pieterman et al. 1999, Bergstrom, Bostedor et al. 2000, Dunford, Thompson et al. 2001). Inhibition of FPPS results in loss of prenylation of these important signaling proteins (Rogers, Frith et al. 1999, Rogers, Gordon et al. 2000, Monkkonen, Ottewell et al. 2007, Russell, Watts et al. 2008). There is also evidence that Zol activity results in accumulation of a cytotoxic analogue of ATP known as ApppI (triphosphoric acid 1-adenosine-5'-yl ester 3-(3-methyl-but-3-enyl) ester) that caused osteoclast apoptosis both *in vitro* and *in vivo* (Monkkonen, Auriola et al. 2006, Monkkonen, Ottewell et al. 2007).

1.9 Clinical applications of Bisphosphonates

Initially, Etidronate was used as a calcification inhibitor in fibrodysplasia ossicans progressive (FOP, also called Stone Man Syndrome), a disease which is characterized by a mutation in the ACVR1 (Activin A receptor, type I) gene resulting in defective repair mechanism of the body as a result of which fibrous tissue such as muscle, tendon and ligament become ossified spontaneously or when damaged (Smith, Russell et al. 1976, Smith 1995, Shore, Xu et al. 2006). In fact etidronate was also employed in patients undergoing hip replacement surgery to improve mobility by preventing the heterotropic ossification (Bijvoet, Nollen et al. 1974).

BPs linked to gamma emitting technetium isotope have been employed in 'bone scanning' as agents that facilitate the bone imaging for detection of bone lesions and bone metastases based on their high affinity for bone mineral at sites of high bone turnover (Fogelman, Bessent et al. 1978, Fogelman and Martin 1983).

However, the most impressive application of BP was as inhibitor of bone resorption in diseases where the key pathological features were excessive osteoclast activity eg Paget's, osteoporosis. A dose dependent inhibition of bone resorption was demonstrated in Paget's using BPs and currently it's the most reliant treatment for this medical condition (Smith, Russell and Bishop, 1971). Patients receive infusions of BPs such as pamidronate or zoledronate with long lasting effects with just a single dose of drug. Although it is known that BPs can reside in bone for long durations but the true mechanism for this long lasting effect are still elusive ((Reid, Miller et al. 2005, Hosking, Lyles et al. 2007, Grey, Bolland et al. 2010, Reid and Hosking 2011).

BPs have also been effectively used in prevention of skeletal complications associated with bone metastasis in myeloma, breast , prostate and lung cancer patients (Table 1.4) (Rosen, Gordon et al. 2004, Saad, Gleason et al. 2004, Coleman 2008). They have also been employed as additional treatment approaches in improvement of bone pain which was otherwise treated with radiotherapy and analgesics (Wong and Wiffen 2002). Bisphosphonates have also been described to have effects on tumor cell adhesion, invasion and proliferation with increased tumor cell apoptosis and impeded matrix metalloproteinase activity in an in vitro setting(Neville-Webbe, Rostami-Hodjegan et al. 2005) (Winter, Holen et al. 2008, Holen and Coleman 2010, Holen and Coleman 2010).

Bisphosphonate schedule	N	Significant findings	Reference
Breast			
Clodronate 1600 mg/day vs. placebo	173	Hypercalaemic events (28 vs. 52, P < 0.01); vertebral fractures (84 vs. 124 per 100 patient years, P < 0.025); overall SREs (218.6 vs. 304.89 per 100 patient years, P < 0.001)	(Paterson, Powles et al. 1993)
Chemotherapy alone vs. chemotherapy plus pamidronate 45 mg iv	295	Time to PD in bone increased by 48% (249 vs. 168 days, P = 0.02). Significant pain relief in 44% vs. 30% (P = 0.025)	(Conte, Latreille et al. 1996)
Pamidronate 90 mg iv vs. placebo	382	Median time to 1st SRE 13.9 vs. 7.0 months (P < 0.001). Proportion of patients experiencing a SRE during 24 months treatment 50% vs. 70% (P < 0.001)	(Hortobagyi, Theriault et al. 1998)
Pamidronate 60 mg iv vs. placebo	404	Time to skeletal progression 11.8 vs. 8.4 months (P < 0.01).	(Hultborn, Gundersen et al. 1999)
Pamidronate 90 mg iv vs. placebo	372	Median time to 1st SRE 10.4 vs. 6.9 months (P < 0.049). Proportion of patients experiencing a SRE during treatment 55% vs. 67% (P < 0.027). SMR 2.4 vs. 3.8 (P = 0.008)	(Theriault, Lipton et al. 1999)
Ibandronate 50 mg po vs. placebo	564	Reduced risk of skeletal event (HR 0.62, 95% CI = 0.48, 0.79, P = 0.0001)	(Body, Diel et al. 2004)
Ibandronate 2/6 mg iv vs. placebo	467	2 mg dose ineffective. With 6 mg SMR 2.18 vs. 1.61 (P = 0.03)	(Body, Diel et al. 2003)
Zoledronic acid 4 mg iv vs. placebo	228	Median time to 1st SRE median not reached vs. 364 days (P = 0.007). Proportion of patients experiencing a SRE during treatment 30% vs. 50% (P = 0.003). 39% reduction in number of SREs.	(Kohno, Aogi et al. 2005)
Myeloma			
Chemotherapy plus clodronate 2.4 g po od vs. chemotherapy plus placebo	350	2 year PFS of bone lesions 24% vs. 12% (P = 0.026)	(Lahtinen, Laakso et al. 1992)
Chemotherapy plus pamidronate 90 mg iv vs. chemotherapy plus placebo	392	Proportion of patients experiencing a SRE during treatment 24% vs. 41% (P < 0.001)	(Berenson, Lichtenstein et al. 1998)
Chemotherapy plus clodronate 1600 mg po od vs. chemotherapy plus placebo	536	Proportion of patients experiencing non-vertebral fracture 6.8% vs. 13.2% (P = 0.04) and vertebral fracture 38% vs. 55% (P = 0.01)	(McCloskey, MacLennan et al. 1998)
Chemotherapy plus clodronate 1600 mg po od vs. chemotherapy plus zoledronic acid	1960	5.5 month improvement in median survival with zoledronic acid (p = 0.04). 16% reduction in risk of death (p = 0.012). 24% risk reduction in SREs with ZOL (p = .0004)	(Morgan and Lipton 2010, Morgan, Davies et al. 2010)
Prostate			
Hormone therapy plus clodronate 2080 mg po od vs. hormone therapy plus placebo	311	Improved overall survival with clodronate (hazard ratio [HR] 0·77, 95% CI 0·60-0·98; p = 0·032).	(Dearnaley, Mason et al. 2009)
Pamidronate 90 mg iv vs. placebo	378	No significant overall benefit regarding bone pain or SREs	(Small, Smith et al. 2003)
Zoledronic acid 4/8 mg vs. placebo	643	Zoledronic acid 4 mg significantly reduces skeletal morbidity: proportion of patients experiencing a SRE during treatment 33% vs. 44% (P = 0.021). Time to skeletal progression median not reached vs. 321 days (P = 0.011).	(Saad 2002)
Other Solid Tumours			
Zoledronic acid 4/8 mg iv q4w vs. placebo	773	Zoledronic acid 4 mg significantly reduces skeletal morbidity: proportion of patients experiencing a SRE during treatment 38% vs. 47% (P = 0.039). Median time to 1st SRE 230 vs. 163 days (P = 0.023)	(Rosen, Gordon et al. 2003)

Table 1.4 Efficacy of bisphosphonates on skeletal morbidity in randomized and placebo-controlled trials of patients with bone metastases secondary to cancer *(SREs = skeletal-related events; PD = progressive disease; SMR = skeletal morbidity rate; PFS = progression-free survival) (Adapted from Coleman 2008)*

In clinical studies, randomized open study of clodronate treatment was associated with a 55% reduction in metastasis in women compared to control groups with better survival effects. These data suggested that effect on survival for several years following 2 years of therapy was analogous to the effects of radiotherapy administered for short courses (Diel, Solomayer et al. 1998, Diel, Fogelman et al. 2007). Powles and colleagues had results mirroring the work of Diel and colleagues in another large double blind placebo controlled study where two years of clodronate treatment (1600mg daily) reduced the risk of bone metastasis by 31% (Powles, Paterson et al. 2002).

In fact an impact on mortality reduction was also observed in clodronate treated patients by as much as 23-26% (Powles, Paterson et al. 2006). Moreover zoledronate i.v. infusion at 4mg every 6 months when administered to post-menopausal women with early breast cancer resulted in a 36% reduction in disease progression and reduced mortality (Gnant, Mlineritsch et al. 2009). Observations in this study also pointed at a potential extra skeletal anti-tumor effect of Zol since a reduction in the contra-lateral breast cancer was seen, consistent with observations made in animal models by Ottewell and colleagues (Ottewell, Deux et al. 2008, Ottewell, Monkkonen et al. 2008). Mode of such mechanisms of bisphosphonates requires further investigations.

1.9.1 Bisphosphonates and mortality

Bisphosphonates have been widely used in treatment of patients with hip fractures or osteoporosis in both men and women. Initial treatment was administered in the form of etidronate (Storm, Thamsborg et al. 1990, Watts, Harris et al. 1990, van Staa, Abenhaim et al. 1998) which was then replaced by alendronate (Liberman, Weiss et

al. 1995, Black, Cummings et al. 1996, Bone, Hosking et al. 2004), risedronate (Reginster, Minne et al. 2000, McClung, Geusens et al. 2001), ibandronate (Chesnut, Skag et al. 2004) and now use of zoledronate (Black, Delmas et al. 2007) is most prevalent as it is the most potent of the BPs. The most interesting and particularly striking feature of studies where BP was administered for treatment of fragility fractures and osteoporosis was the effect on mortality and lifespan.

Fractures are the key cause of morbidity and mortality in patients with age and nearly 10-20% patients die within a year of hip fractures and mortality is associated with vertebral and non-vertebral fractures (Center, Nguyen et al. 1999, Sambrook and Cooper 2006). Systemic reviews on osteoporosis treatments to fracture patients have proven effective in reducing the incidence of these vertebral and non-vertebral fractures however they also had a positive effect on mortality in patients (MacLean, Newberry et al. 2008).

The study by Lyles et al showed that annual treatment with zoledronate administered within first 3 months after hip fracture reduced the incidence of any new clinical fracture by 35% in patients compared to placebo. Secondary analysis revealed that 101 of 1054 patients in Zol treatment died compared to 141 out of 1057 in placebo group, defining a 28% reduction in mortality (Lyles, Colon-Emeric et al. 2007, Lyles, Colon-Emeric et al. 2007). In this study, only 8% of the observed mortality benefit was explained by prevention of subsequent fractures suggesting that fracture prevention plays only a small proportion in the reduced mortality rates and that there must be other mechanisms contributing to survival (Eriksen, Lyles et al. 2009, Colon-Emeric, Mesenbrink et al. 2010). Although the occurrence of infectious disease like pneumonia, cancer, and cardiovascular disease was similar in both the placebo and

Zol treated patients; however the Zol treated group had decreased mortality rates that were in part also attributed to reduced occurrence of these conditions. One of the main limitations of this study was that study patients were younger and healthier than the general population of patients with hip fractures. Moreover no evaluations of spinal radiographs were carried out, suggesting an underestimated prevalence of vertebral fractures. Despite the limitations, these data establish the possibility of Zol's ability to improve an individuals' ability to cope with and endure under conditions of stress and acute illness (Colon-Emeric, Mesenbrink et al. 2010).

In favor of the above clinical study, a meta-analysis of randomized placebo controlled trial, showed a 10% reduction in mortality in older patients (high risk of fragility fractures) who received osteoporotic treatments (Bolland, Grey et al. 2010).

Center et al performed a longitudinal study in older women with and without fractures and demonstrated a 69% reduction in mortality patients on BPs, however in case of men there was a non-significant reduction in mortality. In fact based on information from randomized control trials, a Bayesian analysis did reveal a true relation between BP therapy and mortality a 35% mortality risk reduction in women and a 29% risk reduction in men associated with BPs was observed (Center, Bliuc et al. 2011). Treatment is this study was not randomly allocated as this was an observational study with the bias of self-selection and varying health. Moreover there were variabilities in BP treated individuals compared to other treatments specifically factors such as age, height, lower BMD, lower weight, fractures in presence of lower BMD and greater height. More importantly since a Caucasian group was considered in this study, the results cannot be generalized to other ethnic groups.

More over hip fracture patients on oral bisphosphonates treatment, over a 5 year follow up; showed a 27 % reduction in mortality risk compared to non-users (Beaupre, Morrish et al. 2011, Sambrook, Cameron et al. 2011). All these data on reduced mortality risks point at a new mechanism of effect of bisphosphonates that requires further investigation.

Extended lifespan has been achieved by other chemical interventions as well. For example, treatment with aspirin has shown to inhibit oxidative damage and extend lifespan in male mice (Hsu and Li, 2002; Strong, Miller et al. 2008). Treatment with Metformin has shown to decrease DNA damage levels, activate ATM and extend lifespan in mice when treated at young and middle age (Anisimov, Berstein et al. 2011). Treatment with rapamycin has late age (600 days) has shown extension of median and maximal lifespan in mice. These could be due to delaying death caused by cancer or due to delay in mechanisms of ageing (Harrison, Strong et al. 2009). Zoledronate in combination with statins has been employed as treatment for Hutchinson-Gilford progeria syndrome (HGPS) which is characterized by accelerated ageing. Varela et al investigated the effects of combined treatment of pravastatin (act on HMGCoA pathway) and zoledronate (act on mevalonate pathway) on *Zmpste24*-null progeroid mice and found a marked improvement in HGPS like symptoms (Varela, Pereira et al. 2008). The drug increased life span of mutant mice by 80% and showed a substantial recovery of the progeroid phenotype i.e. an increase in fat deposit and body weight, restoration of bone density and a reduction in curvature of spine and hair loss (Varela, Pereira et al. 2008). At cellular level this was shown to be associated with reduction in occurrence of DNA damage. This raised the possibility of BPs having an effect at cellular level, not only in a diseased condition like in HGPS but

also in normal cells specially the stem cells that are known to maintain tissue homeostasis in response to stress/ injury or disease. This study raised the possibility of a new role of BPs as anti-ageing drug which may explain some of the clinical observations made on mortality. In fact mesenchymal stem cells are progenitors of osteoblasts and adipocytes and an essential component of the hematopoietic stem cell (HSC) niche (Sacchetti, Funari et al. 2007, Mendez-Ferrer, Michurina et al. 2010). MSC undergo cellular ageing with accumulation of DNA damage. This leads to impairment of osteoblastic differentiation both in vitro and *in vivo* (Alves, Munoz-Najar et al. 2010) (Cmielova, Havelek et al. 2012, Wang, Chen et al. 2012) and therefore they can be considered a good model to test our hypothesis.

1.10 Hypothesis

The main hypothesis of this thesis was that:

'Bisphosphonates extend stem cell lifespan by protecting them from incidence of DNA damage and enhance tissue regeneration'

To test this hypothesis my objectives were:

To determine whether BPs extend lifespan of human MSC used as a model of ageing by enhancing DNA repair

To determine signaling pathways associated with lifespan and enhanced repair

To determine whether the action of BPs on stem cells was present *in vivo* in zebrafish and mice models

Chapter 2: Materials and Methods

All equipment, reagents and protocols used in this research were assessed for health and safety precautions following guidelines for Control of Substances Hazardous to Health (COSHH) assessments. All appropriate safety measure was employed including latex examination gloves and laboratory coat.

2.1 Chemical treatments

Zoledronate (Zol), alendronate (Aln) and risedronate (Ris), was dissolved in phosphate buffer saline (PBS, Gibco, Paisley, UK) at 1.45mg/ml to obtain a 5mM concentration and further diluted to the required concentrations in mesenchymal stem cell medium (described in section 2.2). Enantiomeric pair of pyrindine bisphosphonates, PG-1014491 (1R,6S)-isomer of cis-2-azabicyclo[4.3.0] nonane-8,8-diphosphonic and PG-1014493 (1S,6R)-isomer of cis-2-azabicyclo[4.3.0] nonane-8,8-diphosphonic (kindly provided by Prof. Robert Boeckman University of Rochester, Rochester, NY) were also dissolved in PBS and further diluted to obtain the required concentration of 1µM in MSC medium for *in vitro* studies. The blinded provision of these drugs helped validate the robustness of the experimental plan assessing specific involvement of farnesyl pyrophosphate synthase enzyme in DNA repair. Trans, trans farnesol (FOH, Sigma Aldrich, Dorset, UK) and Geranylgeraniol (GGOH, Sigma Aldrich, Dorset, UK) were dissolved in ethanol (VWR International, Leicestershire, UK) at 7.33mg/ml and 9.58mg/ml respectively to obtain a 33mM concentration and further diluted to a final concentration of 33µM in MSC medium for *in vitro* studies. The mammalian target of Rapamycin (mTOR) inhibitors; Rapamycin (Enzo Life Sciences, Exeter, UK) and KU-0063794 (Selleck, Suffolk, UK) were dissolved in di-methylsulfoxide (DMSO, Sigma) and used at a final concentration of 10nM for *in vitro* and *in vivo* studies while the

phospho-inositol-3 kinase (PI3K/AKT) inhibitor, LY294002 (Cell Signalling, Denver, USA) was dissolved in DMSO and further diluted to a final concentration of 10µM.

2.2 Culture of human mesenchymal stem cells

2.2.1 Isolation of mesenchymal stem cells from bone marrow

Human mesenchymal stem cells (hMSC) were derived from bone marrow (BM) harvested from the iliac crest of young patients (2-15 years old) undergoing osteotomy for reasons other than metabolic disorders at Sheffield Children's Hospital, UK (provided by Mr Sanjeev Madan and Mr James Fernandes). Bone marrow was obtained following informed written parental consent in accordance with local research ethical committee approval and the declaration of Helsinki. The bone marrow was collected in MSC medium composed of Dulbecco's Modified Eagle's Medium (DMEM; Gibco) supplemented with 10% Fetal Bovine Serum (FBS Hyclone, Thermo Scientific, Northumberland, UK). Additional supplement of 0.01% of penicillin/streptomycin (Sigma, Dorset, UK), and 0.1% heparin was used in the collection of bone marrow. Bone marrow mononuclear cells (MNC) were isolated by density gradient centrifugation at 800g for 20mins using lymphocyte separation medium (1.077g/L, PAA Laboratories, Somerset, UK). Based on increasing density, the top layer is the plasma and platelets, followed by the mononuclear cells (MNC) concentrate also called the 'buffy coat', then there is the transparent layer of the lymphocyte separation medium and finally the basal layer of the red cells and granulocytes (Fig 2.1). The MNC fraction was collected and after two washes with PBS (Gibco, UK) the cells were counted using 5% acetic acid on a haemocytometer. Cells were plated at 8000 MNC/cm^2 in MSC medium without any antibiotics and incubated at 37˚C in 5% carbon dioxide in air. After 48hrs the non-adherent cells

were removed. The cells were regularly monitored and the medium was changed twice weekly until cells were confluent.

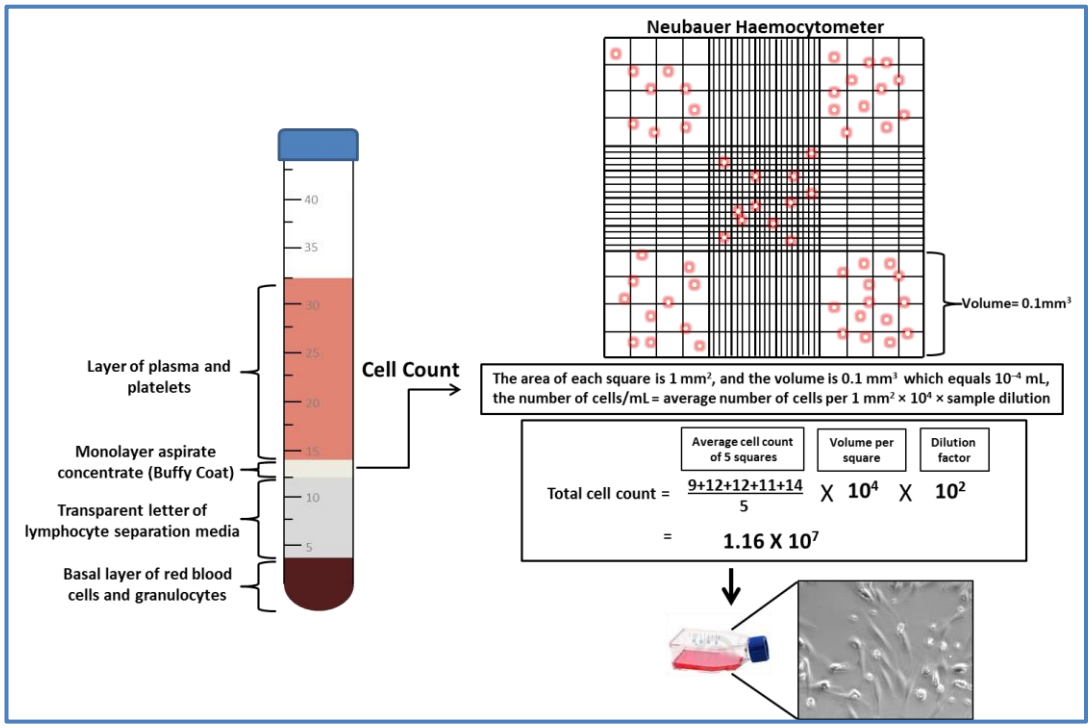


Figure 2.1 Isolation and culture of human mesenchymal stem cells from bone marrow

Total bone marrow aspirate was centrifuged and based on density the different layers were obtained of which the hMSC were isolated from the 'buffy coat' layer. Cells were counted using a Neubauer Haemocytometer and seeded in a tissue culture flask. The cells are plastic adherent and spindle shaped as shown in the image.

2.2.2 Human MSC Culture and Growth kinetics

When hMSC cultures were 90% confluent, they were harvested by incubating in 0.5% Trypsin-1mM ethylene diamine tetra-acetic acid (EDTA; Gibco) and live cells were counted using the trypan blue exclusion method using a Neubauer haemocytometer (Fig 2.1). The dead cells take up the blue dye and were excluded from the count. The cells were re-plated at 1000cells/cm^2 and grown to confluence. This constituted one passage (p1). Cultures were fed twice weekly. Depending on experimental plans

media was supplemented with or without the addition of different treatment groups that were diluted in media to obtain the desired final concentrations.

In case of growth kinetics experiments the number of population doublings (PD) was calculated as log N/Log2, where N is a ratio of number of cells at confluence to the number of cells at the start of culture.

2.2.3 Storage of hMSC

For storage of hMSC, cells were frozen in freezing medium contained 90% fetal calf serum (Gibco) with 10% DMSO and stored in cryo-vials in the -80˚C overnight and twenty fours later transferred for long-term storage in liquid nitrogen. When needed, the frozen vials were defrosted in a 37˚C water bath after which the cells were spun down at 800g for 5mins at room temperature and re-plated.

2.3 Immuno-staining of hMSC and Fluorescent activated cell sorting (FACS)

The identity of hMSC was confirmed by employing cluster of differentiation (CD) markers. Briefly, 10^5 hMSC cells were incubated for 30mins on ice in presence of PBS + 5% FCS (Gibco) with optimized amount of antibody or isotype matched control (Table 2.1). The cells were washed twice with 4 ml of ice cold PBS + 5% FCS by centrifugation at 800g for 5 minutes at 4°C. Thereafter cells re-suspended in PBS containing 2% paraformaldehyde (VWR) and 2% fetal bovine serum (FBS) (Gibco) were stored at 4°C until FACS was performed. For flow cytometry analysis, the cells were acquired using FACS calibre (Becton, Dickinson) and analyzed employing the Cell Quest Software. Prior to acquisition, the required events and parameters were assigned depending on the experiment. A dot plot was selected for information on

the forward scatter (measure of cell size) and side scatter (measure of cell granularity). These were set in linear scales.

Antigen	Fluorochrome	Isotype	Quantity	Company
CD45	Allophycocyanin (APC)	APC conjugated Immunoglobulin G1 (IgG1)	1µg/10^6 cells	Caltag, UK
CD29	R-Phycoerythrin (R-PE)	R-PE conjugated IgG1	1µg/10^6 cells	Caltag, UK
CD31	Fluorescein isothiocyanate (FITC) conjugated	FITC conjugated IgG1	2µg/10^6 cells	BD Pharmingen
CD34	R-Phycoerythrin (R-PE)	R-PE conjugated IgG1	1µg/10^6 cells	Caltag, UK

Table 2.1 Antibodies used for the characterization of human MSC

For the fluorescence markers the respective FL parameters set on log scale were selected as follows: FL1- for measure of Fluorescein isothiocyanate (FITC) fluorescence intensity, FL2- for measure of R-phycoerythin (R-PE) fluorescence intensity and FL4- for measure of Allophycocyanin (APC) fluorescence intensity. Once all parameters were selected the cells containing the isotype were first acquired repeatedly to adjust voltage such that in the histogram the selected FL parameter appeared within the first log. The compensation was assessed only if required as it allowed adjusting the spectral overlay when samples stained with two or more fluorophores would appear as signal in the detector for another fluorochrome. After acquisition, analysis was performed by overlaying the isotype and test histograms and selecting histogram stats option to obtain percentage overlay.

2.4 Colony forming unit-fibroblast (CFU-F)

The assay for the number of colony forming units fibroblast (CFU-F) generated from established cultures was obtained by plating hMSC at 10cells/cm^2 in duplicates. Plates were incubated for 14 days at 37°C in 5% carbon dioxide in air. Cultures were stained using Wright's Giemsa stain (VWR, UK). Briefly, the cells were fixed with methanol (Fisher Scientific, Loughborough, UK) for 5mins followed by Wright's Giemsa stain for 5mins. The stain was washed under running tap water and plates were left to dry in air. The purple stained colonies consisting of a minimum of 50 cells (Mohanty, Cairney et al. 2012) with a center of origin were counted as individual colony forming unit-fibroblast (CFU-F) under an inverted light microscope (Fig 2.2A).

2.5 Colony forming unit-osteoblast (CFU-O)

The assay for the number of colony forming unit osteoblasts (CFU-O) was obtained by plating 20 cells/cm^2 from established cultures in duplicates in MSC medium supplemented with osteogenic supplements composed of 0.05 mM L-Ascorbic Acid (Sigma Aldrich, St. Louis, USA), 10mM β glycerol-phosphate (Sigma Aldrich, St. Louis, USA) and 100nM dexamethasone (Sigma Aldrich, St. Louis, USA). Cells were maintained for 14 days at 37^{o}C in 5% CO_2 in air and fed twice weekly. At day 14 colonies were stained for alkaline phosphatase (ALP) enzymatic activity using 86R alkaline phosphatase kit (Sigma Aldrich) according to the manual instructions. Briefly, the cells were fixed with citrate-acetone formaldehyde fixative. Thereafter the cells were incubated in the dark for 15mins after addition of alkaline dye constituting of dizonium salt solution (sodium nitrate solution added to FRV alkaline solution) mixed with naphthol AS BI solution. The cells were rinsed with water and counterstained with haematoxylin (Sigma). The colonies comprising of at least 40 cells (Mohanty,

Cairney et al. 2012) with a definite center of origin and stained for ALP were considered as one CFU-O and scored under an inverted light microscope (Fig2.2 B).

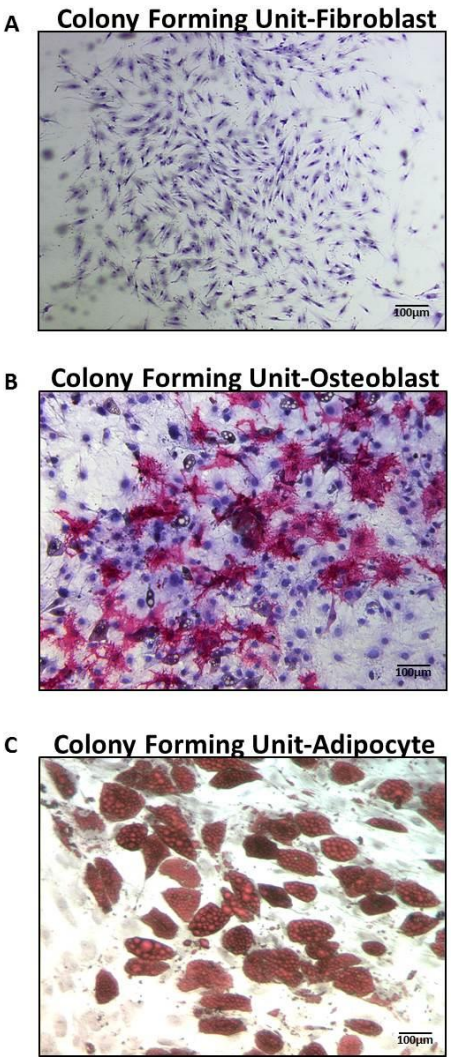


Figure 2.2 Clonogenic assays for hMSC

Representative examples of colony forming unit (A) fibroblast, (B) osteoblast and (C) adipocyte.

2.6 Colony forming unit-adipocyte (CFU-A)

Progenitors with adipogenic potential were enumerated by Colony forming unit adipocyte (CFU-A) assay. Human MSC from established cultures were plated at

limiting dilutions ranging from 10^5 to $6.25X10^3$ (8wells/dilution) at final volume of 100µl/well of MSC medium in a 96 well plate and cultured for 3 days at 37°C in 5% CO_2 in air. Thereafter, medium containing adipogenic supplements constituting of 100nM dexamethasone (Sigma Aldrich) and 100 µg/ml 3-isobutyl-1-methylxanthine (IBMX, Sigma Aldrich) was added and cultures were fed twice weekly for up to 2 weeks. The cells were stained with Oil Red O (Sigma) to detect lipid vacuoles. Briefly, the cells were fixed with 10% formaldehyde for 1 hour. Oil Red O solution was added on cells and left for 10mins after which cells were counterstained with haematoxylin. The plates were viewed under an inverted light microscope for presence of lipid vacuoles (Fig 2.2C). A well was considered positive if it contained more than 20 red stained cells. The percentage of negative cells was scored at different dilutions of the cells and this data was used to calculate the frequency of CFU-A following Poissons distribution using the formula $F_0=e^{-x}$ where F_0 is the fraction of colony negative wells, e is the constant whose value is 2.71 (Wu, Liu et al. 2005) and x is the number of colony forming units per well.

2.7 Differentiation of hMSC

Human MSC seeded at $1.2X10^3$ and $2.8X10^3$ per cm^2 were induced to undergo osteogenic and adipogenic differentiation respectively using osteogenic and adipogenic supplements as specified in sections 2.4 and 2.5 (Mohanty, Cairney et al. 2012). Cells were fed twice weekly either with osteogenic medium or with adipogenic medium and after 2 weeks, total ribonucleic acid (RNA) was extracted to prepare cDNA and carry out quantitative Polymerase Chain Reaction (qPCR) to assess markers of osteogenic and adipogenic differentiation.

2.7.1 Total RNA extraction

Total RNA was extracted using RNAqueous 4PCR Kits (Ambion, Warrington, UK) and following manufacturer's instructions. Briefly, hMSCs differentiated towards osteogenic and adipogenic lineages were treated with lysis solution and equal volume of 64% ethanol to precipitate the nucleic acids. The lysate/ethanol mix was poured through a filter cartridge by centrifugation. The filter cartridge was washed once with wash solution #1 and twice with wash solution #2. The RNA was finally eluted from the cartridge by centrifugation in a microfuge at 13,000 rotations per minute (rpm) into a fresh collection tube by adding pre-heated elution solution. To completely remove traces of DNA, the extracted RNA was treated with DNase 1 and incubated at 37^{o}C for 30 minutes. DNase inactivation reagent was added and thereafter the mixture was centrifuged for a minute to pellet the DNase inactivation reagent and RNA was transferred to a new tube.

The concentration and purity of total RNA obtained was quantified by measuring its absorbance (A) at 260nm and 280nm using a Nano-drop 1000 spectrophotometer (Thermo Scientific, Northumberland, UK). The concentration of nucleic acid was calculated using the Beer-Lambert law. The equation for absorbance (A) is 'A= εlc' where 'A' is the absorbance 'ε' is the specific extinction coefficient, 'c' is the sample concentration and 'l' is the length of the pathway the light travels through the absorber which is specific for each spectrophotometer. An absorbance reading of 1 at A_{260} is equivalent to 40ug/ml hence this absorbance was used to determine the RNA concentration in an unknown sample. A ratio of A_{260} to A_{280} values was used as an indicator of RNA purity and the sample was considered 'clean' or free of contamination when the ratio was in the range of 1.8 to 2.1.

2.7.2 Reverse transcription and cDNA preparation

Total RNA was used for reverse transcription using the 1st Strand complementary DNA (cDNA) kit (GE Healthcare, Amersham, UK). Briefly, 2ug of RNA sample was mixed with RNAse-free water to make a final volume of 20ul and heated at 65°C for 10 minutes followed by incubation on ice. Following denaturation of RNA the bulk first strand cDNA reaction mix containing 4 different deoxynucleotide triphosphates (dNTPs), 1ul dithiothreitol (DTT) solution and 1ul Not I-d (T) 18 primer (1 in 25 dilution in RNAse-free water) was added and mixed thoroughly by pipetting. The mixture was incubated at 37°C for 1 hour to allow cDNA strand synthesis. The cDNA was stored at -20°C for a short period and long term storage was achieved at -80°C. For each sample, an RNA sample without reverse transcriptase (RT) was prepared to control for DNA contamination. A no RNA control was also included in which RNAse free water substituted the RNA in order to detect any contamination in the reagents.

2.7.3 Primer

The Taqman primer design system (Primer express, Applied Biosystems) was used to obtain primer sequence. All sequences were checked using blast programmes in NCBI for potential non-specific binding (Genbank-NCBI BLAST, Nucleotide BLAST) and were found to be unique to the gene of interest in humans. The list of primer sequences tested in this project is enlisted in table 2.2. All primers were ordered from Invitrogen (Paisley, UK) and were stored as a concentrated stock of 1mM in DNase RNase free water (Gibco) at -20°C and used at a final concentration of 0.1µM.

2.7.4 Real time quantitative polymerase chain reaction (qPCR)

Quantitative real-time polymerase chain reactions were performed using SYBR green PCR Master Mix (Eurogentec, Romsey, UK) and 0.1 µM primers (Table 2.2).

PRIMERS	SEQUENCE
L-32 forward	5'-GGGAGAGACACCGTCTGAACA-3'
L-32 reverse	5'-GAACCACGATGGTCGCTTTC-3'
RUNX2 forward	5’-CACTATCCAGCCACCTTTACTTACAC-3'
RUNX2 reverse	5’-TAGTGAGTGGTGGCGGACATAC-3'
OSX forward	5'-CATCTGAGCCGGGTGGAA-3'
OSX reverse	5'-TTGGCAAGCAGTGGTCTAGAGA-3'
OPN forward	5’-AATTGCAGTGATTTGCTTTTGC-3'
OPN reverse	5’-GAACTTCCAGAATCAGCCTGTTTAA-3'
OC forward	5’-CAATCCGGACTGTGACGAGTT-3'
OC reverse	5’-CCTAGACCGGGCCGTAGAAG-3'
ALP forward	5’-CCCGTGGCAACTCTATCTTTG-3'
ALP reverse	5’-GCCATACAGGATGGCAGTGA-3'
PPAR-γ forward	5’-TGGGTGAAACTCTGGGAGATT-3'
PPAR-γ reverse	5’-TTTCTTGTGATATGTTTGCAGACAGT-3'
LPL forward	5’-TTGTGAAATGCCATGACAAGTCT-3'
LPL reverse	5’-CATGCCGTTCTTTGTTCTGTAGA-3'

Table 2.2 Sequence of primers used in real time quantitative PCR

For the standard curve known concentrations (50ng, 5ng, 0.5ng and 0.05ng) of human genomic DNA (Promega, Southampton, UK) were prepared by serial dilutions and stored in aliquots to avoid freeze/thaw cycles. Real time-qPCR was performed in triplicates at final volume of 10ul volume that contained 2ul template DNA, 1ul each

of forward and reverse primer at 1µM concentration, 5ul SYBR GREEN 2X qPCR Mix (Eurogentec, Belgium) and 1ul distilled water. Each reaction mix was added to a 384 well PCR plate that was sealed after preparation with a transparent film. The plate was inserted into the 7900HT Real-Time-PCR System (Applied Biosystems). The reaction conditions employed for the PCR involved 1 cycle of 50˚C for 2 min, 95˚C for 10 min; 40 cycles of denaturation at 95˚C for 15 seconds and annealing at 60˚C for 1 min. This was followed by a dissociation stage where the conditions were 95ºC for 15 seconds, 60˚C for 15 seconds and 95˚C for 15 seconds to generate a melting curve for verification of specificity of the amplification product.

Presence of a single peak suggested a single amplification product. In the control wells in absence of template no product formation signified by absence of peak suggested no primer dimer formation (Fig 2.3 A). Every qPCR experiment was conducted 3 times. Analysis was performed using SDS 2.2.1 software utilizing the standard curve method following the guidelines under ABI prism 7700 Sequence Detection System (Fig 2.3 B). A standard curve was determined using the genomic DNA serial dilutions. The amplification plot generated showed the number of cycles of PCR reaction on the X-axis and the change in fluorescence on Y-axis. The cycle threshold (C_T) is defined as the cycle number at which the fluorescence from a sample passes the fixed threshold. Therefore a lower C_T value indicates that there is higher amount of the target and therefore the sample passes threshold level much sooner. High C_T values meant that higher amplification cycles were required to reach the fluorescence intensity threshold due to lower amount of sample cDNA. The C_T threshold was set to lie in the middle of the linear region of the logarithm phase where the increasing amplification was exponential. The baseline was kept at the

setting of 3-15 cycles where no change in fluorescence above the background level was observed. The reaction efficiency was calculated from the Standard genomic DNA curve with log of amount of DNA on X axis and mean Ct on Y axis was in the expected range of -3.3 to -3.8. The reaction efficiency (E) is given by

$$\boldsymbol{E} = 10^{\left(-\frac{1}{gradient}\right)}$$

The relative expression quantities of all the genes were normalized to housekeeping gene L32 to plot the graphs.

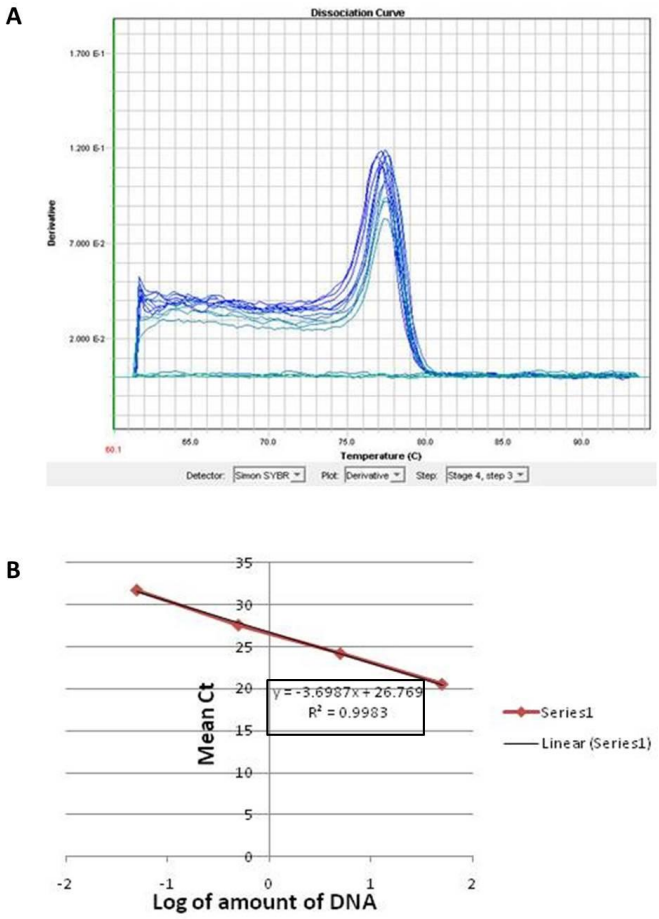


Figure 2.3 Analysis of qPCR efficiency

(A) Single peak dissociation curve obtained while testing primer binding ability. This is obtained when primers anneal at a single point on template resulting in single amplification product. (B) Standard genomic DNA curve with log of amount of DNA on X axis and mean C_T on Y axis showing a good curve with efficiency of reaction measured to be -3.6.

2.8 Culture of cancer cells

Murine myeloma cell lines 5T33 and 5TGM1 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 media with Glutamax (Gibco) supplemented with 10% FCS (Sigma), penicillin (1units/ml) and streptomycin (1µg/ml) (Sigma), 0.01mM sodium pyruvate (Gibco) and 1mM non-essential amino acids (NEAA). Human prostate cancer cell line PC3 cells were maintained in DMEM with Glutamax supplemented with 10%FBS, penicillin (1units/ml) and streptomycin (1µg/ml). Murine prostate cancer cell line 178-2 BMA cells were maintained in DMEM with Glutamax

supplemented with 10%FBS, penicillin (1units/ml), streptomycin (1µg/ml), 0.01mM sodium pyruvate and 1% of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid or HEPES (10mM, Life Technologies, Inc. Gaithersburg, MD). Human breast cancer cell line MDA-MB-231 cells were cultured in RPMI 1640 supplemented with 10% FBS (Sigma), 0.01mM sodium pyruvate and penicillin (1units/ml)/streptomycin (1µg/ml). All cells were incubated at 37°C in 5% CO_2 in air.

2.9 Clonogenicity of cancer cells

Colony-forming ability of non-adherent 5TGM1 and 5T33 myeloma cancer cell line was assessed by incubation of 10^6 cells in Roswell Park Memorial Institute (RPMI) 1640 complete media for 72hrs. The drugs were washed off and the cells were then added to methylcellulose medium (StemCell Technologies, Manchester, UK). The cell suspension was plated in 24 well plates and maintained in a 37˚C 5% CO_2 in air. After 14 days, colonies consisting of more than 40 cells were directly scored using an inverted microscope. In case of the other cancer lines (PC3, 178-2 BMA and MDA-MB-231) colony forming unit (CFU) assay was performed as described in section 2.4 plated at 35cells/cm^2 in their respective media and incubated at 37˚C in 5% carbon dioxide in air. At day 14 cultures were stained with Wright's Giemsa stain (VWR International) and the purple stained colonies were counted using an inverted light microscope (Leica Microsystems, UK). Colonies containing a minimum of 40 cells were considered as one colony forming unit.

2.10 Induction of DNA Damage in hMSC and cancer cells by Ionizing Irradiation

Five thousand hMSC or adherent cancer cells were suspended in 200µl of respective medium and seeded on sterile cover slips in petridish. All the petridish were placed

on a sterile tray and incubated for 1 hour at 37˚C in 5% carbon dioxide in air. The cells were then covered with respective medium with addition of appropriate treatments and incubated for 3 days at 37˚C in 5% carbon dioxide in air. In case of non-adherent cancer cells, 10,000 cells were fixed on slides using a cytospin centrifuge (1000rpm, 1min soft spin at acceleration and brake set on 6) after fixing the cells in 4% PFA for 15mins on ice.

DNA damage was induced by exposing the cells to ^{137}Cs Gamma source. The cells were subjected to different doses of irradiation (1Gy, 3Gy, 5Gy and 7Gy) and after irradiation the cells were incubated at 37˚C in 5% carbon dioxide in air for different durations (4hrs, 12hrs, 24hrs, 48hrs and 72hrs) depending on the experiment.

2.10.1 Detection of DNA damage by immuno-staining

DNA damage was induced by exposing cells to a ^{137}Cs Gamma source. DNA double strand breaks were identified by phosphorylation at serine 139 site on histone2AX (γH2AX) staining. Briefly, cells were fixed with 4% para-formaldehyde, permeabilized with 0.5% Triton-X (Sigma, UK) and blocked with 5% normal goat serum (DAKO, Glostrup, Denmark) in PBS, followed by incubation overnight at 4°C with primary antibody, anti-phospho-histone H2AX (Ser139) (Millipore, Massachusetts, USA) used at 1:800 in 5% normal goat serum. Cells were then incubated in anti-mouse Fluorescein Isothiocyanate (FITC) conjugated secondary antibody (Insight Biotechnology, Santa Cruz, USA) at 1:200 in PBS for 1 hour at room temperature. Slides were mounted using VectaShield containing 4', 6-diamidino-2-phenylindole (DAPI) to stain the nuclei blue. Cells were viewed using a 63X objective in an Inverted Zeiss LSM 510 NLO microscope (Fig2.4). To compile the distribution of gamma-H2AX throughout the nuclei in one image, 15 2D images for each field with a 0.3µM z-axis

between two slices was obtained. Maximum intensity projection was established to determine the number of foci per nuclei which was manually counted by two independent operators, unaware of the various treatments. The DAPI stained nuclei helped established a mask for the region within which the foci were enumerated. Seventy five randomly selected nuclei were imaged and DNA damage γH2AX foci were scored using ImageJ 1.45 software (http://rsbweb.nih.gov/ij/). Initial experiments to enumerate foci following irradiation were also repeated by 3 independent students as well, to confirm the image acquisition and quantification methodology. All experiments were performed in duplicates using three or more independent MSC cell cultures from different donors.

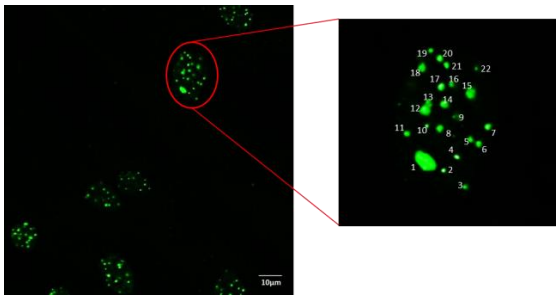


***Figure* 2.4 Enumerating DNA damage foci using confocal microscopy**

Panel on the left represents a field of hMSC with γH2AX green foci and the panel on the right shows the enumeration of one highlighted nucleus using ImageJ software.

2.10.2 Comet Assay

Human MSC and cancer cells were irradiated at 3Gy and DNA damage was assessed by single-cell gel electrophoresis alkaline comet assay using Trevigen assay kit (Abingdon, UK) according to manufacturer's instructions. Briefly, cells were washed in cold PBS and re-suspended in 0.5% low melting point agarose (37˚C) spread evenly at a density of 500 cells on comet slides, lysed and subjected to electrophoresis under alkaline condition on a horizontal electrophoresis apparatus (1V/cm) for 30 mins. Slides were stained with ethidium bromide and imaged with a fluorescent

microscope. The Comet IV software was used to quantify the percentage of DNA in tail and tail moment, from which the Olive tail moment was determined. DNA damage was scored by classification of tail content in five categories (CC1-CC5) based on increasing tail moment. Overall score was calculated as percentage of cells in each comet category for each treatment with CC5 signifying maximum DNA damage.

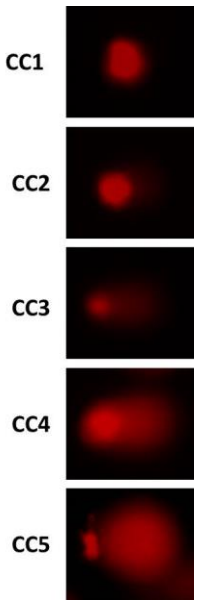


Figure 2.5 Comet tail categories

A representation of the categories of comet tails divided into CC1-CC5 groups based on measure of the comet tail moment starting with the shortest tail signifying lowest content of damaged DNA in CC1 and highest in CC5.

2.11 Protein expression by Western Blotting

2.11.1 Extraction of total protein lysates from hMSC

Three times 10^6 hMSC were washed with PBS and spun at 3000 rpm for 3 minutes. The cell pellet was re-suspended in 500 µl of mammalian cell lysis buffer (Mammalian cell lysis kit, Sigma-Aldrich, Dorset, UK) containing 250mM Tris- 5mM EDTA, 750mM sodium chloride, 0.5% sodium dodecyl sulphate, 2.5% deoxycholic acid and 5% Igepal supplemented with 10 µl of proteinase inhibitors cocktail (Sigma-Aldrich, Dorset, UK) containing 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF),

pepstatin A, bestatin, leupeptin, aprotinin and trans-epoxysuccinyl-L-leucyl-amido(4-guanidino)-butane (E-64) and 10 µl phosphatase inhibitors cocktail (Sigma) containing sodium orthovanadate, sodium molybdate, sodium tartrate and imidazole. The lysate was incubated for 15 minutes on a rotating shaker at 4°C and then spun at 12,000 rpm for 10 minutes. The supernatant was collected in a pre-chilled Eppendorf and stored at -20°C in aliquots for further use.

2.11.2 Extraction of nuclear and cytoplasmic protein lysates

Human MSC were harvested from flasks in 1ml of PBS by scraping with a rubber scraper and collected in Eppendorf tubes to centrifuge for 5mins at low speed (3000rpm). The respective buffers used for compartmentalizing the nuclear and cytoplasmic extracts are described in table 2.3. After two consecutive washes with buffer A (w/o NP40, a detergent) the pellet was re-suspended in buffer A (w/ NP40) and incubated for 10mins at 4˚C. The lysate was spun down at 15,000g for 5mins and supernatant was collected as cytoplasmic extract and stored in buffer C at -20˚C. The pellet was re-suspended in buffer C and incubated for 15mins at 4˚C. The lysate was spun down at 15,000×*g* for 10 min and the resulting supernatant was saved as first nuclear fraction.

BUFFER A (w/o NP40)	BUFFER A (w/ NP40)	BUFFER C
Hepes (10mM)	Hepes (10mM)	Hepes (20mM)
KCl (10mM)	KCl (10mM)	KCl (500mM)
Dtt (0.5mM)	Dtt (0.5mM)	Dtt (0.5mM)
H_2O	NP 40(1%)	Glycerol (20%)
--	H_2O	EDTA (0.2mM)
--	--	PMSF (0.5mM)
--	--	$MgCl_2$ (1.5mM)
--	--	H_2O

Table 2.3 Composition of buffers for extraction of nuclear and cytoplasmic compartment

2.11.3 Bicinchonic Acid (BCA) protein quantification Assay

The BCA assay was performed to quantify the concentration of proteins in each sample. Briefly BCA (Sigma Aldrich) was diluted with copper II sulphate ($CuSO_4$; Sigma Aldrich; 1:50) and this solution was added to the sample protein containing lysis buffer (1:2 dilution). The copper Cu^{2+} ions reduce to Cu^{+} ions in the presence of proteins and each Cu^{+} ion is chelated by two molecules of BCA. This resulted in a green purple color product which was quantified on a plate reader at 560nm wavelength (Spectramax M5, Sunnyvale, USA) and values extrapolated to the standard protein curve. The standard curve was generated using different concentrations of bovine serum albumin (BSA) (Life Technologies, Paisley, UK) ranging from 0 to 10 μg/ml diluted in mammalian cell lysis buffer (Sigma) to a final volume of 10μl in duplicates in a 96-well plate.

2.11.4 Western blotting

Protein lysates (40 μg) were diluted in equal volume of 2X Laemmli buffer (Gibco) containing Dithiothreitol (DTT, Sigma, UK). The samples were heated at 95°C for 5 minutes, after which they were quickly transferred on ice ready to be loaded on a 10% or 12% Tris-glycine gel (Table 2.4). The gel was casted on mini protean 3 cell equipment (Biorad Laboratories, Hempstead, UK). Briefly, separating gel consisting of distilled water, 1.5M Tris-HCL pH 8.8 (Sigma), 10% sodium dodecyl sulphate (SDS) (Sigma), 30% acrylamide (Sigma), 10% Ammonium persulphate (APS) and N,N,N'N' Tetra-methylethane-1,2-diamine (TEMED) was loaded in gel casting stand and allowed to set for 10-15 minutes. Thereafter stacking gel composed of distilled water, 0.5M Tris-HCL pH 6.8, 10% SDS, 30% Acrylamide, 10% APS and TEMED was added and a 9 or 10 well comb was inserted to prepare wells. The gel was allowed to set for 10-15mins. Thereafter the comb was removed and samples were loaded and run at 150V for 60 minutes in running buffer (0.25M Tris, 150mM Glycine, 1% SDS).

Separating gel

REAGENTS	10%	12%
Distilled water	4.05	3.35
1.5M Tris-HCL; pH-8.8	2.5	2.5
10% SDS	0.1	0.1
30% Acrylamide	3.3	4
10% APS	0.05	0.05
TEMED	0.05	0.05

Stacking gel

REAGENTS	12%
Distilled water	6.1
0.5M Tris-HCL; pH-6.8	2.5
10% SDS	0.1
30% Acrylamide	1.3
10% APS	0.1
TEMED	0.01

Table 2.4 Volume of reagents (ml) for 10% and 12% tris-glycine gels

Electro-blotting was carried out by wet transfer using either nitro-cellulose membrane (Amersham Biosciences, UK) or Immobilon-P membranes (Millipore Corp., Billerica, MA, USA). The membrane and gel were inserted between 4 sponges, 3 pieces of 3mm papers (Whatman Schleicher and Schuell, UK) and soaked in transfer buffer (0.25M Tris, 150mM Glycine and 10% methanol). The membrane was blotted at 70V for 70 minutes.

After electro-blotting, membranes were blocked using 5% dry milk in 0.1% Tween 20 in PBS (PBS-T; BDH, Poole, UK) for all antibodies with the exception of RAP1A, γH2AX p-FOXO3A, FOXO3A, p-ATM and ATM, which were blocked in 5% Bovine Serum Albumin (Sigma, UK) in 0.1% Tween 20. Membranes were then incubated with primary antibodies anti-mTOR, AKT, P70S6K, FOXO3A and their phosphorylated forms p-mTOR, p-AKT, p-P70S6K, p-FOXO3A plus p-ATM (Cell Signaling Technology, Denver, USA) at 1:1000. Glyceraldehyde 3 phosphate dehydrogenase (GAPDH, Abcam, Cambridge, UK) was used at 1:30000, Lamin B1 (Invitrogen, Paisley, UK) and RAP1A (SantaCruz, Middlesex, UK) at 1:3000 and βACTIN (Abcam, Cambridge, UK) at 1:10000. Secondary HRP-conjugated anti-mouse and anti-rabbit IgG were use at 1:30000 and 1:1000 respectively (DAKO, Glostrup, Denmark). Detection was performed using ECL reaction kits according to manufacturer's instruction (GE Healthcare, Buckinghamshire, UK) and quantification of the signal was performed using ImageJ 1.45 software (http://rsbweb.nih.gov/ij/).

2.12 Knockdown of FOXO3a using small interfering RNA

Human MSC were cultured in 24 well plates ($5X10^4$ cells/well) up to 50% confluence and transfected with FOXO3a siRNA (5'-CAUGCAAAGUGACUAACAA-3'; 3'-UUGUUAGUCACUUUGCAUG-5') at 100nM (Cell Signalling) or non-silencing siRNA (5'-CGUACGCGGAAUACUUCGA-3'; 3'-UCGAAGUAUUCCGCGUACG-5') using transfection reagent Lipofectamine 2000 (Invitrogen, Paisley, UK) according to manufacturer's instructions. Cells were either treated with Zol (1µM) or PBS 6 hours following addition of siRNA and 72 hr later sacrificed to confirm knockdown of FOXO3a by either western blotting as described above or immunofluorescence. For analysis by confocal microscopy hMSC were fixed with 4% paraformaldehyde for 10mins at room temperature. Cells were permeabilised in 0.5% TritonX-100, blocked with PBS containing 2% BSA, and incubated with antibody specific to FOXO3a (1:300) or ATM-pS1981(1:200) followed by Cy3 (red) conjugated anti-rabbit (1:200) and Alexa488 (green) conjugated anti-mouse (1:200) secondary antibodies. Slides were mounted using Vecta Shield containing 4', 6-diamidino-2-phenylindole (DAPI) to stain the nuclei blue. Cells were viewed using a 63X objective in an Inverted Zeiss LSM 510 NLO microscope. The images were analysed using the NIH ImageJ 1.45 software (http://rsbweb.nih.gov/ij/) Co-localisation of the two proteins was shown as the merged images.

2.13 *In vivo* studies using Zebrafish embryos

Wild-type zebrafish from AB background were maintained in the zebrafish aquarium facility of the University Of Sheffield, UK and raised as specified in The Zebrafish Book (Monte Westerfield, 1993, University of Oregon Press). All assays were terminated within 5.2 days post fertilization due to Home Office regulations. The developmental

stages of zebrafish are shown in Fig 2.6. After spawning embryos were collected at the 16 cell stage (~1.5 hours post fertilization, hpf) and grown in E3 embryo medium (15mM NaCl, 0.5mM KCl, 1mM $CaCl_2$, 1mM $MgSo_4$, 1.5mM KH_2PO_4, 0.05mM Na_2HPO_4, and 0.7mM $NaHCO_3$) at 28.5°C in petridishes. The chorionic layer was punctured carefully using a needle when embryos were incubated with either zoledronate (1µM), FOH (33µM), GGOH (33µM), rapamycin (10nM), KU-0063794 (10nM) or LY294002 (10 µM). Dechorionation or removal of the chorion layer was carried out using forceps under the microscope.

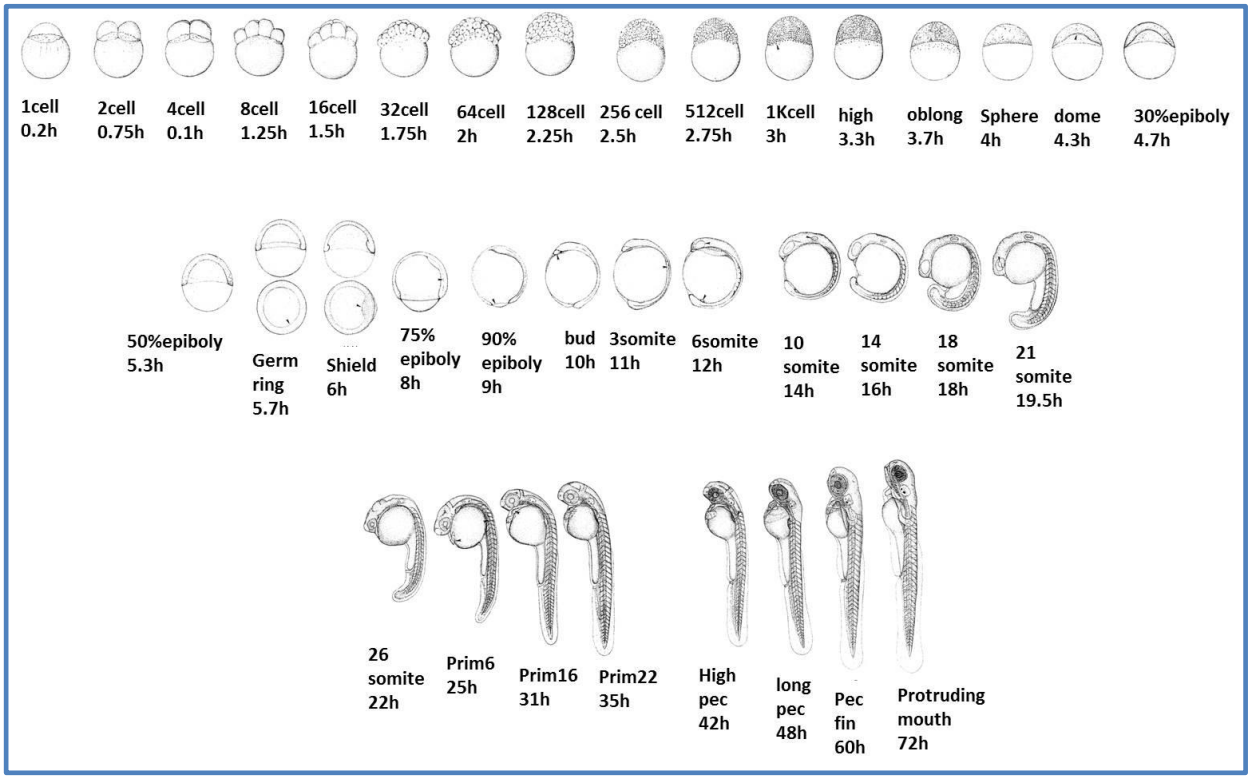


Figure 2.6 Developmental stages of zebrafish embryo

Adapted from The Zebrafish Book (Monte Westerfield, 1993, University of Oregon Press)

DNA damage was induced by exposing the embryos to different doses of gamma irradiation (1Gy, 3Gy, 5Gy using ^{137}Cs source). Post irradiation zebrafish tail was amputated using a sharp blade. The embryos were left to incubate at 28.5°C and 120hpf the embryos were anaesthetized, fixed and image was captured using

ProgRes® C14plus camera (Jenoptik AG, Jena, Germany). Embryos were anaesthetized by adding 4% Tricaine solution (400mg Tricaine powder 97.9ml H_2O, 2.1ml 1M Tris pH9, buffered to pH7) before being fixed in 4% paraformaldehyde overnight and stored in 100% glycerol. Tail length was measured taking the anal region as the starting reference point and the end of fin fold as the end point of measurement (Fig 2.7). The measurements were carried out by two independent operators and the treatments were kept unknown at the time of measurement.

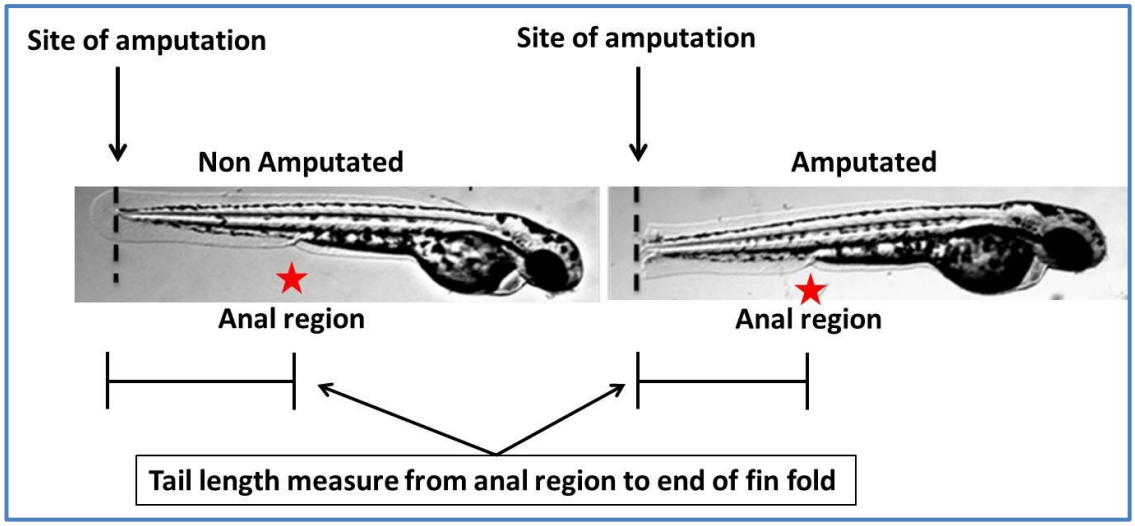


Figure 2.7 Measurement of zebrafish tail fin length following amputation

A representative image of the zebrafish non-amputated (left panel) and amputated (right panel) at the site of amputation (dashed line). The tail length was measured from the anal region (red star mark) to the end of the fin fold.

Preparation of protein lysates from embryos involved removal of yolk sacs using deyolking buffer composed of cold Ringer's solution (Calcium Free; 116mM NaCl, 2.9mM KCl, 5mM HEPES, set pH at 7.2), 0.3mM PMSF and 1mM EDTA. By pipetting the yolk sacs were removed and embryos were washed in Ringer's solution and snap frozen in liquid nitrogen followed by homogenization in lysis buffer containing

protease and phosphatase inhibitors. Following quantification of protein, western blots were performed as described in Section 2.11.

2.14 *In vivo* studies using C57Bl/6 mice

All *in vivo* mice experiments were performed according the protocols approved on the personal and project license. Different study plans were submitted and after approval from the home office the experimental procedures were carried out.

2.14.1 Detection of tissue unprenylation *in vivo* in C57Bl/6 mice

C57Bl/6 mice were injected with zoledronate (125µg/kg) or PBS and 3 days later sacrificed by concussion to the brain accompanied with cervical dislocation as a confirmation of death. All tissues were snap frozen in liquid nitrogen and collected for extraction of protein lysates following homogenization in lysis buffer containing protease and phosphatase inhibitors. Western blotting was carried out against RAP1A antibody as described in section 2.12.

2.15 Immuno-staining of γH2AX in murine tissue following irradiation

Mice (C57BL6/J) were injected with either zoledronate (single dose, i.p.,125µg/kg) or PBS. On day 3, post-injection, mice received whole body irradiation (3Gy, n=6/group) using ^{137}Cs Gamma source. Twelve hours post irradiation mice were sacrificed for all soft tissues and bone tissue that were fixed in 4% paraformaldehyde for 24 hours followed by storage in 70% ethanol. When mice were injected with zoledronate (125µg/kg) or PBS and 3 days later irradiated at 12Gy using 137Cs Gamma source, two batches were sacrificed at either 24 hours post irradiation (n=6) or 4 days later. The mice sacrificed 4 days post irradiation required a bone marrow transplant within

24 hours post irradiation as per protocol guidelines on the project licence. Post sacrifice intestine tissue was fixed, wax embedded and sectioned.

All specimens (including soft tissues and decalcified bone) were placed in histological cassettes, washed in PBS and processed following standard histology protocols (performed by Bone Analysis Lab, Mellanby Centre for Bone Research, Sheffield). Tissues embedded in paraffin wax were sectioned at 3-5µm thickness and mounted onto HEPES coated glass microscope slides. In case of intestines, the tissues were cut into lengths of 0.5cm per piece and cross sections of five intestinal pieces were taken representing 5 levels or regions along the length of the intestine. Each section was about 3-5µm thick. The slides were dewaxed in xylene (BDH, Leister, UK) and rehydrated by passing through a series of ethanol dilutions. Heat induced antigen retrieval in citrate buffer was carried out. The sections were then probed with primary antibody, anti-phospho histone H2AX (Ser139) (Millipore, Massachusetts, USA) at 1:800 in diluent, and incubated overnight at 4°C. Secondary anti mouse IgG Fluorescein Isothiocyanate (FITC) conjugated (Insight Biotechnology, Santa Cruz, USA) was used at 1:200 in PBS for 1 hour incubation at room temperature. The cover-slips were mounted on slides with mounting media (VectaShield) containing 4', 6-diamidino-2-phenylindole (DAPI) to stain the nuclei. Cells with double stranded breaks showed green foci in the nuclei. Tissue sections were viewed using an Inverted Zeiss LSM 510 NLO microscope equipped with Argon (Ar) laser (488nm) 30mW to image the fluorescent marker FITC and UV lamp to image DAPI stained nuclei. Eight to ten randomly selected fields consisting of 400 cells in total were randomly selected from a tile scan (20X) and then scored for cells containing γH2AX DNA damage foci, using

ImageJ 1.45 software (http://rsbweb.nih.gov/ij/). Cells were considered positive for presence of DNA damage when they showed >5 foci/cell.

To stain for detection of LGR5 expressing crypt stem cells, tissue sections were treated similarly but blocked in 5% donkey serum followed by incubation in LGR5 primary antibody (Abgent, Maidenhead, UK) 1:50 dilution overnight at 4˚C. The sections were incubated in donkey anti rabbit Cy3 secondary antibody at 1:200 dilution. Nucleus was stained using DAPI in mounting media and viewed under the confocal microscope. The number of LGR5 positive cells present per crypt was enumerated in total of 150 crypts over 5 levels. The number of crypts containing LGR5 positive stem cells was also enumerated in 175 crypts over 5 levels.

In case of double staining for γH2AX and LGR5 C57BL6 mice were treated as described above but subjected to 3Gy irradiation. This was due to the high level of damage in vehicle treated animals at 12Gy, which was too high and made difficult the enumeration of DNA damage foci. Twelve hours following irradiation intestinal tissues were collected. Following heat induced antigen retrieval using Citrate buffer pH6.0 sections were incubated in special Mouse on Mouse IgG blocking reagent (Vector Labs, Peterborough, UK) followed by blocking solution containing 3% donkey and 2% goat serum in PBS. Stained was then performed as described above.

2.16 Enumeration of CFU-F and CFU-O in C57Bl/6 mice following irradiation

Mice were injected with zoledronate (125µg/kg) or PBS and 3 days later irradiated at 12Gy using ^{137}Cs Gamma source. After 24 hours mice were sacrificed and bone marrow was flushed out from the femurs and tibiae. Briefly, the femur and tibiae were dissected and collected in DMEM (Gibco) supplemented with 10% FBS (Gibco),

10% heparin (Royal Hallamshire Hospital pharmacy, Sheffield, UK) and 0.25% penicillin-streptomycin (Gibco). The bones were stripped of muscle using sterile forceps and gauze cloth in a laminar air flow cabinet. Once the bones were cleaned, the ends were trimmed to facilitate the flushing of BM cells from the bone. Using a sterile syringe and 21G needle, the bone marrow was flushed into murine MSC complete medium (Stem Cell Technologies, Vancouver, Canada). To assay the number of murine CFU-F and CFU-O, freshly isolated murine bone marrow cells were plated in 6 well culture plates at a density of 52 x 10^3 cells/cm^2 per well in duplicates in 4 ml of murine MSC complete medium or osteogenic differentiation medium respectively. Plates were incubated for 14 days at 37°C in 5% carbon dioxide in air with medium change every 3 days for two weeks. At day 14 the plates were stained as described earlier.

2.17 Statistical Analysis

Statistical analysis was performed on GraphPad Prism 6 version 6.0.4. To obtain statistical significance, all *in vitro* experiments were repeated with n=3 or more donors of MSCs cultures, with the exception of human dermal fibroblasts and cancer cell lines. In case of cell lines, the repeats were carried out with different passages of the cells from the same source. In case of clonogenic assays, immunostaining, western blotting and qRT-PCR, each of the biological repeats were carried out in duplicates or triplicates and the average of the internal repeats were represented as n=1. To obtain statistical significance 3 or more biological repeats were done. In case of *in vivo* experiments, 3-6mice or 15-45 zebrafish embryos were employed to attain statistical significance. Data were analyzed for normal distribution using D'Agostino and Pearson normality test. For multiple comparisons data were analyzed using one

way analysis of variance (ANOVA) test. This was followed by Bonferroni's post-test. When a two way ANOVA test was used, a Sidak's multiple comparison post-hoc tests was performed. For two group comparisons an unpaired student's t-test was performed. All data were expressed as mean ± standard deviation (SD). A difference was stated to be statistically significant if the p value was <0.05 (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).

Chapter 3: Bisphosphonates extend life span in stem/progenitor cells and mature fibroblast by enhancing DNA repair

3.1 Introduction

Mesenchymal stem cells undergo a process of cellular ageing with loss of spindle shaped cellular morphology, proliferation and differentiation potential when undergoing long term expansion in vitro (Baxter, Wynn et al. 2004, Wagner, Bork et al. 2009, Yew, Chiu et al. 2011, Mohanty, Cairney et al. 2012). Ultimately these cells succumb to replicative senescence. This can be triggered by multiple mechanisms, one of which is accumulation of DNA damage which also correlates with loss of multi-potency (Alves, Munoz-Najar et al. 2010, Cmielova, Havelek et al. 2012, Wang, Chen et al. 2012). These changes in MSC following *in vitro* expansion have also resulted in a decline in engraftment potential or heterotopic bone formation capacity (Rombouts and Ploemacher 2003, Agata, Asahina et al. 2010). Since MSC are clinically important in transplants and stem cell based therapies for their ability to maintain bone and blood, it is therefore important to understand mechanisms leading to cellular ageing in MSC and identifying new interventions to delay this process.

Zoledronate, a nitrogen-containing bisphosphonate has shown an extension in lifespan in progeroid mice models of accelerated ageing with remarkable recovery of progeroid phenotypes which was associated with an unexplained reduction in DNA damage at cellular level (Varela, Pereira et al. 2008). This raised the question that can zoledronate extend lifespan in stem cells more so the mesenchymal stem cells which are progenitors of osteoblast and chondrocytes and undergo cellular ageing with loss of multi-potency and accumulation of DNA damage. In this chapter I have tested

whether 'bisphosphonates extend lifespan of hMSC used as a model of cellular ageing following in vitro expansion by enhancing DNA repair'.

3.2 Characterization of human MSC antigenic profile and differentiation ability

Human MSC were isolated and cultured from human bone marrow as described in section 2.1 and 2.2 according to procedures routinely used in our laboratories. To confirm their identity they were tested for their antigenic profile and differentiation ability at passage 2. As expected they (n=5) showed an antigenic profile typical of MSCs i.e. negative for haematopoietic CD45 and endothelial CD31 markers and positive for CD29 and CD105 (SH2, endoglin) by flow cytometry (described in section 2.3). A representative example of antigenic profile of an MSC culture is represented in Fig 3.1 A-D.

Human MSC are multi-potent and therefore should possess the ability to differentiate towards osteogenic and adipogenic lineages. To determine whether the hMSC cultures were able to differentiate to osteogenic and adipogenic lineage, they were exposed to osteogenic and adipogenic supplements respectively (described in section 2.5 and 2.6). After 2 weeks exposure to osteogenic supplements, hMSC cultures (n=5) showed expression of alkaline phosphatase (ALP), a marker upregulated during early osteoblastogenesis. Un-differentiated hMSCs did not stain for alkaline phosphatase expression (Fig 3.1 Ei) whereas a positive ALP expression was characterised by dark pink/red colour staining (Fig 3.1 Eii). Likewise hMSC cultures that were exposed to adipogenic supplements developed lipid filled vacuoles that stained for Oil Red O (Fig 3.1 Fii), whereas cultures that received no supplements just took up the counterstain and had no morphological changes (Fig 3.1 Fi).

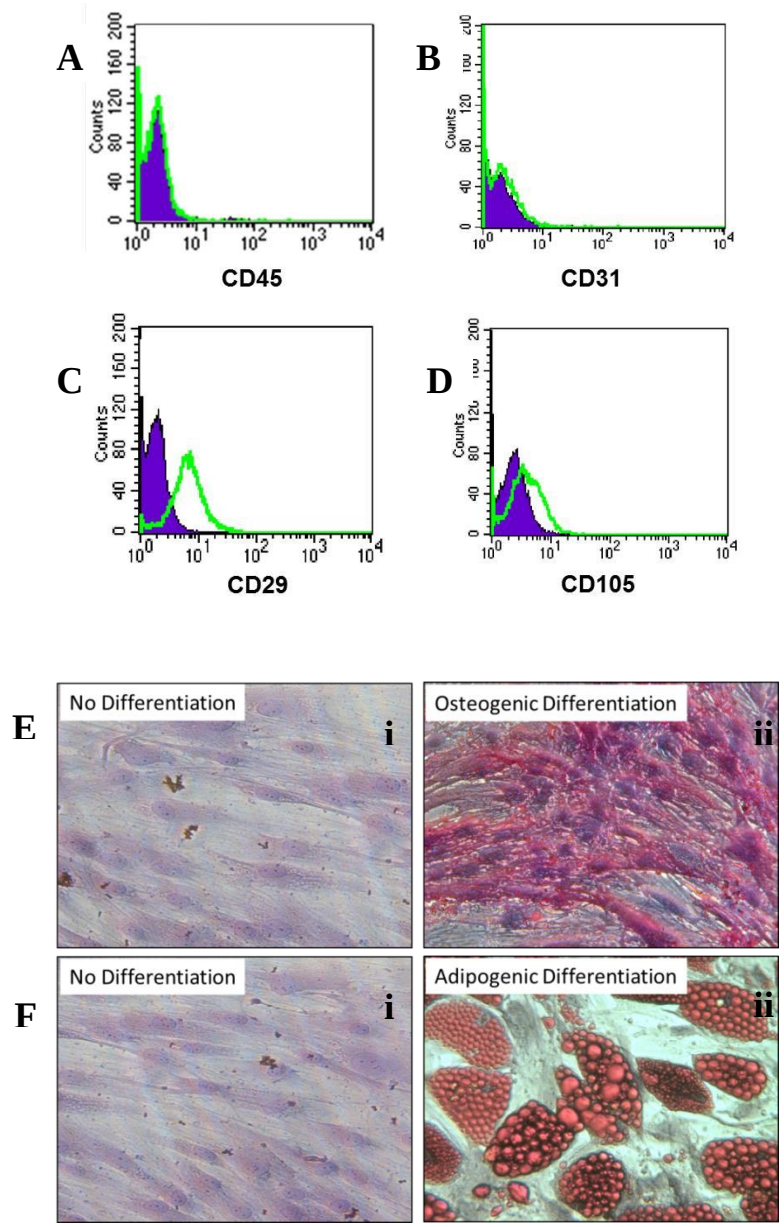


Figure 3.1 Human bone marrow cultures have properties of mesenchymal stem cells

(A-D) A representative example of cluster of differentiation (CD) markers used to identify hMSC isolated from bone marrow. Histograms represent an overlay of hMSC stained with antibodies for (A) CD45, (B) CD31, (C) CD29, (D) CD105 respectively (shown in green) and hMSC cells stained with matched isotype as control (shown in purple) (n=5). (E-F) Representative example of (Ei & Fi) hMSCs in normal medium and those differentiated to (Eii) osteogenic and (Fii) adipogenic lineages with the addition of respective supplements for 14 days (n=5).

3.3 Growth kinetics of hMSC treated with Zoledronate shows extension of lifespan.

To determine whether zoledronate (Zol) extended lifespan of progenitor cells, hMSC (n=9) were expanded in presence or absence of zoledronate (1µM) until they stopped

proliferating for 3 weeks, after which they were considered senescent (Misra, Mohanty et al, 2015). Whilst control cultures (PBS) stopped proliferating after 27-31 population doublings (PD), cultures grown in the presence of Zol proliferated up to 37-42 PD (Fig 3.2 A), thus showing an extension of lifespan. Moreover hMSC cultured in absence of Zol lost their morphological characteristics with time in culture while hMSC expanded in presence of Zol retained the spindle shaped fibroblast morphology for longer time (Fig 3.2 B). In fact Zol treated hMSC achieved 38.4%±9.9% more PD when compared to PBS treated hMSC (n=9; Fig 3.2 C). This was equivalent to over 40 fold increase in the total number of cells generated by hMSC in Zol compared to those maintained in medium with PBS (Fig 3.2 D).

3.4 Zoledronate delays loss of clonogenic progenitors and differentiation ability

Human MSC cultures are heterogeneous and only a fraction of cells is able to show high proliferation ability and form colony forming unit fibroblast (CFU-F) or progenitors with osteogenic and adipogenic potential. All of these progenitors are usually lost with time in culture (Mohanty, Cairney et al. 2012) together with their ability to differentiate. To determine whether Zol preserved the presence of those clonogenic progenitors with time in culture, CFU-F were enumerated by re-plating at low density, hMSC cultures at passage 8. Human MSC cultured in presence of Zol showed a higher content of clonogenic cells (n=6; Fig 3.3 A) when expanded in presence of Zol (CFU-F; Fig 3.3 A) compared to untreated hMSCs. Similarly hMSC cultured in presence of Zol (n=6) at passage 8 showed a significant increase in number of CFU-O and CFU-A when compared to PBS treated hMSC (Fig 3.3 B-C).

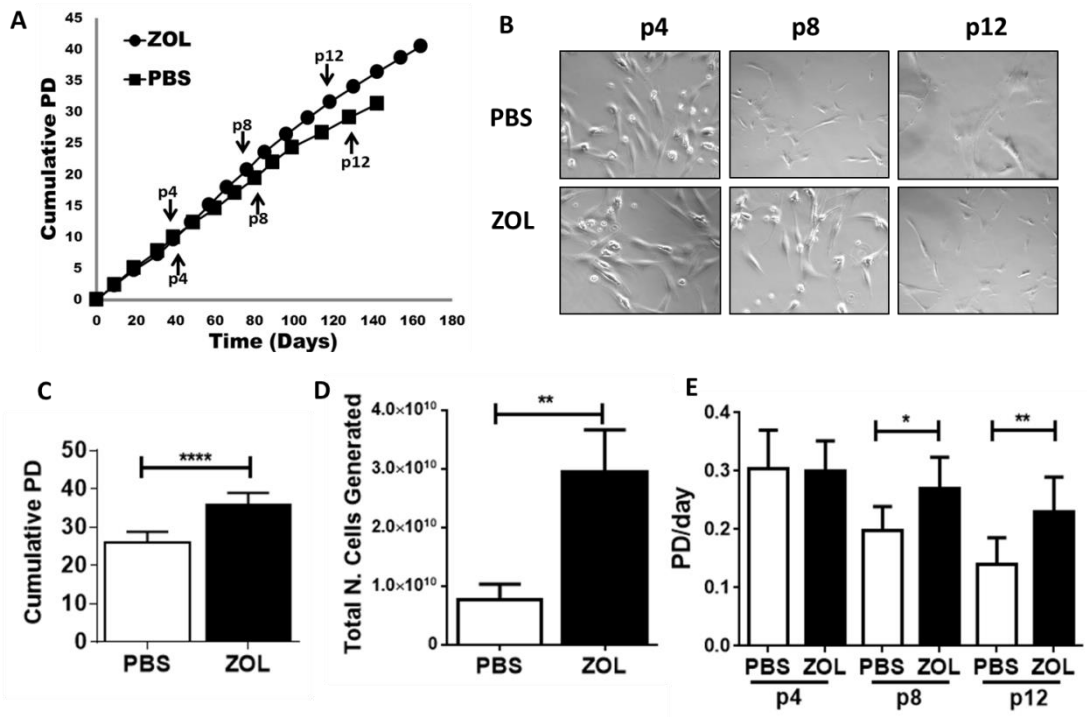


Figure 3.2 Human Mesenchymal Stem Cells (hMSC) cultured in the presence of zoledronate (Zol) show an extension of life span

*(A) A representative example of growth kinetic of hMSC shown as cumulative population doublings (PD) of hMSC with time in culture expanded in presence (circle) or absence of Zol at 1µM (square) (n=9). (B) Representative example of the morphological appearance of hMSC in presence or absence of Zol at passage (p) 4, 8 and 12 respectively as marked with arrow on panel A. (C) Total number of population doublings accomplished by hMSC cultures (n=9) in presence or absence of Zol. (D) Total number of cells generated from the expansion of hMSC cultures (n=9) in PBS or Zol treatment. (E) Population doubling per day in hMSC cultures (n=9) at passage 4, 8 and 12 in PBS or Zol treatment. Data presented as mean ± SD and analysed by student t-tests with Mann Whitney post-hoc test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

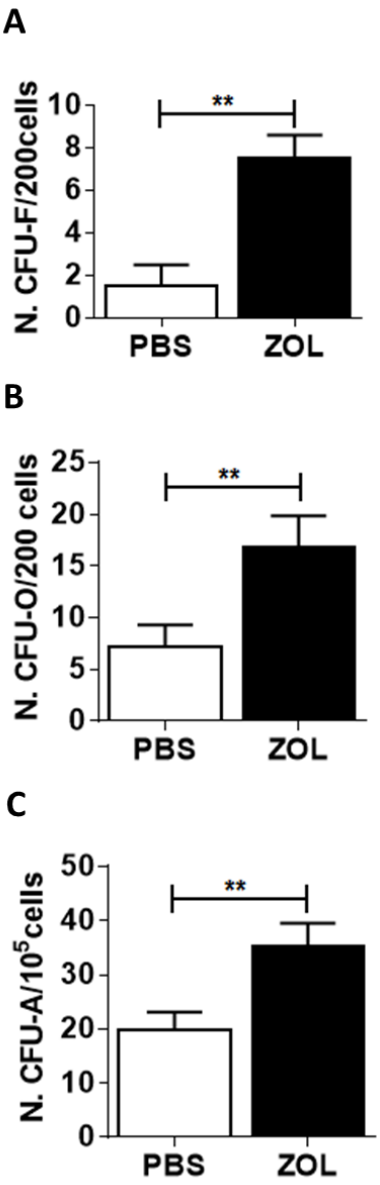


Figure 3.3 Clonogenic potential of hMSC was enhanced with Zol treatment

*(A) Number of Colony Forming Unit-Fibroblast (CFU-F) in hMSC cultures (n=6) at passage 8 in the presence or absence of Zol (1µM) and replated at low density. (B) Number of Colony Forming Unit-Osteoblast (CFU-O) in hMSC cultures (n=6) at passage 8 in presence or absence of Zol and re-plated at low density with osteogenic supplements. (C) Number of Colony Forming Unit-Adipocytes (CFU-A) in hMSC cultures (n=6) at passage 8 in presence or absence of Zol and re-plated with adipogenic supplements. Data presented as mean ± SD and analysed by student t-tests with Mann Whitney post-hoc test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

To determine whether hMSC that were expanded in presence of Zol retained higher ability to differentiate to osteogenic and adipogenic lineages, hMSC (n=3) at passage 8 expanded in presence or absence of Zol were plated in osteogenic and adipogenic supplements and assessed for markers of osteogenesis and adipogenesis by qRT-PCR. A significant increase in expression levels of osteoblast markers, core-binding factor subunit alpha-1 (CBFA1), alkaline phosphatase (ALP), osteopontin (OPN) and osteocalcin (OC) (Fig 3.4 A-D), was seen in cultures expanded in the presence of Zol and induced to differentiate to the osteogenic lineage. Similarly the adipogenic differentiation markers, peroxisome proliferator-activated receptor gamma (PPARγ) and lipoprotein lipase (LPL), were significantly increased in Zol expanded cultures compared to PBS when cultures were exposed to adipogenic supplements (Fig.3.4 E-F). These results suggest that zoledronate extends the life span of hMSC following in vitro expansion and delays the loss of clonogenic and differentiation ability observed with cellular ageing.

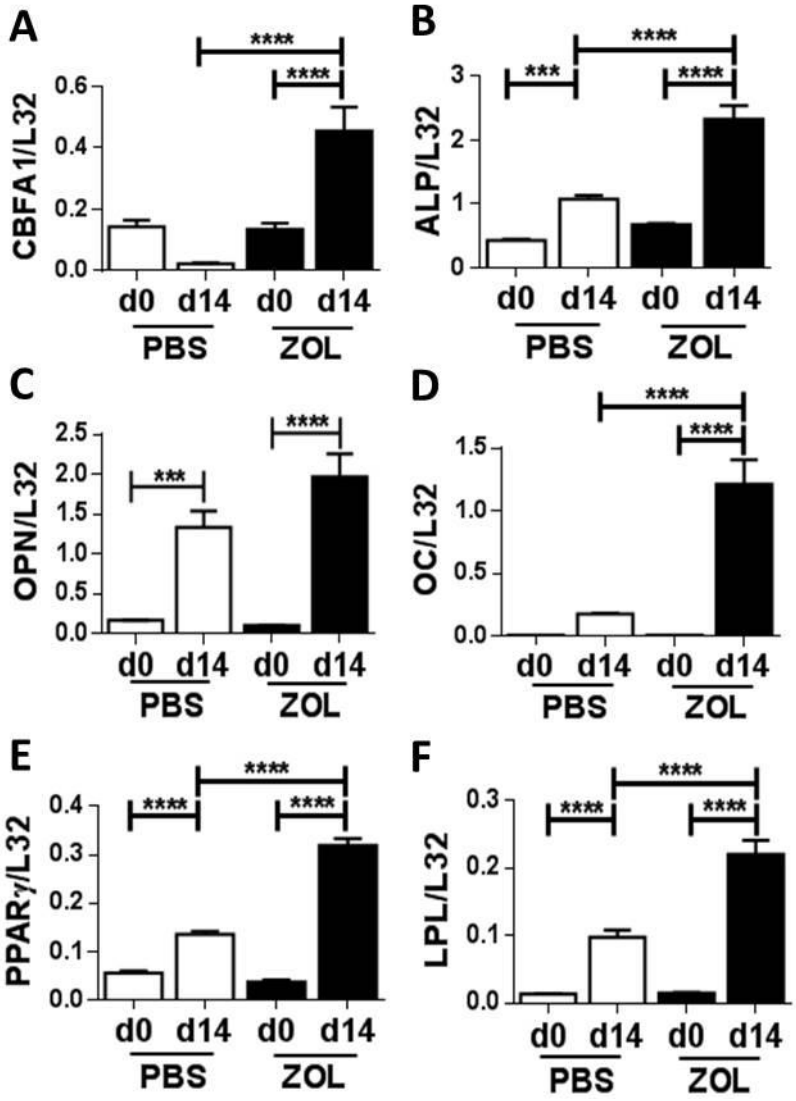


Figure 3.4 Human MSC expanded in presence of Zol showed increased differentiation capacity

*Human MSC cultures (n=3) at passage 8 expanded in presence or absence of Zol (1µM) were re-plated in presence of osteogenic (A-D) or adipogenic (E-F) differentiation supplements for 14 days and assessed for expression of osteogenic differentiation markers (A) core-binding factor subunit alpha-1 (CBFA1); (B) alkaline phosphatase (ALP); (C) osteopontin (OPN); (D) osteocalcin (OC), and adipogenic differentiation markers (E) peroxisome proliferator-activated receptor gamma (PPARγ); (F) lipoprotein lipase (LPL) respectively by qPCR. All markers were normalised to ribosomal protein L32. Data presented as mean ± SD and analysed by one way ANOVA with Bonferroni multiple comparison post-hoc test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

3.5 Zoledronate prevents DNA damage accumulation in hMSC with cellular ageing

In order to determine whether hMSC expanded in the presence of Zol showed decreased accumulation of DNA damage, I quantified the amount of DNA double strand breaks present in the cultures at two different time points. Gamma H2AX (γH2AX) DNA damage foci were enumerated at passage 3 (8-10 PD) and after 10 passages (24-29 PD). Both PBS and Zol treated cultures showed low levels of DNA damage foci at early passage (n=6; Fig 3.5 A-B). Whereas at late passage a significant increase in the number of foci was observed in PBS treated cultures, but not in Zol treated hMSC. These data confirm that hMSC accumulate DNA damage with cellular ageing and show that zoledronate can prevent it.

3.6 Zoledronate enhances DNA repair in hMSC following irradiation and rescues their clonogenicity

To determine whether Zol protects from incidence of DNA damage or enhances the repair process, hMSC were cultured (n=3) for 3days in either zoledronate at 1μM concentration or equivalent amount of PBS. These cells were then subjected to gamma irradiation by exposure to Cs-137 source at doses of 1Gy, 3Gy, 5Gy or 7Gy. Following irradiation, hMSCs were fixed and stained for DNA damage at different time points to enumerate the number of γH2AX foci. No viable cells were observed when cells were exposed at 7Gy and therefore no further work was done at this dose. High levels of DNA damage foci were observed immediately after irradiation at all three doses of irradiation compared to cells that were unirradiated (Fig 3.6 A-D; Appendix Figure 1). No significant difference was found between hMSC treated with either Zol or PBS (n=3). A significant and progressive reduction in the number of foci was observed at 1Gy and 3Gy by as much at 84±2.76% and 40±6.42% respectively. In

contrast at 5 Gy the progressive decrease in foci was significant starting at 12h after irradiation. The decrease was not as pronounced and was 19±2.30%. These results suggested that zoledronate enhanced repair of DNA following damage.

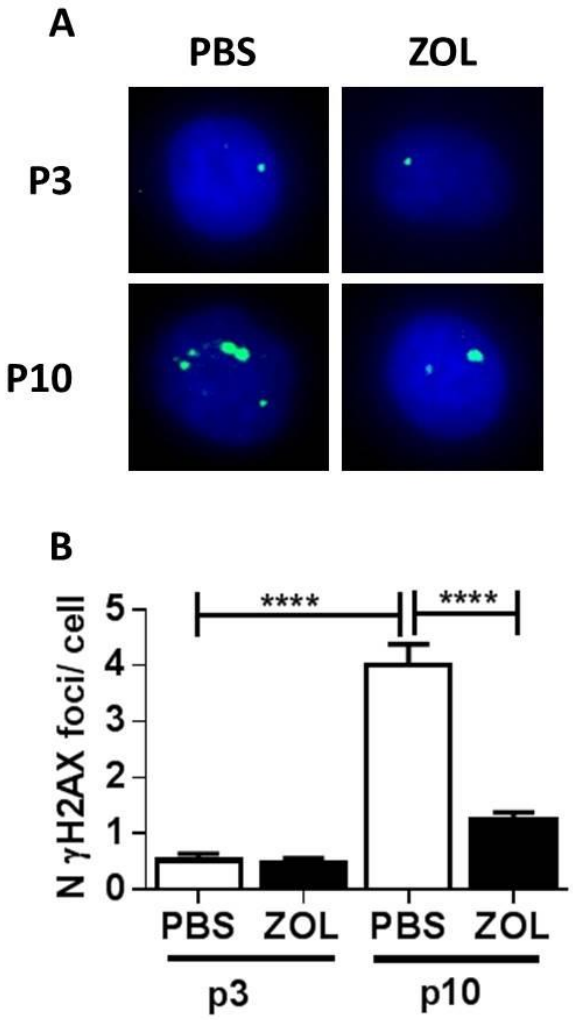


Figure 3.5 Zol reduced incidence of DNA damage accumulation with time in culture

*Human MSC cultures (n=6) at passage 3 and 10 cultured in the presence or absence of Zol (1µM) were fixed and stained for γH2AX DNA damage foci. (A) A representative example of γH2AX foci (green) and DAPI stained nuclei (blue) in PBS and ZOL treated hMSC at early passage (p3) and late passage (p10). (B) Number of DNA damage foci enumerated at passage 3 and 10 in hMSC cultured in the presence or absence of Zol (n=6). Data presented as mean ± SD and analysed by one way ANOVA with Bonferroni multiple comparison post-hoc test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

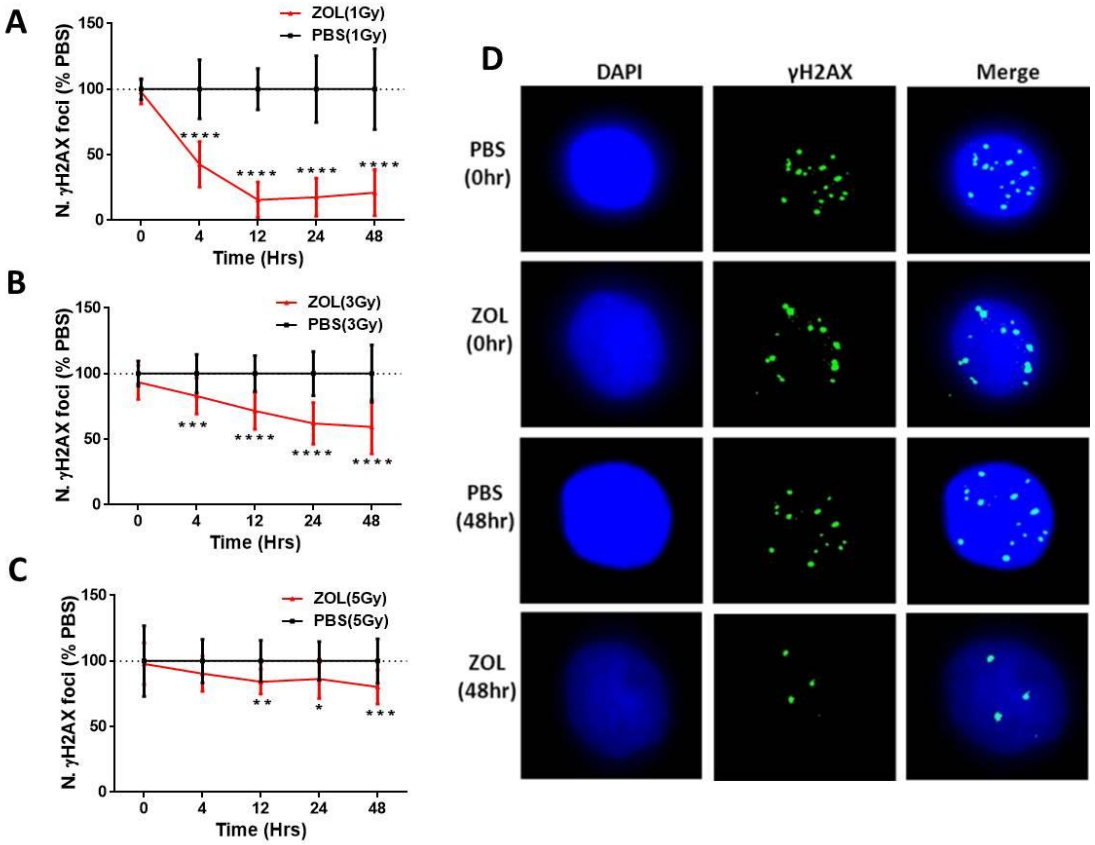


Figure 3.6 Zoledronate enhanced repair of DNA damage induced by exposure to irradiation

*(A-C) Number of γH2AX foci enumerated in hMSC (n=3) cultured for 3 days in the presence or absence of Zol (1μM). Cells were irradiated at (A) 1Gy; (B) 3Gy or (C) 5Gy and assessed at time points 0, 4, 12, 24 and 48h post irradiation at any of the doses. (D) A representative example of γH2AX foci (green) detected in hMSC nuclei (DAPI stained blue) in presence or absence of Zol, immediately (0h) and 48hours post irradiation (1Gy). Data presented as mean ± SD and analysed by one way ANOVA with Bonferroni multiple comparison post-hoc test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

To determine whether the enhanced DNA repair was associated with protection of MSC clonogenicity, CFU-F assay was performed. Human MSC (n=3) from established cultures were plated at low densities and cultured in presence or absence of zoledronate (1µM) for 3 days. The cells were subjected to irradiation at 1Gy, 3Gy or 5Gy. Following irradiation cells were incubated for another 12 hr in presence of zoledronate and then incubated for 14 days in CFU-F medium (Fig 3.7 A-C). Clonogenic ability of hMSC was significantly reduced in hMSC when cells were exposed to 1Gy, 3Gy and 5Gy (Fig 3.7 A-C; Appendix Figure 2) irradiation. However no loss of CFU-F activity was observed if hMSC were irradiated in presence of Zol thus suggesting that Zol protected their clonogenic ability.

3.7 Zoledronate protects hMSC proliferation and function following gamma irradiation

To determine whether Zol was able to rescue hMSC lifespan following irradiation, hMSC cultured (n=3) in presence or absence of Zol for 3 days were either subjected to 3Gy dose of irradiation or left un-irradiated. Twelve hours post irradiation the cells were washed free of treatment and expanded in hMSC medium till cultures stopped proliferating for almost 3 weeks when they were considered senescent. Untreated hMSC undergoing irradiation (triangle) showed reduced lifespan (equivalent to 17-20 PD) compared to non-irradiated hMSC (cross, equivalent to 29-31 PD). In contrast Zol treated hMSC (circle, equivalent to 39-42 PD) in absence of irradiation had extended life span compared to un-irradiated hMSC (cross, equivalent to 29-31PD) by 34% (Fig 3.8 A). Moreover hMSC undergoing irradiation in presence of Zol (diamond, equivalent to 31-34 PD) extended lifespan compared to irradiated hMSC (triangle equivalent to 17-20 PD) by 32%. These data together suggested a consistent

extension of lifespan of hMSC in presence of Zol in both presence and absence of irradiation exposure.

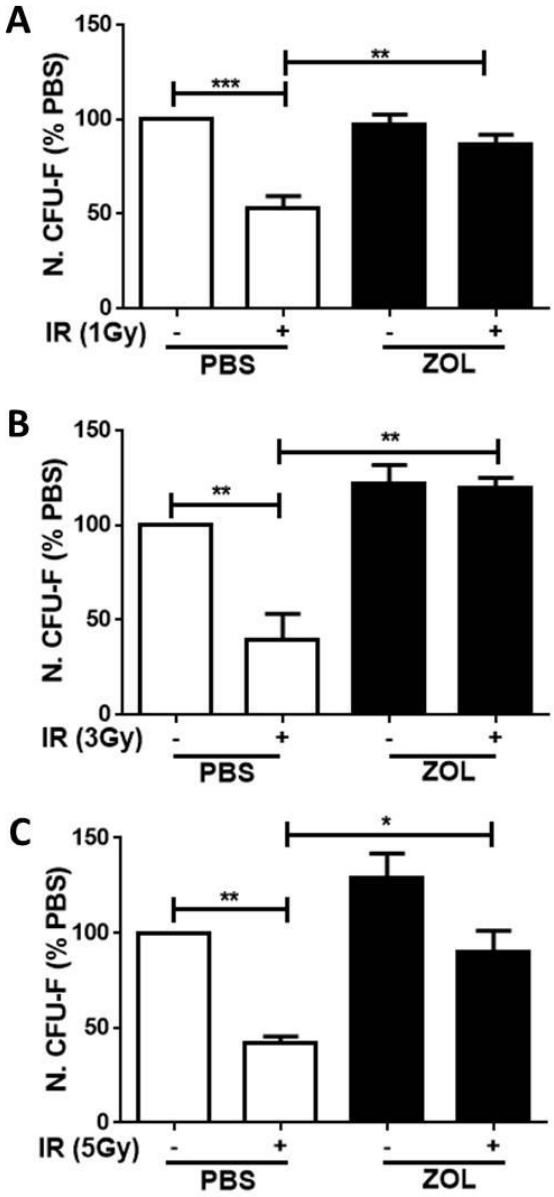


Figure 3.7 Zoledronate rescued hMSC clonogenicity following exposure to irradiation

*(A-C) Number of CFU-F obtained from hMSC (n=3) seeded at low density and exposed to irradiation at either (A) 1Gy; (B) 3Gy or (C) 5Gy in the presence or absence of Zol (1μM) and left to grow for 14 days at 37˚C, 5% CO_2 in air. Data presented as mean ± SD and analysed by one way ANOVA with Bonferroni multiple comparison post-hoc test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

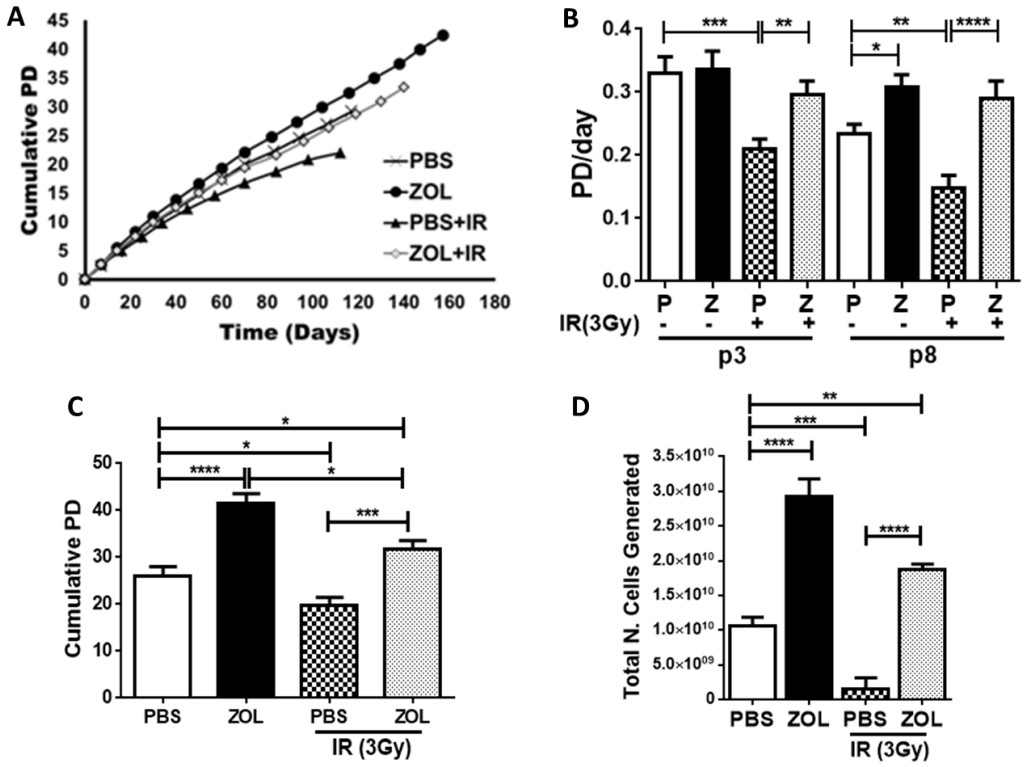


Figure 3.8 Zoledronate extended hMSC lifespan following irradiation

*(A)A representative example of a growth curve showing number of cumulative population doublings (PD) of hMSC (n=3) with time in culture. Cultures were either left non-irradiated or irradiated at 3 Gy and grown in the presence (non-irradiation circle; plus irradiation triangle) or absence of Zol (non-irradiation cross; plus irradiation diamond) for 3 days, and 12h later cultures were washed free from Zol and expanded in hMSC medium. (B) Total PD/day at passage (p) 3 and 8 in hMSC treated with or without Zol and irradiation (3Gy). (C) Total number of population doublings accomplished by hMSC cultures (n=3) in presence or absence of Zol and irradiation (3Gy). (D) Total number of cells generated from the expansion of hMSC cultures (n=3) in PBS or Zol treatment with or without irradiation. Data presented as mean ± SD and analysed by one way ANOVA with Bonferroni multiple comparison post-hoc test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

At passage 3 no significant difference was seen in proliferation rates of hMSC in either treatment in absence of irradiation, however at passage 8 there was a significant decline in PD/day in vehicle (PBS) treated hMSC although in Zol treated hMSC PD/day was significantly higher (n=3; Fig 3.8 B). Following irradiation there was a significant decline in the PD/day in vehicle (PBS) treated hMSC compared to Zol at

passage 3 which was more pronounced at passage 8. In presence of Zol treatment following irradiation, there was also a significant increase in the total number of cumulative population doublings compared to controls (n=3; Fig 3.8 C). Moreover Zol treated hMSC had a significant increase in total number of cells generated from expansion in both presence and absence of irradiation compared to vehicle treated hMSC (Fig 3.8 D). Thus Zol not only enhanced the lifespan of hMSC following irradiation but extended it beyond those of the controls.

To determine if Zol protected hMSC ability to differentiate following irradiation, cultures at passage 8 (Fig 3.8 A) were re-plated and exposed to osteogenic and adipogenic supplements and assessed for the expression of differentiation markers by qRT-PCR. A significant increase in expression levels of osteoblast markers, core-binding factor subunit alpha-1 (CBFA1), alkaline phosphatase (ALP), osteopontin (OPN) and osteocalcin (OC), was seen in un-irradiated hMSC treated with Zol in comparison to vehicle cultures at day 14 (Fig 3.9 A-D; n=3). Following irradiation hMSC control cultures lose their ability to differentiate as shown by the absence of expression of osteogenic markers, however in presence of Zol treatment following irradiation there was a significant increase in expression of osteogenic markers (Fig 3.9 A-D). Similarly the adipogenic differentiation markers, peroxisome proliferator-activated receptor gamma (PPARγ) and lipoprotein lipase (LPL), were significantly increased in Zol treated hMSC compared control cultures with or without exposure to irradiation (Fig 3.9 E-F).

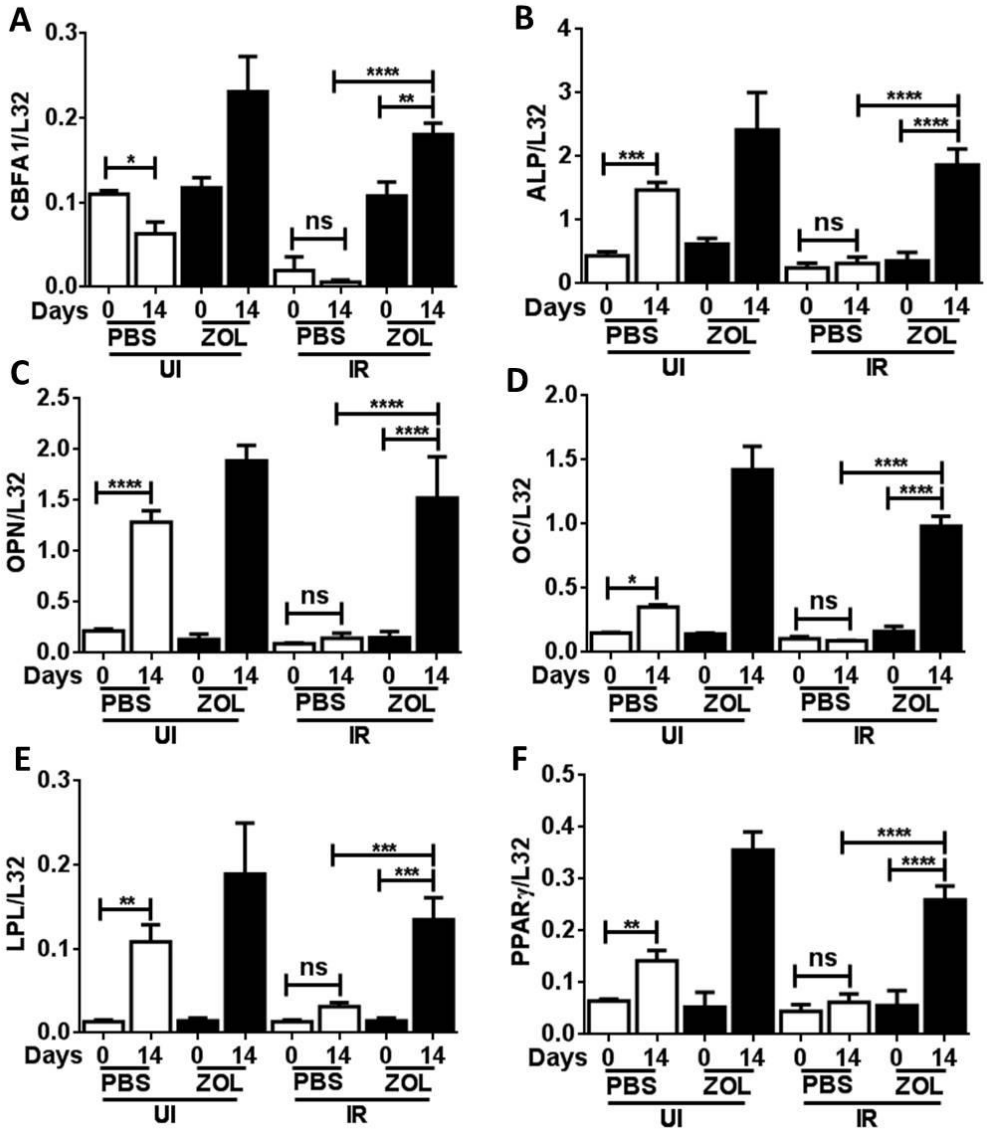


Figure 3.9 Zoledronate rescued hMSC differentiation potential following irradiation

*(A-F) Human MSC cultures (n=3) at passage 8 undergoing irradiation in presence or absence of Zol (1μM) were re-plated and exposed to osteogenic (A-D) and adipogenic (E-F) differentiation supplements for 14 days and assessed for expression of osteogenic differentiation markers (A) core-binding factor subunit alpha-1 (CBFA1); (B) alkaline phosphatase (ALP); (C) osteopontin (OPN); (D) osteocalcin (OC), and adipogenic differentiation markers (E) peroxisome proliferator-activated receptor gamma (PPARγ); (F) lipoprotein lipase (LPL) by qPCR. All markers were normalised to ribosomal protein L32. Data presented as mean ± SD and analysed by one way ANOVA with Bonferroni multiple comparison post-hoc test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

3.8 Post irradiation treatment with Zoledronate enhanced DNA damage repair

In an attempt to explain why Zol prompted extension of lifespan in cells which were treated only for 3 days we hypothesized that Zol may have repaired pre-existing DNA damage as well as repairing what was there. To test that, hMSC were cultured for 3 days in culture medium and exposed to 1Gy radiation following which Zol was administered at two time points: 0hr (immediately after irradiation) and 24hr respectively, and hMSC were thereafter fixed and stained for DNA damage foci detection at different time points (Fig 3.10A). Immediately after irradiation there was a significant increase in γH2AX foci in hMSC compared to those unirradiated (n=3; Fig 3.10 B-C). DNA repair was significantly achieved at 72 h post irradiation when hMSC were administered with Zol at 0h after irradiation time and in case of cells that received Zol 24 hours post irradiation, a significant reduction in DNA damage foci was seen only at 120hours post irradiation. These data suggest that Zol repaired DNA damage even when it was added after irradiation was administered but it took 4 days to see a significant decrease in the number of DNA damage foci.

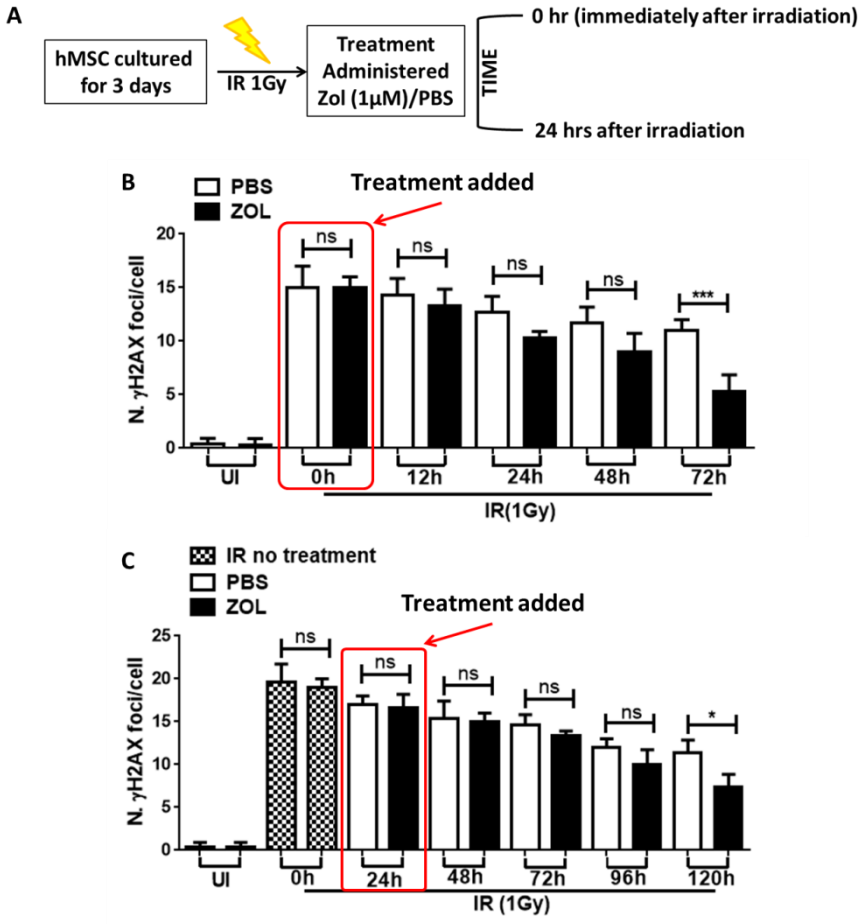


Figure 3.10 Treatment with zoledronate following irradiation enhanced DNA damage repair

*(A) A Schematic representation of the experimental plan. (B-C) Number of γH2AX foci in hMSC at different time points following irradiation when Zol was administered either 0h (B) or 24 hrs (C) after exposure to irradiation. Data presented as mean ± SD and analysed by one way ANOVA with Bonferroni multiple comparison post-hoc test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

3.9 Alendronate and Risedronate extend life span of hMSC and rescue clonogenic function

To determine the effect of other nitrogen containing bisphosphonates like Alendronate (Aln) and Risedronate (Ris) on hMSC, these cells (n=3) were expanded in presence or absence of either Alendronate (1µM) or Risedronate (1µM) until they stopped proliferating for 3 weeks, after which they were considered senescent. Whilst control cultures (n=3) grown in PBS stopped proliferating after 21-25 population doublings (square symbols), cultures grown in presence of Ris stopped proliferating after 29-34 PD (triangle symbols) from the start of the treatment and cultures grown in presence of Aln (circle symbols) stopped proliferating after 27-30 PDs (Fig 3.11 A). Moreover in presence of BP treatment there was a significant increase in the total number of population doublings accomplished by hMSC cultures when compared to PBS cultures (n=3; Fig 3.11 B). Ris and Aln treated hMSC also showed a significant increase in total number of cells generated from the expansion of hMSC cultures (n=3) when compared to those maintained in medium with PBS (Fig 3.11 C).

To determine whether Ris and Aln enhanced the clonogenic ability of hMSC cultures (n=3), the number of CFU-F, CFU-O and CFU-A were enumerated at passage 8 by re-plating at low densities. Human MSC cultures in presence of Ris and Aln showed an increase in clonogenic cells (CFU-F; Fig 3.12 A) compared to PBS treated hMSC. Similarly hMSC cultures in presence of Ris or Aln showed a significant increase in the number of CFU-O and CFU-A when compared to PBS treated hMSC (Fig 3.12 B-C). Together these data suggested that other nitrogen containing bisphosphonates like

Risedronate and Alendronate also extended life span and maintained quality of hMSC culture by protecting their clonogenicity.

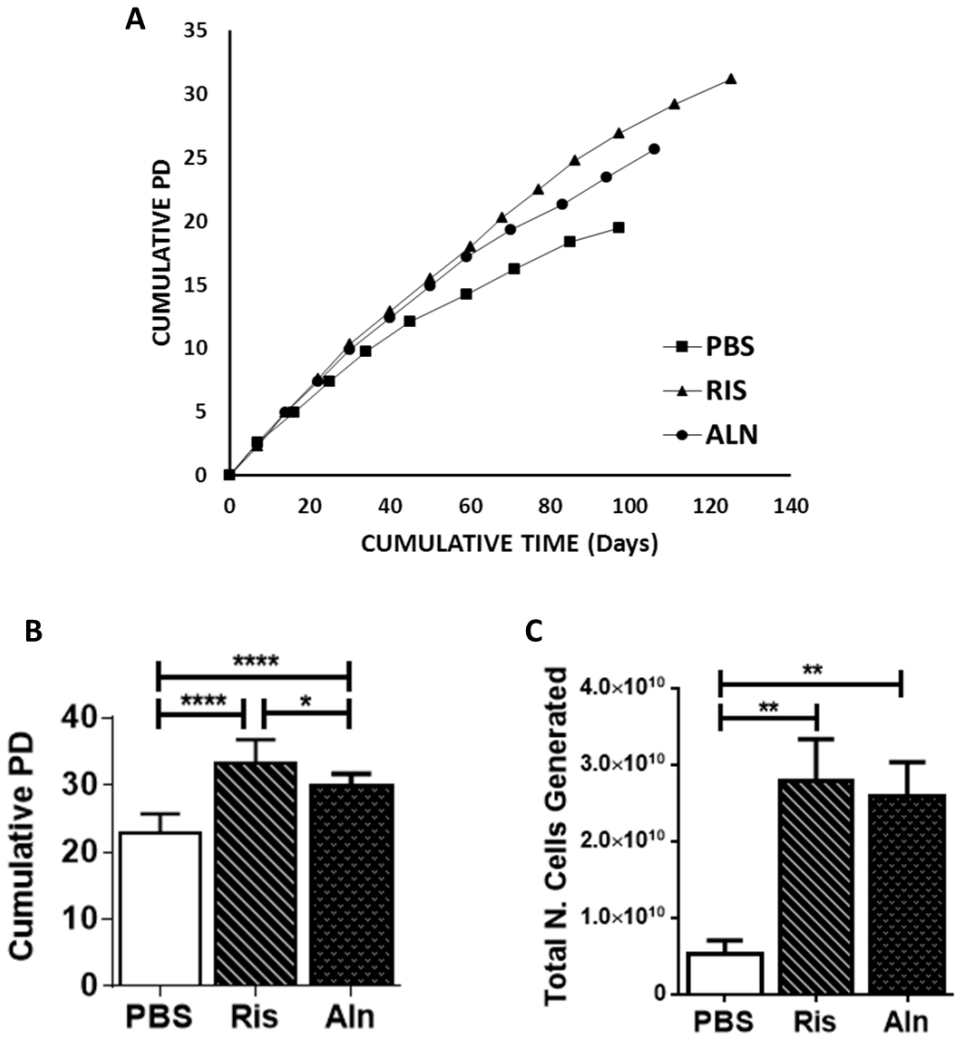


Figure 3.11 Human MSC cultured in the presence of Risedronate or Alendronate show an extension of life span

*(A) A representative example growth kinetics of hMSC shown as cumulative population doublings (PD) of hMSC with time in culture grown in media in the absence (square) or presence of Ris (triangle) or Aln (circle) at 1µM (n=3). (B) Total number of population doublings accomplished by hMSC cultures (n=3) in presence or absence of Ris or Aln. (C) Total number of cells generated from the expansion of hMSC cultures (n=3) in PBS or Ris or Aln treatment. Data are expressed as mean ±SD and were analysed by one way ANOVA and Bonferroni post-test for multiple comparisons *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

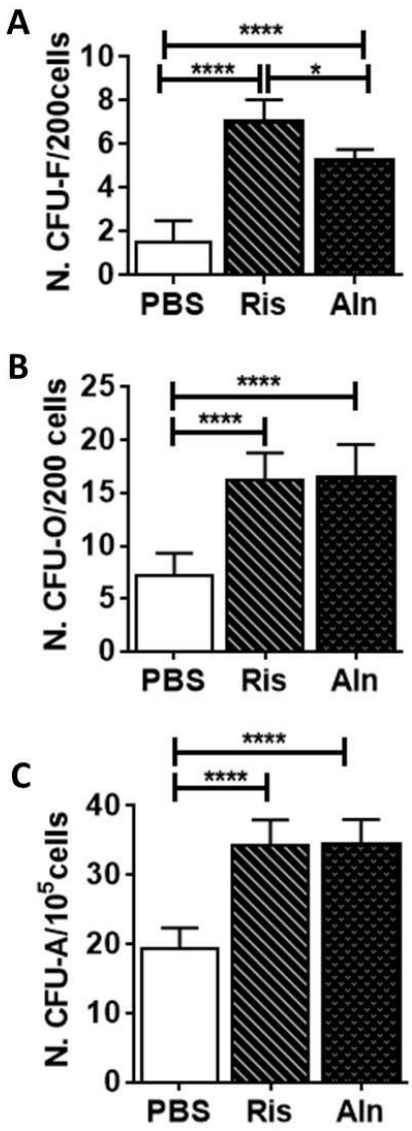


Figure 3.12 Risedronate and Alendronate delay loss of clonogenic progenitors during hMSC expansion

*(A-C) Number of Colony Forming Unit-Fibroblast (Panel A; CFU-F); Colony Forming Unit-Osteoblast (Panel B; CFU-O) and Colony Forming Unit-Adipocytes (Panel C; CFU-A) in hMSC cultures (n=3) at passage 8 grown in the presence or absence of Ris and Aln (1µM) and re-plated at low density with respective differentiation supplements. Data are expressed as mean ±SD and were analysed by one way ANOVA and Bonferroni post-test for multiple comparisons *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

3.10 Alendronate and Risedronate enhance repair of DNA damage following irradiation

To determine whether Ris and Aln were able to enhance DNA repair following exposure to irradiation, hMSC (n=3) cultured in presence or absence of either Zol, Ris or Aln for 3 days were exposed to 1Gy irradiation and DNA damage foci were quantified at 0h, 4h and 12h following irradiation (Fig 3.13A). Immediately after irradiation (0h) a significant increase in DNA damage foci was seen in all treatment groups. However 4 h later there was a significant repair of DNA damage in all three bisphosphonate treatments (Zol, Ris and Aln) when compared to PBS treated cultured. This was more pronounced 12 hrs later suggesting enhanced repair (Fig 3.13 A; Appendix 3).

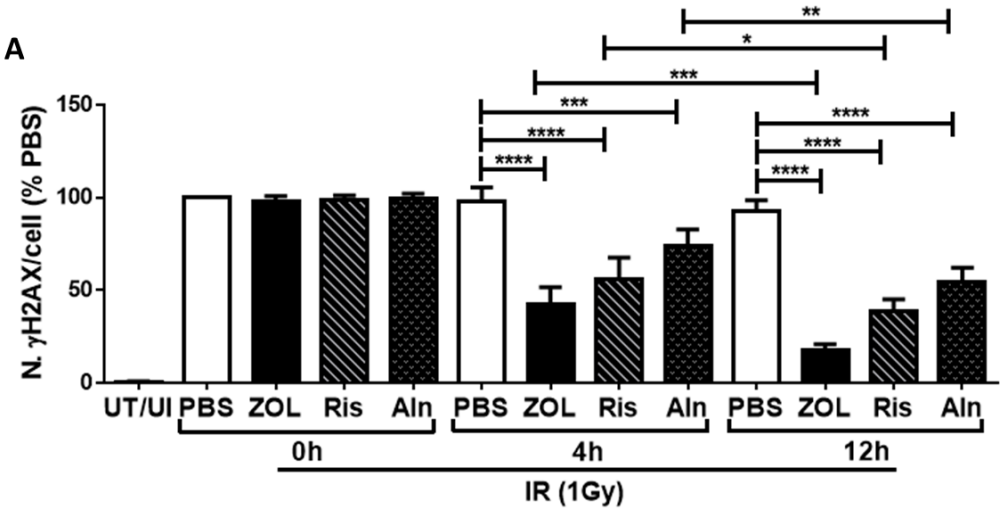


Figure 3.13 Human MSC cultured in the presence of Risedronate or Alendronate have enhanced repair of DNA damage

*(A) Number of γH2AX foci enumerated in hMSC (n=3) exposed to irradiation (1Gy) in presence or absence of different bisphosphonates treatments (Zol, Ris or Aln) at time points 0h, 4h and 12h following irradiation. Data are expressed as mean ±SD and were analysed by one way ANOVA and Bonferroni post-test for multiple comparisons *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*

3.11 Zoledronate extends lifespan and repairs DNA damage in normal human dermal fibroblast

To determine whether Zol's ability to repair DNA damage is restricted to stem cells or it occurs in mature cells, I used primary human dermal fibroblasts (hDF; n=3) and expanded them in culture in presence or absence of Zol (1µM) until they stopped proliferating for three weeks in culture. Cultures grown in PBS (square symbols) senesced after 17-21 population doublings whereas those grown in presence of Zol (triangle symbols) stopped proliferating after 27-34 PDs (Fig 3.14 A). Zol treatment significantly increased the total number of population doublings (Fig 3.14 B) and the total number of cells (Fig 3.14 C) accomplished by expansion of hDF when compared to PBS treated cultures. I next wanted to determine whether Zol could repair DNA damage in these hDF following irradiation. Human DF (n=4) were cultured in the presence or absence of Zol and exposed to 1Gy gamma-irradiation. Cells were fixed for staining at 12h following irradiation (Fig 3.14 D). Untreated hDF exposed to IR had high amounts of DNA damage foci. These were significantly reduced when hDF were cultured in presence of Zol. These data together suggest that Zol enhances DNA damage repair not only in human stem cells but also in mature human dermal fibroblasts.

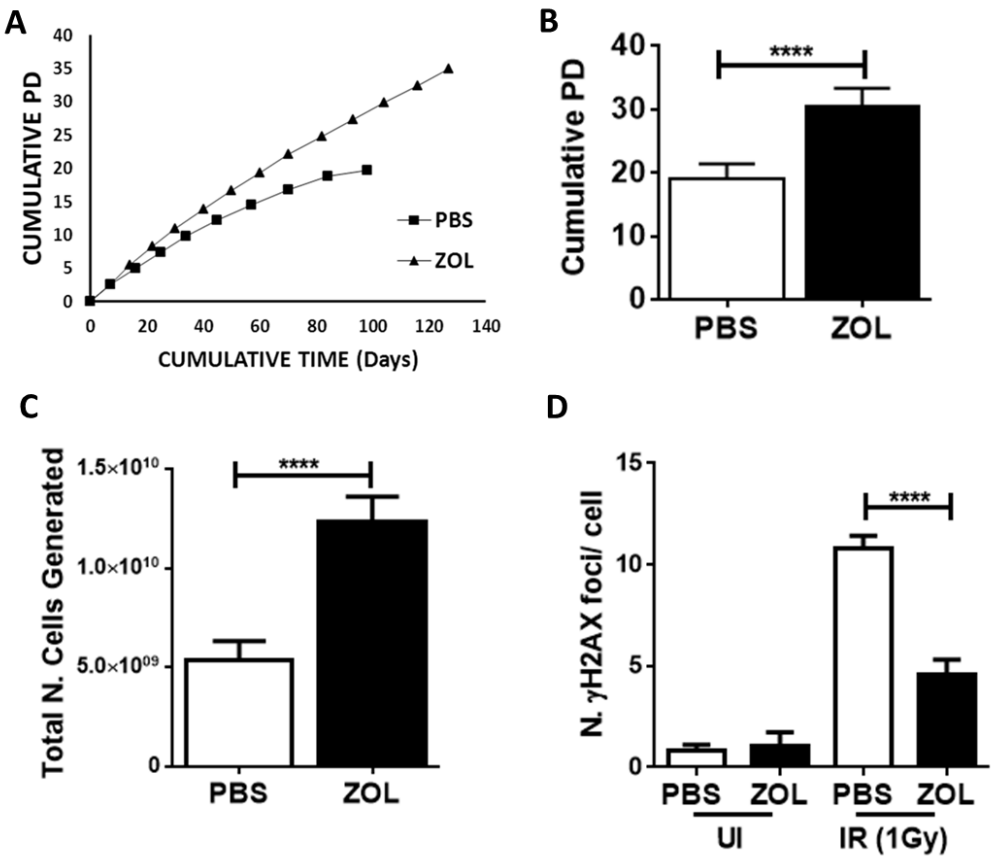


Figure 3.14 Zoledronate extended lifespan of human Dermal Fibroblasts and enhances DNA damage repair

*(A) A representative example of growth kinetics of hDF shown as cumulative population doublings (PD) with time in culture. Human DF were grown in media in the presence (triangle) or absence of Zol at 1μM (square) (n=3). (B) Total number of population doublings accomplished by hDF cultures (n=3) in presence or absence of Zol. (C) Total number of cells generated from the expansion of hDF cultures (n=3) in PBS or Zol treatment. (D) Number of γH2AX foci in hDF not irradiated (UI) or exposed to 1Gy of irradiation in the presence or absence of Zol (1μM). Data presented as mean ± SD and analysed by either student t-tests or one way ANOVA with Bonferroni multiple comparison post-hoc test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

3.12 Discussion

In this chapter I investigated whether bisphosphonates extended hMSC life span and delayed cellular ageing. To address the aims of this chapter I first isolated human MSC and characterised them. I established cultures of hMSC that were positive for CD105 and CD29 and negative for marker of haematopoietic and endothelial contaminants (Barry, Boynton et al. 1999, Pittenger, Mackay et al. 1999, Deans and Moseley 2000, Zhou, Huang et al. 2003). I characterised their morphology and multi lineage potential by triggering osteogenic and adipogenic differentiation. The plastic adherent hMSC had spindle shaped morphology and were capable of proliferating, however with age in culture; there was a decline as they stopped proliferating. This has been reported by others as well (Friedenstein, Chailakhjan et al. 1970, Friedenstein, Gorskaja et al. 1976, Bruder, Jaiswal et al. 1997, Pittenger, Mackay et al. 1999, Dominici, Le Blanc et al. 2006). In fact it has been previously demonstrated in our research group by Mohanty et al (2012).

Treatment with Zoledronte at 1 µM resulted in extension of lifespan of hMSC by delaying cellular ageing. Zoledronate was used at a concentration of 1µM or below as concentrations above this were detrimental to cell viability. In fact doses 10-100 fold higher than this have been associated with apoptosis and growth inhibition in tumour cells and osteoclasts (Shipman, Rogers et al. 1997, van Beek, Pieterman et al. 1999, Dunford, Thompson et al. 2001, Croucher, Jagdev et al. 2003, Croucher, De Hendrik et al. 2003, Baulch-Brown, Molloy et al. 2007). Moreover studies on effect of bisphosphonates on osteoblasts that results from MSC differentiation have reported a significant induction of apoptosis at concentrations higher than 5 µM (Idris, Rojas et al. 2008, Ebert, Zeck et al. 2009). Varela et al in their study used Zol in combination

with statins at a concentration of 1μM which resulted in extension of lifespan in progeroid mice, hence the choice of this dose was well calculated (Varela, Pereira et al. 2008). In clinical applications, Zol is administered intravenously at a dose of 4 mg over 15 min. and interestingly, deductions from pharmacokinetic data analysis showed that estimated maximum plasma concentration (Cmax) of Zol is about 1μM therefore justifying the clinical relevance of the chosen dose(Chen, Berenson et al. 2002).

Spontaneous immortalization has been reported only in one report of adipose derived MSC although in my study this was not observed in MSC cultures from bone marrow of at least 9 separate donors. Although in presence of zoledronate there was a 10 population doubling increment (38%) in long term expansion with maintenance of cell morphology and proliferation rate but this did not immortalize the cells as they eventually stopped proliferating after 37-42 PD. My observations in untreated hMSC are supported by a number of other studies (Stenderup, Justesen et al. 2003, Bonab, Alimoghaddam et al. 2006, Siddappa, Licht et al. 2007). Moreover in studies by Izadpanah et al (2008) following long term expansion in vitro, human MSC did not develop polyploidy throughout the culture with absence of any chromosomal rearrangements at any passage (Izadpanah, Kaushal et al. 2008). Although immortalization was not observed, expansion of cells resulted in decrease in proliferation rate (PD/day) with time in culture and this was a common trend observed in both untreated and Zol treated cultures with Zol cultures declining much slower than vehicle. This difference could be attributed to delay in cellular ageing by Zol in hMSC.

Cellular ageing may be triggered by various stresses including such as oxidative stress, oncogene overexpression, telomere shortening and accumulation of DNA damage. Cellular response to damage is arrest of cell cycle to trigger DNA repair mechanisms, and beyond a certain threshold cells either undergo senescence or apoptosis. Various studies in MSC have reported replicative senescence and functional implications in differentiation potential (Stenderup, Justesen et al. 2003, Baxter, Wynn et al. 2004, Fehrer and Lepperdinger 2005, Bonab, Alimoghaddam et al. 2006, Wagner, Horn et al. 2008). In accordance with literature my studies too showed that with time in culture hMSC had stopped proliferating (Mohanty, Cairney et al. 2012). But long term treatment with bisphosphonates resulted in extension of lifespan and enhanced clonogenic potential assessed by CFU-F, CFU-O and CFU-A assays. Accumulation of DNA damage as a key driver of cellular ageing has also been established in other stem cells (Lopez-Otin, Blasco et al. 2013). Studies in adult hematopoiteic stem cells (HSC) have demonstrated age-related accumulation of DNA damage and a functional decline of the regenerative capacity of stem cells (Nijnik, Woodbine et al. 2007, Rossi, Seita et al. 2007, Rube, Fricke et al. 2011). Evidence on low levels of DNA damage at early and late passage on chronic treatment with Zol suggested a delay in cellular ageing thus explaining the extension in lifespan and protection of function. It is interesting to note here that even short term treatment of only 3 days with zoledronate also resulted in extended lifespan of hMSC both in un-irradiated and irradiated conditions. One plausible explanation could be that zoledronate may have repaired pre-existing DNA damage as well as new DNA damage caused by irradiation thus allowing cells to delay cellular ageing. My results on zoledronate treatment post

administration of radiation and their ability to still enhance DNA repair nearly 4 days after injury further corroborates this hypothesis.

No studies to my knowledge have investigated chronic BP treatment to study extension of lifespan and enhanced clonogenic or differentiation potential in MSC including their effect on delaying cellular ageing. I found that both following prolonged treatment as well as 3 day treatment there was enhanced potency of hMSC to differentiate towards both adipogenic and osteogenic lineages. In fact pulse treatment of bisphosphonates for 3 or 6 hrs has shown increased potential of hMSC to differentiate towards osteogenic lineage (Ebert, Zeck et al. 2009). Studies by von Knoch et al (2005) have also shown that treatment with BPs (Alendronate, Risedronate and zoledronate) increased their potential towards osteogenic differentiation at concentration of 100nM which is 10 times lower that what I have used (von Knoch, Jaquiery et al. 2005). Interestingly in their study they have pre-treated the hMSC with zoledronate and then induced differentiation with no addition of drugs in the osteogenic induction media similar to what I have done. However in contradiction to my study, other studies assessing the effect of Risedronate or zoledronate on hMSC differentiation to osteoblasts have shown that bisphosphonate treatment results in apoptosis and reduced osteogenic differentiation (Fujita, Kurokawa et al. 2011)Idris, 2008 #409}. One plausible explanation for this difference is the fact that they administered the drugs continuously throughout the process of differentiation and at doses higher than 1µM. Moreover Fujita et al (2011) used only one immortalized hMSC clone to conclude the detrimental effects of risedronate on MSC differentiation. Whether immortalization of hMSC causes differential response to differentiation induction warrants further investigation.

With respect to adipogenic differentiation, studies by Duque and Rivas (2007) showed that treatment of Alendronate resulted in decline in adipogenic differentiation yet again their studies investigated the effects on differentiation by addition of the bisphosphonate in the induction medium (Duque and Rivas 2007). Similar effects were observed in rat BMSCs that too had reduced levels of Oil red O staining (Fu, Tang et al. 2008). In their study they have shown that ERK and JNK pathways are associated with osteogenic differentiation. Inhibition of these pathways results in reduced expression of osteogenic markers and increase of adipogenic markers in cells continuously exposed to Alendronate in respective induction medium. In absence of treatment there are high levels of ERK in osteogenic media. This is further enhanced on treatment with Alendronate during induction of differentiation. This boost of ERK may explain enhanced osteogenic differentiation. Literature suggests that ERK is down regulated in adipogenic differentiation, therefore if Alendronate is added in differentiation media there is increase in ERK levels which provides a potential explanation to the reduced levels of adipogenic potential (Fu, Tang et al. 2008, Fuentes, Acuna et al. 2010, Scioli, Bielli et al. 2014). Unfortunately the levels of ERK in cells in adipogenic induction media in absence of Alendronate have not been investigated. It is interesting note here that all studies assessing the detrimental effects of bisphosphonates on adipogenic and osteogenic differentiation have been performed in presence of BP treatment with induction supplements, which on their own modulate signalling pathways to trigger the process of differentiation. Therefore in reality presence of drug treatment during the differentiation process in these cases may not allow appreciating the actual difference in differentiation potential. Since long or short treatment with Zol resulted in both adipogenic and osteogenic

differentiation potential, these data suggest that the heterogeneous population of MSC in BP treatment retained more MSC stem/progenitors with multi-lineage potential that were protected from cellular ageing. This research thus raises specific interest on chemical interventions in MSC expansion *in vitro* to alleviate the issues of cellular ageing.

MSC have been reported to have higher resistance to the accumulation of DNA damage induced by irradiation than other cell types (Prendergast, Cruet-Hennequart et al. 2011), however DNA damage can accumulate in MSC and lead to decreased ability to initiate osteoblastic differentiation both in vitro and *in vivo* (Alves, Munoz-Najar et al. 2010, Cmielova, Havelek et al. 2012, Wang, Chen et al. 2012). Studies on adult MSC isolated from dental pulp (dental pulp stem cells, DPSC) respond to IR by cell cycle arrest in G2 phase, apoptosis is not induced, but cells enter stress induced premature senescence (SIPS) (Muthna, Soukup et al. 2010). Studies on irradiated MSC indicate quite high resistance of mesenchymal stem cells to radiation-induced apoptosis accompanied with no significant increase in caspase activity however there was induction of SIPS (Chen, Lin et al. 2006, Schonmeyr, Wong et al. 2008, Muthna, Soukup et al. 2010, Cmielova, Havelek et al. 2012). In fact Cmeilova et al show that exposure to 2 Gy significantly inhibited the proliferation of BM MSC, but while the cells lose the proliferative capacity, they remain viable (metabolically active) (Cmielova, Havelek et al. 2012). In my study as well, when hMSC were exposed to irradiation 3Gy, their proliferative capacity declined and with time in culture and cells stopped proliferating after 17-21 PD. It is however interesting to note here that results available on radiation induced senescence in MSC is very controversial. Bone marrow MSC has increase in β –galactosidase, 3 and 7 days after IR by the doses of 7

and 12 Gy. In my study irradiation at 7Gy was lethal to the cells. Muthná et al. (2010) detected SIPS following 20Gy irradiation after 3-13 days in the DPSC whereas Chen et al (2006) did not detect increase in β -galactosidase in BM MSC even 5 days after 9Gy irradiation, however studies by Cmeilova et al (2012) detected increased β -galactosidase at 20Gy in BM –MSC 3-13 days after irradiation. It would be advantageous to know where my study fits in with the available literature on irradiated MSC in terms of senescence activity however, it is important to note that most of the studies had senescence induction after a short time lapse following irradiation whereas in my study cells stopped proliferation much later (17-21PD) which could be due to two reasons, first being the dose of irradiation administered in my study was really low and second the source of irradiation may vary and result in different levels of damage induction.

Accumulated DNA damage may affect stem cell functions and in order to test this DNA damage was induced by irradiation. Clonogenic function of stem/progenitor cells was assessed by the number of CFU-F in presence or absence of irradiation. Following irradiation at the different doses, hMSC suffered from loss of clonogenic function as expected and this has been demonstrated by other studies in response to irradiation or chemotherapy by reduction in number of CFU-F (Xu, Hendry et al. 1983, Galotto, Berisso et al. 1999). Human MSC that were treated with zoledronate for 3 days and then exposed irradiation however had a rescue of CFU-F. This is attributed to ability of Zol to repair damaged DNA thereby rescuing the clonogenicity. It is interesting to note that although repair at 24hrs post irradiation in cells irradiated with 3Gy was less than irradiated with 1Gy yet ability to form colonies was completely rescued in both cases. The most plausible explanation for this may the time duration after which the

respective assessments are done. In case of DNA damage staining at 24 hr post irradiation there is higher amount of DNA damage recorded at 3Gy than 1Gy, but when the assessment for CFU-F is done, the cells are allowed 14days following which colonies are enumerated, giving a larger time frame for repair to occur.

DNA damage in telomeric regions that result in telomere attrition may contribute to cellular ageing. In fact studies by Hewitt et al (2012) suggest that DNA damage foci induced by radiation are located at the telomeric regions(Hewitt, Jurk et al. 2012). In human fibroblasts the DNA damage foci would repair rapidly but the telomere associated foci (TAF) remained stable and these TAF had a fraction increase with time. Whether the DNA damage foci seen in hMSC following irradiation are in the telomeric regions or not remains to be determined. Zoledronate may actually prevent accumulation of these kinds of telomere associated DNA damage foci and therefore it may be useful to investigate the effect of Zol on TAFs. In order to achieve this Immuno-fluorescent in situ hybridization (Immuno-FISH) can be employed to determine the existence of γH2AX foci co-localizing with telomeric region. Moreover kinetic live cell microscopy can also be employed using a combination of AcGFP-53BP1c fusion protein which quantitates DNA damage foci and telomere PNA probes that tag telomere repeats to determine the formation and disappearance of DNA damage foci in telomeric regions following zoledronate treatment (Molenaar 2003, Nelson, Buhmann et al. 2009). It would be exciting to know if zoledronate repairs DNA damage foci in telomeres as telomere regions repair DNA by inhibiting the NHEJ (error prone) pathway and if we see repair what would be the mechanism of action.

The amount of DNA damage/repair by zoledronate has been assessed by counting the frequencies of γH2AX foci. The ease of counting these foci formed following gamma

irradiation may lead to an occasional over interpretation of this being used as an ideal surrogate marker for DSB. One major limitation to note is that not all γH2AX may represent DSBs (de Feraudy et al. 2010; Revet et al. 2011). The phosphorylation of H2AX can also be caused by collision of replication forks (Bonner, Redon et al. 2008; Mogi and Oh, 2006). One may argue that improved repair of DNA DSB by Zoledronate cannot be interpreted by use of only one marker of DNA damage. 53BP1 is another key player in DSB repair and processing and is potentially recruited at site of DSB downstream of γH2AX (Noon, Goodarzi et al. 2011; Callen, Virgilio et al. 2013; Zimmermann, Lottersberger et al. 2013; Fradet-Turcotte, Canny et al. 2013). Therefore investigating the response of this marker could strengthen our claims. Moreover reduction in frequency of γH2AX foci can be established by supressing nutrient signalling as was seen in studies where late passage cells containing high levels of DNA damage foci showed reduced foci counts in response to serum starvation (Satyanarayana, Greenberg et al. 2004). Interestingly, pseudo DNA damage response such as γH2AX has been seen in senescent cells without detection of 53BP1 co-localisation with γH2AX and absence of DNA damage in comet assay. A reduction in these foci following inhibition of mTOR by rapamycin indicates the possibility of γH2AX as a pseudo DDR marker of senescent cells (Pospelova, Demidenko et al. 2009). Reduction in oxidative stress may also result in decrease in γH2AX (Passos, Nelson et al. 2010). Reduction in foci by zoledronate may therefore be due to enhanced turnover of DDR foci components or improved mitophagy, reduced levels of oxidative stress or reduced senescence associated secretory phenotype. Most importantly none of these mechanisms involve improved DNA repair, therefore it is possible that Zol may repair by modulating one of the many multiple possibilities

mentioned above. To this end I employed another assay to test the robustness of DNA damage and repair by Zoledronate. The comet assay demonstrated similar results of reduced DNA damage following Zol treatment suggesting the possibility of enhanced repair (Misra, Mohanty et al, 2015; also described in Chapter 6, Fig 6.10). Moreover recruitment of KU70/KU80 and RAD51 following treatment with Zol add evidence in favour of DNA repairing ability (Misra, Mohanty et al, 2015).

In conclusion I have shown that zoledronate and other bisphosphonates extended hMSC life span in culture by enhancing repair of damaged DNA both following cellular ageing and exposure to damaging agent such as irradiation. In fact zoledronate was also able to extend human dermal fibroblast lifespan and repair DNA following damage in these mature cells. Post irradiation treatment with zoledronate still resulted in enhanced DNA repair although time take for repair mechanism to kick in in presence of Zol was longer in contrast to pre-treatment. However these results highlight the importance of identifying the mechanism of action of this clinically safe drug which is what I investigated in my next chapter.

Chapter 4: Zoledronate enhances DNA repair via partial inhibition of mevalonate and mTOR signalling pathways

4.1 Introduction

In the previous chapter I have shown that zoledronate extended lifespan and enhanced DNA repair in hMSC undergoing either cellular ageing or exposure to irradiation, however the mechanism of action on DNA repair is unknown. Evidence suggests that BPs like zoledronate inhibit the mevalonate pathway by inhibiting the enzyme farnesyl pyrophosphate synthase and a direct outcome is inhibition of prenylation (farnesylation and geranylgeranylation) of protein GTPases such as Ras, Rap, Rheb, Rho, Roc, Rac among others (Dunford, Thompson et al. 2001) (Rogers, Jou et al. 2003, Roelofs, Thompson et al. 2006). Interestingly inhibition of prenylation has been implicated in improved cardiac health and lifespan in drosophila (Spindler, Li et al. 2012, Spindler, Li et al. 2012). Moreover inhibited protein prenylation resulted in recovery of the ageing phenotype in Hutchinson Guilford Progeroid Syndrome (HGPS) (Gordon, Kleinman et al. 2012).

Prenylation of proteins is an important process that affects different pathways. One pathway that requires prenylated proteins is the mammalian target of rapamycin (mTOR) which is downstream of the phosphatidylinositol 3 kinase (PI3K) pathway. mTOR signalling encompasses two signalling complexes, TOR complex-1 (TORC-1) and TOR complex-2 (TORC-2) (Vezina, Kudelski et al. 1975, Loewith, Jacinto et al. 2002, Wullschleger, Loewith et al. 2006). More importantly mTOR has been implicated in extension of life span in several organisms. Both genetic and pharmacological inhibition of mTOR has resulted in extension of lifespan in yeast

(Kaeberlein, Powers et al. 2005, Powers, Kaeberlein et al. 2006) worms (Vellai, Takacs-Vellai et al. 2003) and flies (Kapahi, Zid et al. 2004). Rapamycin induced inhibition of mTOR has also resulted in extension of lifespan in mice (Chen, Liu et al. 2009, Anisimov, Zabezhinski et al. 2010, Anisimov, Zabezhinski et al. 2011). Studies by Selman et al have also shown that knockout of S6 kinase 1 that is one of the mTORC1 mediators resulted in increased lifespan of C57Bl/6 mice similar to studies with Rapamycin (Selman, Tullet et al. 2009).

Apart from lifespan, mTOR plays a role in regulating or rather promoting senescence. Evidences from human fibrosarcoma cell line HT-p21 with increased SA-β-gal activity show diminished senescence markers following treatment with rapamycin (Demidenko and Blagosklonny, 2008). In WI-38 human fibroblast cells, treatment with rapamycin has shown to attenuate senescence induced by doxorubicin (Demidenko and Blagosklonny, 2008). Moreover in ARPE-19 cells (human retinal pigment epithelial), treatment with rapamycin resulted in decreased senescence and also protected from the loss of proliferation capacity (Demidenko, Zubova et al. 2009). These data are suggestive of a delay in cellular senescence progression by inhibition of mTOR signalling. In fact inhibition of mTOR has also shown to increase the clonogenic capacity of primary human oral keratinocytes and their resident self-renewing cells by preventing stem cell senescence (Iglesias-Bartolome, Patel et al. 2012). Studies have also shown that prolonged activation mTOR has been associated with exhaustion of stem cell pool due to diminished self-renewal or premature ageing or loss of quiescence (Castilho, Squarize et al. 2009, Chen, Liu et al. 2009, Gan and DePinho 2009, Lee, Budanov et al. 2010, Chakkalakal and Brack 2012, Chakkalakal, Jones et al. 2012, Johnson, Rabinovitch et al. 2013).

All these evidences in lifespan extension therefore pose mTOR as potential pathway to explore in order to explain the extension of lifespan seen in hMSC following treatment with zoledronate. Moreover this pathway is also associated with DNA repair as its downstream effectors such as the ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) are important molecules of DNA repair machinery (Zhou and Elledge 2000, Shiloh 2003, Kastan and Bartek 2004) therefore understanding their status upon activation or inhibition of this signalling pathway is essential for dissecting out repair mechanisms following damage. Based on these findings it was therefore logical to hypothesise that '**Zol extended lifespan of hMSC and mediated DNA repair via mevalonate and mTOR signalling pathways** (Fig 4.1)'.

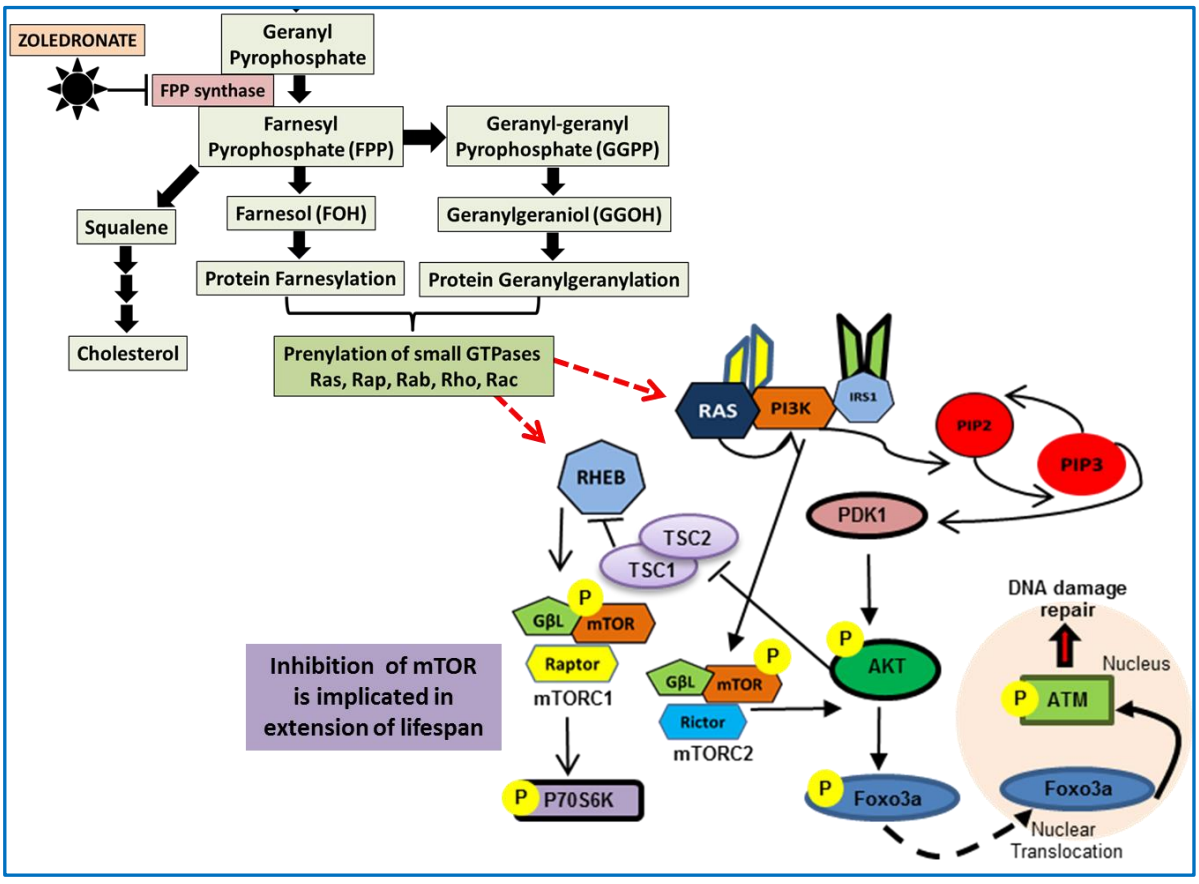


Figure 4.1 Schematic representation of the mevalonate and mTOR pathway

4.2 Zoledronate inhibit mevalonate pathway in hMSC

To determine whether Zol and other amino-bisphosphonates Ris and Aln internalised hMSC and resulted in inhibition of the mevalonate pathway, hMSC (n=3) were cultured for 3 days in presence or absence of drugs at 1µM concentration and sacrificed for detection of unprenylated RAP1A (GTPase). In the vehicle (PBS) treated cultures there was no unprenylated RAP1A detected by western blotting, however hMSC cultures that were treated with either of the amino-bisphosphonates (Zol, Ris or Aln) showed expression of unprenylated RAP1A suggesting inhibition of the mevalonate pathway (n=3; Fig 4.2 A-B).

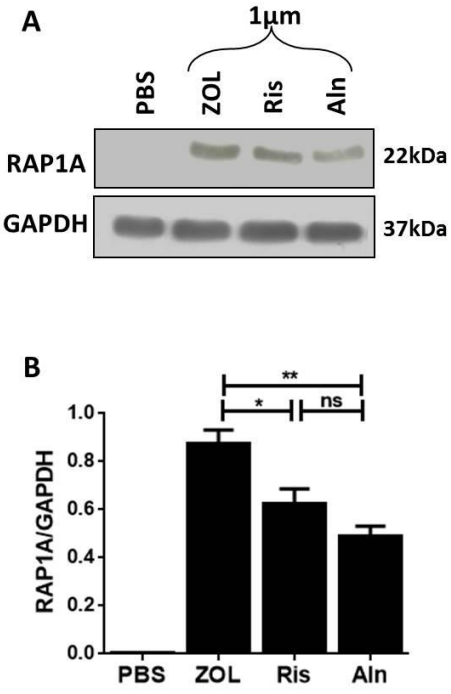


Figure 4.2 Zoledronate and other amino-bisphosphonates unprenylated RAP1A in hMSC

(A) A representative example of western blot showing expression of unprenylated Rap1A (top panel) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; bottom panel) in hMSCs

*following treatment with either Zol, Ris or Aln (1µM) (B) Quantitation of un-prenylated RAP1A proteins normalised to GAPDH in 3 hMSC cultures exposed to Zol, Ris or Aln. Quantification was done using ImageJ. Data presented as mean ±SD were analysed by one way ANOVA and Bonferroni post-test for multiple comparisons *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*

4.2.1 Inhibition of Mevalonate pathway by Zol is dose dependent

To determine whether Zol enhanced DNA repair through the inhibition of the Mevalonate pathway in a dose dependent way, I first cultured hMSC (n=3) in presence or absence of Zol at increasing concentrations (100nM-1µM) for 3 days and sacrificed for detection of unprenylated Rap1A. A dose dependent increase in unprenylated RAP1A was observed as shown by a significant positive correlation between Zol concentrations and expression of unprenylated RAP1A (Fig 4.3 A-C).

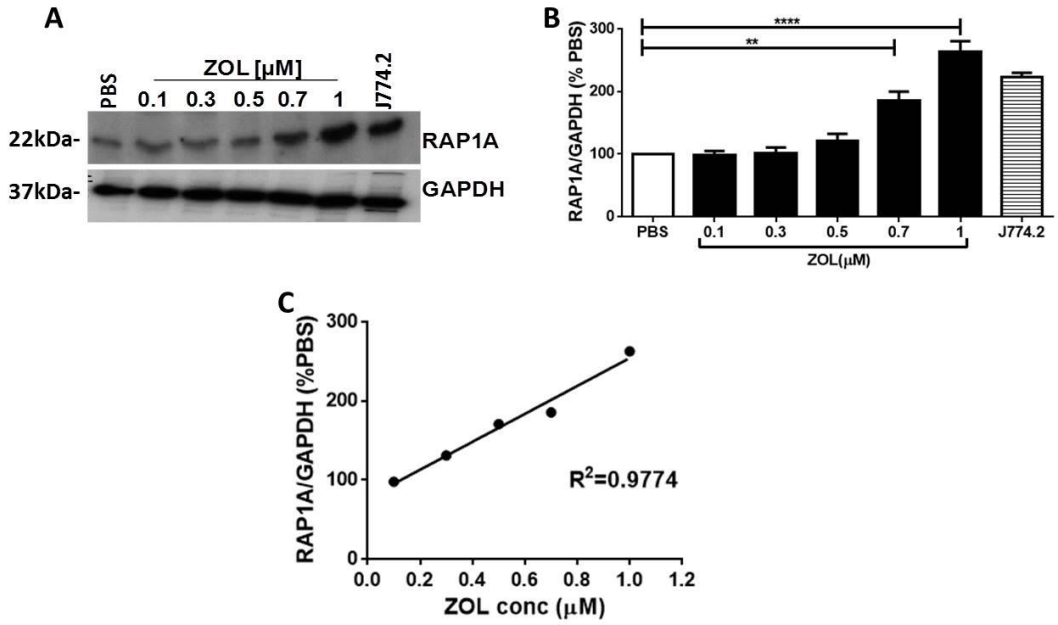


Figure 4.3 Zoledronate increased expression of unprenylated RAP1A in a dose dependent manner

*(A) A representative example of expression of un-prenylated Rap1A (top panel) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; bottom panel) in hMSC (n=3) following treatment with increasing concentrations of Zol ranging between 0.1-1μM (B) Quantitation of un-prenylated RAP1A proteins normalised to GAPDH. Quantification was done using ImageJ. (C) The graph represents the correlation between expressions of un-prenylated RAP1A (expressed as percentage of PBS) and concentration of Zol. Data presented as mean ± SD and analysed by one way ANOVA with Bonferroni multiple comparison post-hoc test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

4.3 DNA damage repair by Zol is dose dependent

To determine whether repair of DNA damage was dose dependent, hMSC (n=3) were treated with Zol at increasing concentrations ranging from 100nm to 1μM for 3 days following which they were subjected to irradiation at 1Gy and 4 hrs later sacrificed for γH2AX foci enumeration. In vehicle treated cultures there was high numbers of DNA damage foci however hMSC treated with Zol had significant and progressive decrease in DNA damage foci with increasing concentrations of drug, thus establishing a negative correlation between the numbers of DNA damage foci in hMSC with increasing concentrations of Zol (Fig 4.4 B).

To determine whether dose dependent changes in number of γH2AX foci was associated with dose dependent changes in unprenylated RAP1A, hMSC treated with zoledronate (100nM-1μM) for 3 days were irradiated at 1Gy and sacrificed for either expression of unprenylated RAP1A or enumerating DNA damage foci, 4hrs following irradiation. A dose-dependent decrease in the number of γH2AX foci was mirrored by a dose-dependent increase in un-prenylated RAP1A. This established a correlation between these two events (Fig 4.4 C).

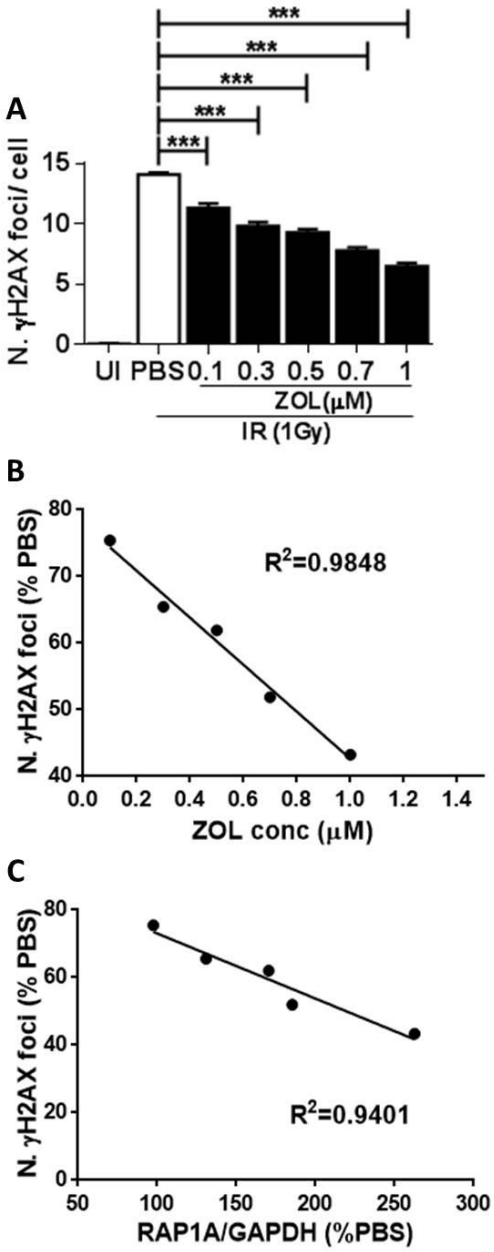


Figure 4.4 Zoledronate repaired DNA damage in a dose dependent manner

*(A) Number of γH2AX foci in hMSC treated with different doses of Zol ranging between 0.1-1μM following irradiation at 1Gy (n=3). (B) Linear decrease in number of DNA damage foci with increasing concentrations of Zol. (C)Linear decrease in γH2AX foci with increasing expression of un-prenylated RAP1A expressed as percentage of PBS. Data presented as mean ± SD and analysed by one way ANOVA with Bonferroni multiple comparison post-hoc test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

4.3.1 DNA damage repair by Zol is mediated by FPPS inhibition

To determine whether inhibition of farnesyl diphosphate synthase (FPPS) was associated with DNA damage repair, downstream metabolites (FOH) and geranyl-geraniol (GGOH) were re-introduced to reverse the inhibition of FPPS by Zol (Fig 4.2A). Human MSC cultured in PBS (vehicle control for Zol) or ethanol (vehicle control for FOH and GGOH) following irradiation (1Gy) had higher number of DNA damage foci in comparison to hMSC that received Zol treatment where a significant reduction was found (n=3; Fig 4.5). Addition of FOH and GGOH substrates reverted Zol's ability to enhance repair of DNA following damage. Together these data suggest that zoledronate enhances DNA repair via inhibition of mevalonate pathway.

To further strengthen the evidence supporting the inhibition of mevalonate pathway an enantiomer pair of octahydro-pyrindine-bisphosphonates with similar structure but different affinity and potency for the FPPS enzyme were employed (Fig 4.6 A) (Ebetino, Hogan et al. 2011). Human MSC (n=3) were cultured for 3 days in presence or absence of the molecules 1R,6S and 1S,6R following which they were irradiated at 1Gy and sacrificed 4hr later for enumerating γH2AX foci (Fig 4.6 B) The enantiomer 1R, 6S (IC50=15) has greater affinity for FPPS compared to 1S, 6R (IC50=359). Following irradiation vehicle treated hMSC had higher DNA damage that was significantly reduced by treating with Zol. Among the enantiomeric pair only the molecule with high affinity for FPPS was able to enhance DNA repair similar to Zol (Fig 4.6 B). These data confirm that the DNA repair activity by Zol depended on degree of FPPS inhibition.

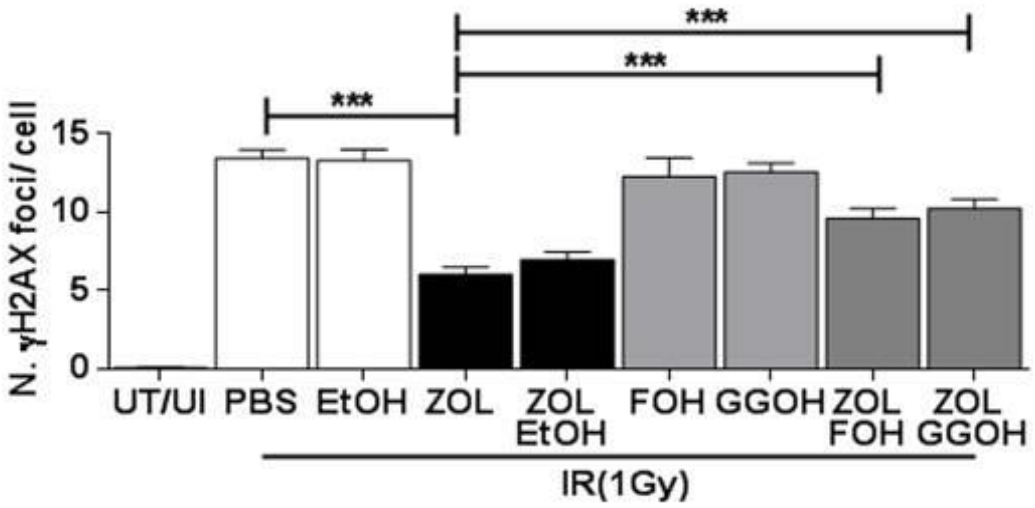


Figure 4.5 Addition of metabolites FOH and GGOH downstream of the FPP synthase abrogated Zol's DNA repair activity

*Number of γH2AX foci in hMSC not irradiated (UI) or exposed to 1Gy of irradiation in the presence or absence of Zol (1μM) and with the addition of farnesol (FOH; 33μM) or geranylgeraniol (GGOH; 33μM) (n=3). Ethanol (EtOH) was added as control with Zol in the same amount used to dissolve GGOH and FOH. Data presented as mean ± SD and analysed by one way ANOVA with Bonferroni multiple comparison post-hoc test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

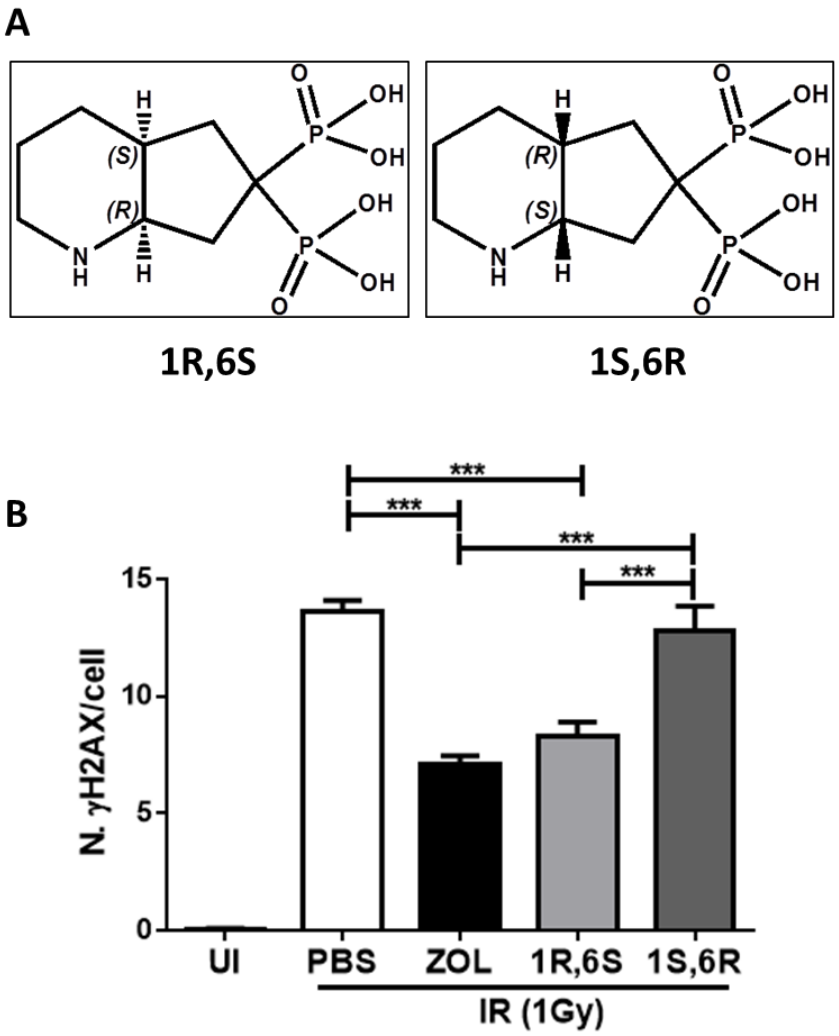


Figure 4.6 Active octahydro-pyrindine bisphosphonate of enantiomeric pair enhanced DNA repair similar to zoledronate

*(A) Structural formula of the octahydro-pyrindine-BP enantiomeric pair (1R,6S is the stronger inhibitor of FPPS with an IC-50 =15nM, whereas the 1S,6R enantiomer is a weaker inhibitor with an IC-50 = 359nM) (B) Number of γH2AX foci in hMSC exposed to irradiation (1Gy) in the presence of the octahydro-pyrindine-BP enantiomeric pair (n=3). Data presented as mean ± SD and analysed by one way ANOVA with Bonferroni multiple comparison post-hoc test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

4.4 Zol inhibits mTOR signaling downstream of the mevalonate pathway

To determine whether Zol inhibited mTOR pathway, hMSC were cultured (n=3) in the presence or absence of Zol for 3 days and analysed for protein expression of mTOR, ribosomal protein S6 Kinase (P70S6K), AKT, mTOR and their phosphorylated forms p-mTOR (Ser2448), p-P70S6K (Thr421/Ser424), p-AKT (Ser473) and p-FOXO3a (Ser 318/321) (Fig 4.7 A-Bi-iv). No difference was observed in expression of mTOR, AKT and P70S6K in both PBS and Zol treated hMSC respectively (Fig 4.7 A). However the phosphorylated forms p-mTOR (Ser2448), p-AKT (Ser473) and p-P70S6K (Thr421/Ser424) and p-FOXO3A (Ser318/321) were significantly reduced on treatment with Zol compared to PBS treated hMSC (Fig 4.7 A-Bi-iv). On addition of FOH and GGOH (to reverse the effects of Zol); no significant reduction in the expression of these phosphorylated proteins was observed (Fig 4.7 A-B). Together these data suggested that zoledronate inhibited both TORC1 and TORC2 mediated mTOR signalling downstream of the mevalonate pathway.

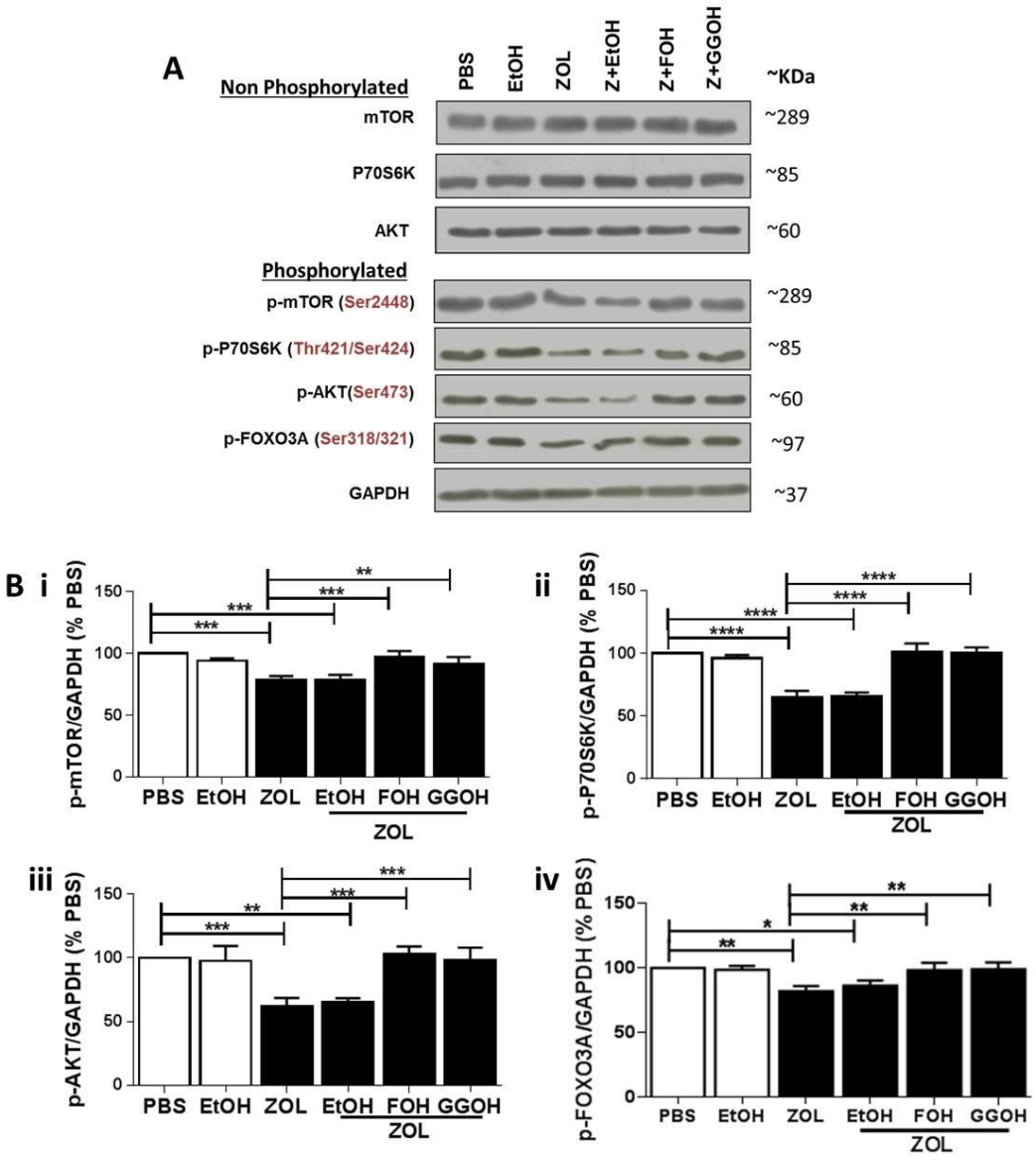


Figure 4.7 Zoledronate inhibited mTORC1 and mTORC2 forks of mTOR signalling

*(A) Representative example of expression of mTOR, P70S6K, AKT, their phosphorylated forms p-mTOR, p-P70S6K, p-AKT and p-FOXO3A by western blot analysis in hMSC exposed to zoledronate (ZOL) alone or in combination with farnesol (FOH) or geranylgeraniol (GGOH). PBS was added in the same amount as used for Zol, and ethanol (EtOH) was added in the same amount as used for GGOH and FOH as controls. (Bi-iv) Quantification of phosphorylated proteins expression normalised to the expression of the 'housekeeping protein', glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using ImageJ in 3 hMSC cultures exposed to Zol and/or FOH, GGOH (n=3). Data presented as mean ± SD and analysed by one way ANOVA with Bonferroni multiple comparison post-hoc test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

4.5 Zol enhances DNA repair by increased nuclear translocation of FOXO3a and increased p-ATM recruitment

Since AKT activity lies downstream of mTORC2 and regulates FOXO3a phosphorylation and DNA repair mechanisms, therefore I wanted to determine whether inhibition of mTORC2 signalling resulted in an enhanced induction of a DNA damage response by nuclear translocation of FOXO3a. I determined expression levels of FOXO3A, p-ATM (Ser1981) in hMSC (n=3), which were not irradiated (UI) or 10 minutes after irradiation (IR, 1Gy) in both cellular and cytoplasmic compartments (Fig 4.8 A-Bi-iii). Nuclear levels of FOXO3A were significantly increased in Zol treated hMSC regardless of irradiation (Fig 4.8 A-Bi-ii). In contrast p-ATM showed increased nuclear expression in Zol treated cultures only following irradiation (Fig. 4.8 A-Biii). On addition of FOH and GGOH the levels of both FOXO3A and p-ATM reverted to control PBS levels confirming that inhibition of mTOR signalling is downstream of the mevalonate pathway (Fig 4.8 A-Bi-iv). Taken together these results suggested that inhibition of mTORC2 signalling resulted in low levels of p-AKT and increased nuclear translocation of FOXO3A, which in turn increases phosphorylation of ATM in the presence of DNA damage, resulting in an activated response to DNA damage.

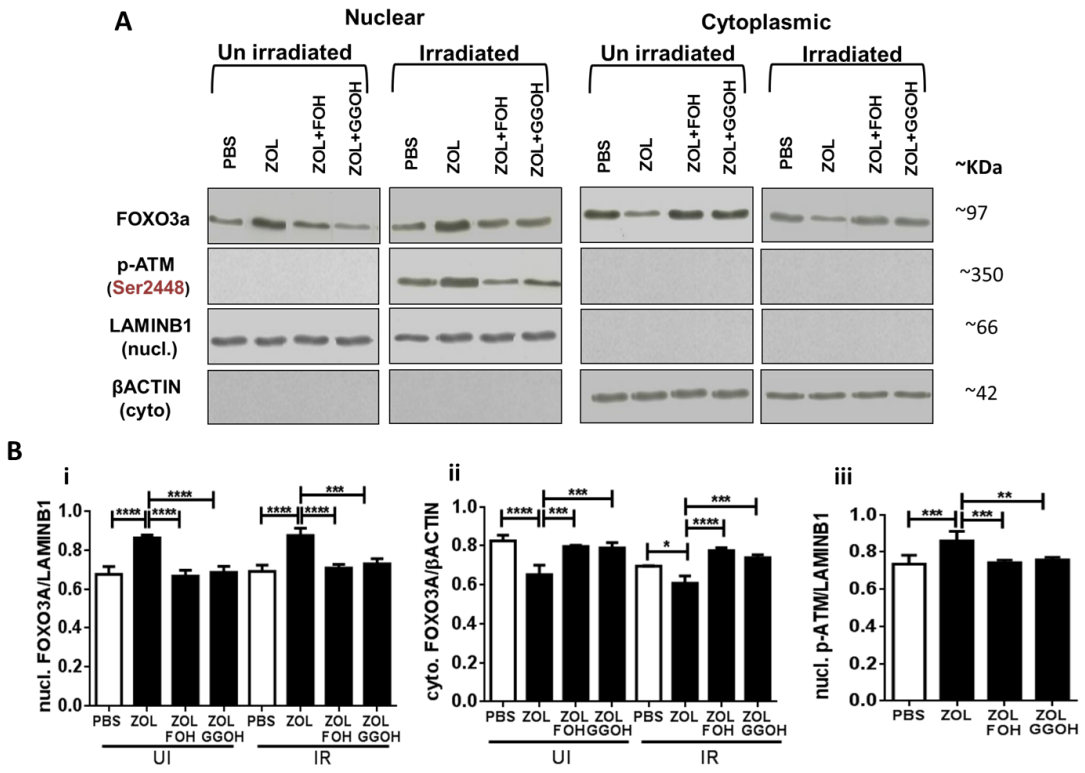


Figure 4.8 Zoledronate increased FOXO3a nuclear translocation in hMSC

*(A) A representative example of expression of nuclear and cytosolic FOXO3A and p-ATM in non-irradiated hMSC (UI) and in hMSC 10 minutes after irradiation (IR) in the presence or absence of Zol normalised to expression levels of LaminB1 and βACTIN respectively. (B) Quantification of nuclear (Bi) and cytosolic FOXO3A (Bii) and nuclear p-ATM (Biii) in non-irradiated hMSC and in hMSC 10 minutes after irradiation in the presence or absence of Zol normalised to LaminB1 and βACTIN and analysed by ImageJ. Data are expressed as mean ±SD and were analysed by one way ANOVA and Bonferroni post-test for multiple comparisons *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

To determine whether increased translocation of FOXO3a in response to Zol was required for the increased DNA damage response, hMSC cultures were treated with siRNA to knockdown FOXO3a as described in section 2.13 (materials and methods). zoledronate or PBS was added to the hMSC cultures after transfection and after 3 days cell cultures (n=3) were subjected to irradiation at 1Gy and 12 hrs later sacrificed for protein expression of FOXO3a and assessment of DNA damage foci by western blotting and confocal microscopy (Fig 4.9 A-B). Following transfection with siRNA FOXO3a, there was an 88.19±4% and 88±3.56% knockdown of FOX3a in respective vehicle and ZOL treated hMSC (Fig 4.9 A-B). This was also seen by immune fluorescent imaging of hMSC where siRNA FOXO3a knockdown resulted in absence of FOXO3a (red) staining in DAPI (blue) stained nuclei when compared to non-silencing siRNA treated hMSC in presence or absence of Zol (Fig 4.10A). Knockdown of FOXO3a resulted in no ATM recruitment in the nucleus of both PBS and Zol treated hMSC confirming the known importance of FOXO3a in recruitment of ATM.

To determine whether FOXO3a is required for DNA damage repair activity by Zol, FOXO3a silenced and non-silenced hMSC (n=3) were irradiated in presence or absence of Zol at 1Gy and 12 hrs later fixed and stained for enumeration of γH2AX foci (Fig 4.11 A-B). As expected in the non-silenced hMSC Zol treated significantly reduced the number of DNA damage foci compared to PBS treated hMSC. However, following FOXO3a knockdown, there was no significant reduction in the damage foci in Zol treated cells compared to PBS (n=3; Fig 4.11 A-B). Together these data suggested that FOXO3a plays a key role in the repair of DNA damage by Zol downstream of the mevalonate and mTOR signalling pathway.

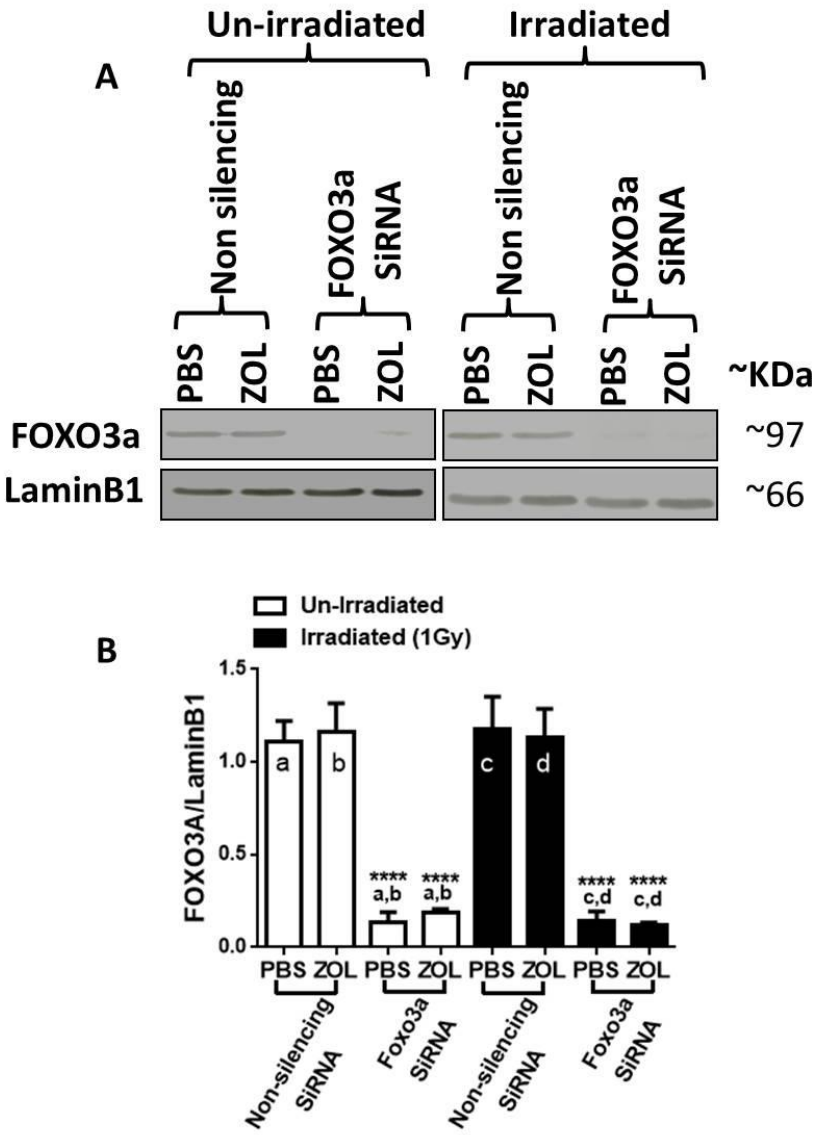


Figure 4.9 Knockdown of FOXO3a in hMSC

*(A) Representative example of expression of FOXO3a (top panel) and LAMINB1(bottom panel) in hMSC transfected with siRNA targeting FOXO3a (FOXO3 siRNA) or non-silencing siRNA, treated with PBS or Zol and gamma irradiation at 1Gy. (B) Quantitation of protein FOXO3a normalised to LAMINB1 using ImageJ in 3 hMSC cultures exposed to Zol and/or irradiation in FOXO3a siRNA transfected or non-silenced cells. Data presented as mean ±SD and were analysed by one way ANOVA and Bonferroni post-test for multiple comparisons *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

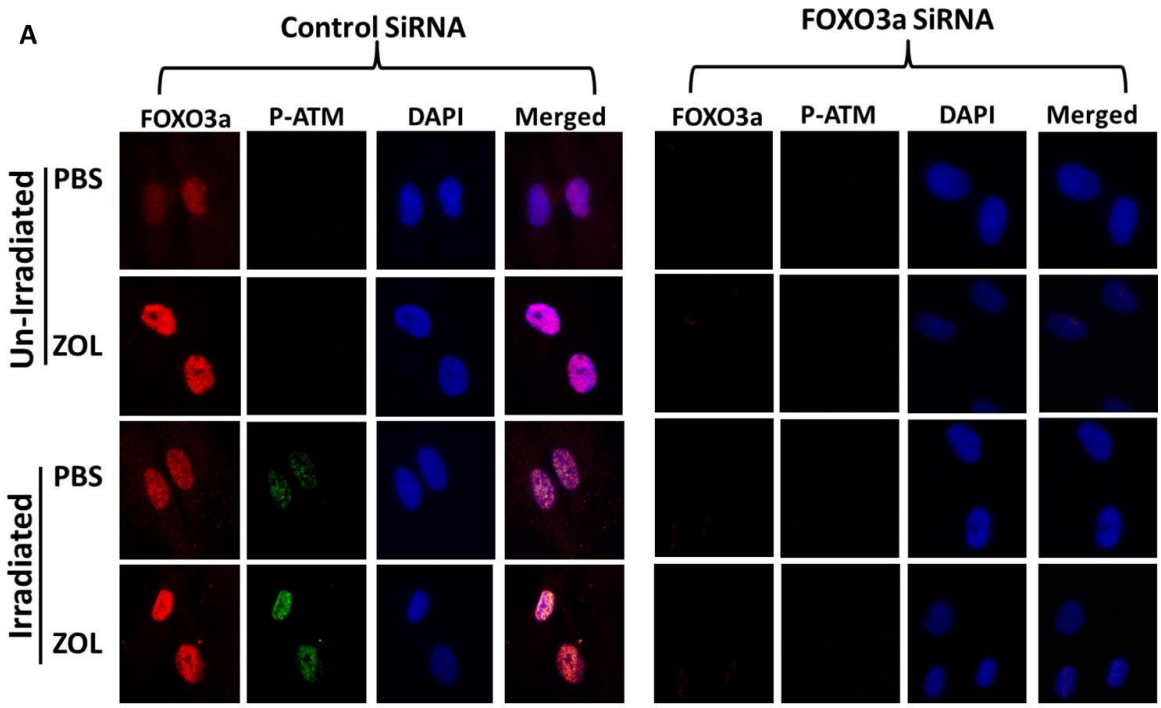


Figure 4.10 Zoledronate increased co-localisation of FOXO3a with p-ATM following irradiation

*(A) Representative examples of FOXO3a (red) and pATM (green) in DAPI stained nuclei (blue) of FOXO3a silenced or non-silenced hMSC cultures (n=3) treated with either vehicle (PBS) or ZOL and irradiation at 1Gy. Co-localization of nuclear FOXO3a with ATM-pS1981 was shown as the merged images in hMSC cultures (n=3). Data presented as mean ±SD and were analysed by one way ANOVA and Bonferroni post-test for multiple comparisons *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

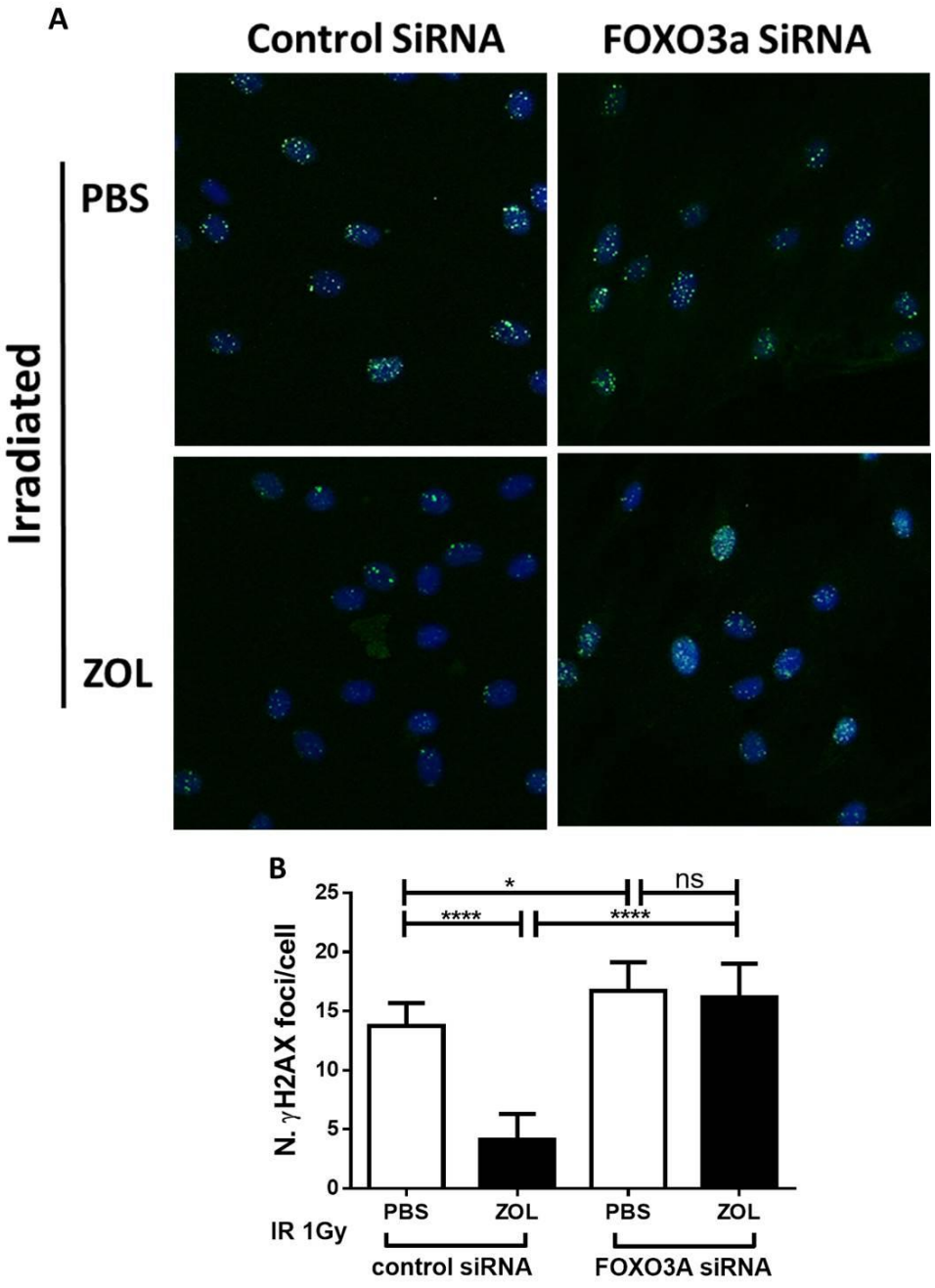


Figure 4.11 FOXO3a is necessary for reduction of foci frequencies by Zol.

*(A) Representative example of DNA damage γH2AX foci (green) in hMSC transfected with FOXO3a siRNA in DAPI stained nuclei (blue). Cultures of hMSC were treated with PBS or ZOL and irradiated at 1Gy (n=3). (B) Quantification of the number of γH2AX foci per cell in hMSC transfected with FOXO3a siRNA in presence or absence of Zol (n=3). Data presented as mean ±SD and were analysed by one way ANOVA and Bonferroni post-test for multiple comparisons *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

4.6 mTORC2 inhibition mediated enhanced DNA damage repair

To further strengthen evidence supporting that enhanced DNA repair by Zol was mediated by mTORC2 inhibition, I determined whether this effect was seen with other mTOR inhibitors. Human MSC were exposed to the inhibitor of Phospho-inositol-3 kinase Ly294002, and to the inhibitor of mTOR KU0063794, both resulting in inhibition of both TORC1 and TORC2. Human MSC were also exposed to rapamycin a known inhibitor of mainly TORC1 upon short term exposure. The expression of the downstream targets of TORC1 and TORC2 p-p70S6K, p-AKT and p-FOXO3a was inhibited by Ly294002 and KU0063794 as shown by western blot analysis (n=3; Fig 4.12 A). In contrast only the expression of p-p70S6K, but not p-AKT and p-FOXO3a, was decreased when cells were exposed to Rapamycin (Fig. 4.12 A). This was in agreement to the conception that rapamycin inhibited only TORC1. Moreover an increase nuclear expression of FOXO3a and p-ATM was seen only in hMSC exposed to Ly294002 and KU0063794 but not to rapamycin (Fig. 4.12 B). This was reflected in a significant decrease in the number of γH2AX foci (to similar levels to Zol) only in cells treated with Ly294002 and KU0063794 but not with rapamycin (n=3 Fig. 4.12 C). Taken together these results suggest that abrogation of mTORC2 signalling by Zol results in low levels of p-AKT and increased nuclear translocation of FOXO3A, which in turn increases phosphorylation of ATM in the presence of DNA damage, resulting in an activated response to DNA damage.

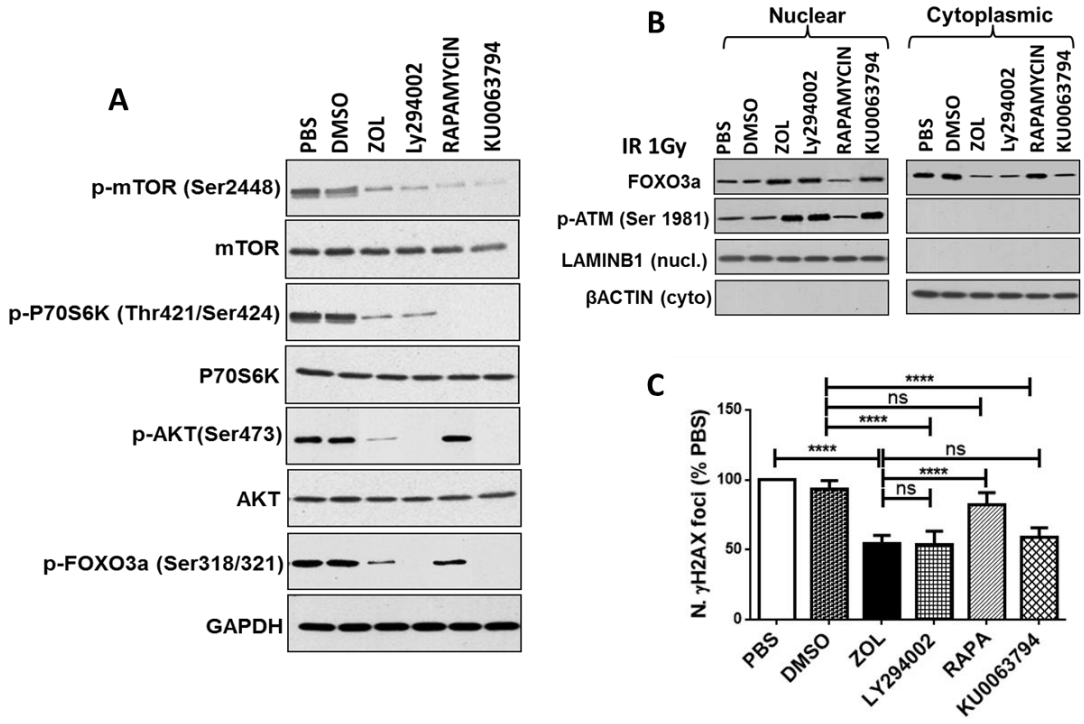


Figure 4.12 Inhibitors of both TORC1 and TORC2, but not rapamycin, promote enhanced DNA repair similar to Zol in hMSC

*(A) A representative example of expression of p-mTOR, mTOR, p-P70S6K, P70S6K, p-AKT, AKT and p-FOXO3A by western blot analysis in hMSC exposed either to zoledronate at 1µM (ZOL), inhibitor of Phospho-inositol-3 kinase Ly294002 (10µM), inhibitor of mTOR KU0063794 (10nM) or rapamycin (10nM). PBS was added in the same amount as used for Zol, and DMSO was added in the same amount as used for the other inhibitors as controls. Protein expression was normalised to the expression of the 'housekeeping protein', glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (B) A representative example of expression of nuclear and cytosolic FOXO3A and p-ATM in hMSC 10 minutes after irradiation (1Gy) in the presence or absence of Zol, Ly294002, KU0063794 or rapamycin normalised to expression levels of LAMINB1 and βACTIN respectively. (C) Number of γH2AX foci in response to Zol, Ly294002, KU0063794 and rapamycin following irradiation at 1Gy.Data presented as mean ±SD and were analysed by one way ANOVA and Bonferroni post-test for multiple comparisons *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

4.7 Discussion

In this chapter I have dissected out the mechanism of action of zoledronate to enhance DNA damage repair in human MSC following exposure to irradiation. I have established correlative evidence that inhibition of mevalonate pathway by Zoledronate results in unprenylation of RAP1A which correlates with fewer foci. I have identified for the first time that Zol repairs via inhibition of mTOR signalling pathways specifically the mTOR-FOXO3a-ATM signalling. Zoledronate inhibited TORC1 and TORC2 signalling and their downstream effector molecules. Specific inhibition of TORC2 resulted in enhanced DNA repair via AKT-FOXO3a regulation and knockdown of FOXO3a resulted in complete abrogation of DNA damage response that was otherwise promoted by Zol. Moreover, the same effect was obtained using other mTOR inhibitors capable of inhibiting the TORC2 complex in hMSC.

Indeed short incubation with the TORC1 inhibitor rapamycin (3 days) induced little or no significant enhancement of DNA damage repair. This appears in contrast with the results of Gharibi et al (2014) and Iglesias-Bartolome et al. (2012). The former showed inhibition by rapamycin of the PI3K/Akt/mTOR pathway and lower levels of DNA damage in mesenchymal stem cells (Gharibi, Farzadi et al. 2014). The latter showed inhibition of p70S6K but not Akt leading to reduced accumulation of reactive oxygen species and decreased accumulation of DNA damage (Iglesias-Bartolome, Patel et al. 2012). The discrepancies may lie in the dose, the time of incubation and the size of the effect. In the former study cells were incubated in the presence of rapamycin for prolonged times (5 weeks), not the 3 days of treatment we used. It is known that chronic administration of rapamycin leads to inhibition of TORC2 as well as TORC1 (Sarbassov, Ali et al. 2006). In the latter a higher dose of rapamycin was used.

Whether our dose was not sufficient to initiate the action by rapamycin leading to reduced DNA damage require further investigation. However, our dose was sufficient to lead to decreased expression of p70S6K downstream of TORC1.

RAS and RHEB are important prenylated proteins of the PI3K-MTOR pathway and thus they establish a plausible link between prenylated GTPases of the mevalonate pathway and the mTOR pathway. Although in this study I have not tested whether RAS or RHEB are the connector between mevalonate pathway and mTOR pathway, there are good incidences that this may be the case Investigations have shown that Rheb, a small GTPase involved in activating the serine/threonine kinase mammalian target of rapamycin (mTOR), requires farnesylation for proper membrane association such that it can activate the mTOR pathway (Clark, Kinch et al. 1997, Aspuria and Tamanoi 2004, Basso, Mirza et al. 2005). Studies have shown that there are elevated levels of Rheb in breast cancer suggesting that inhibitors of Rheb or mTOR are useful in cancer treatment and there is several evidences available for this using farnesyl transferase inhibitors and mTOR inhibitors (deGraffenried, Friedrichs et al. 2004, Basso, Mirza et al. 2005, Johnston 2006, Beeram, Tan et al. 2007). Definitely Rheb plays an important role in regulating mTOR (Yu, Li et al. 2012). I propose that since zoledronate inhibits the prenylation of small GTPases like Rho, Rac and Rab it may even inhibit prenylation of RHEB. As a result there is inactivation of the mTOR pathway and this may establish the connection between prenylation pathway and mTOR signalling to explain the extension in lifespan and DNA damage repair in presence of Zol. One of the future experiments could include the transduction of Rheb constructs (RHEB M184L) into hMSC that are pre-treated with Zol and have inhibition of mTOR signalling (Ding, McDonald et al. 2014). These constructs become

membrane associated after geranylgeranylation and activate the mTOR signalling once transduced. If the connection of RHEB as a link between the pathways is true then they should reverse the effects of Zol mediated mTOR inhibition. Alternatively knockdown of RHEB in hMSC should result in inactivation of mTOR signalling similar to what Zol does in these cells and this would also confirm a direct association of RHEB to the two pathways.

FOXO3A is involved in several functions fundamental to cell survival and longevity. In addition the general importance of FOXO3A in longevity is supported by studies involving centenarians and non-agenerians, in whom particular variants of the human FOXO3A gene have been linked with longevity traits (Willcox, Donlon et al. 2008). FOXO3 has also been shown to enhance longevity by regulating ROS detoxification in several tissues as found by activities of mitochondrial enzyme superoxide dismutase 2 (SOD2) (Kops, Dansen et al. 2002), Catalase (CAT) (Tan, Wang et al. 2008), antioxidant enzyme peroxiredoxin III (Chiribau, Cheng et al. 2008) or the sestrin 3 (SESN3) redox enzyme (Chen, Jeon et al. 2010, Hagenbuchner, Kuznetsov et al. 2012).

FOXO3a is important in autophagy, whose inhibition has been shown to induce degenerative changes in mammalian tissues that resemble those associated with ageing (Rubinsztein, Marino et al. 2011). Deletion of FOXO3A led to a marked down regulation of the pro-autophagic program and to the inability of HSC to mount an adaptive autophagy response upon metabolic stress, suggesting that FOXO3A is responsible for protecting HSC survival through an autophagy mechanism under conditions of metabolic stress (Warr, Binnewies et al. 2013). Besides regulating metabolism and oxidative stress by promoting the expression of antioxidant enzymes or through proautophagic programming, FOXO3a also enhances recruitment of the

DNA repair machinery (Salih and Brunet 2008). Responses of FOXO3a to DNA damage was first established in *C.elegans* where FOXO3 homologue DAF-16 regulated stress response and extended lifespan during a nutrient deprived state (Braeckman and Vanfleteren 2007). In fact FOXO transcription factors also contribute to cells survival and stress resistance by triggering growth arrest and activating DNA damage repair enzymes GADD45a in response to oxidative stress (Wang, Gorospe et al. 1999, Zhan, Antinore et al. 1999, Furukawa-Hibi, Yoshida-Araki et al. 2002). GADD45 participates in cellular responses to DNA damage and is associated with activation of cell cycle checkpoints and DNA repair thus helping in maintenance of genomic integrity (Smith, Chen et al. 1994, Smith, Kontny et al. 1996, Hollander, Sheikh et al. 1999, Jin, Antinore et al. 2000). In a state of stress induced by DNA damage or accumulation of ROS, the inactivation of FOXO induced by growth factors is overruled by phosphorylation of Jun-N-terminal kinase (JNK) and mammalian STE20 like protein kinase 1 that either phosphorylate FOXO3 or its 14-3-3 protein binding partner resulting in nuclear accumulation (Essers, Weijzen et al. 2004, Sunayama, Tsuruta et al. 2005, Lehtinen, Yuan et al. 2006, Sunters, Madureira et al. 2006). Moreover FOXO3a phosphorylation by Akt results in the inactivation of FOXOs and in their accumulation in the cytoplasm (Greer and Brunet 2005). This negative regulation of FOXO3A by PI3K-AKT signalling has also been recognized as important effector for the recruitment of DNA damage response protein ATM (Tsai, Chung et al. 2008). Interaction of FOXO3 and ATM has also been reported in human neuroblastoma cells where the FOXO3-ATM complex resulted in activation of CREB a downstream target associated with caspase-8 activation (Geiger, Hagenbuchner et al. 2012). These

studies highlight the possible mechanisms by which FOXO3a associates with stress management, DNA repair and longevity.

FOXO3a and ATM are important for stem cells survival. Depletion of FOXO3A leads to depletion of HSC and neural stem cells (Miyamoto, Miyamoto et al. 2008, Paik, Ding et al. 2009, Renault, Rafalski et al. 2009) and can do this through inhibition of multiple anti-ageing defence mechanisms. In fact in haematopoietic stem cells FOXO3a modulation of ATM is associated with repair of oxidative stress induced damage thus maintaining the HSC self-renewal (Yalcin, Zhang et al. 2008). Indeed loss of the DNA damage sensors ATM and FOXO3a has been shown to deplete HSC (Ito, Hirao et al. 2004, Miyamoto, Miyamoto et al. 2008). This has also shown to aggravate the loss of melanocyte stem cells in response to low dose radiation (Inomata, Aoto et al. 2009), and promote the loss of undifferentiated spermatogonia (Takubo, Ohmura et al. 2008). Thus several studies have reported interaction of FOXO3a and ATM with ROS and DNA repair mechanisms leading to cell survival (Tsai, Chung et al. 2008, Yalcin, Zhang et al. 2008, Chung, Park et al. 2012). In fact even in my study I have found that FOXO3a is associated with recruitment of ATM, and when FOXO3a is knocked down, there is no recruitment of ATM which confirms their association similar to what others have reported. In this study in mesenchymal stem cells I find extension of lifespan and DNA repair to be mediated via mTORC2/AKT/FOXO3a/ATM signalling.

Genes involved in innate immunity, inflammation and apoptosis are controlled by NF-κB transcription factor and inhibition of NF-κB has using pharmacological interventions has shown to cause extended median lifespan in drosophila (Moskalev and Shaposhnikov, 2011). Moreover NF-κB inhibition delays DNA damage-induced senescence and aging in mice (Jeremy, Tilstra et al. 2012). Zoledronate is known to

inhibit NF-κB by inhibiting phosphorylation of the active subunit RelA (Schech, Kazi et al, 2013). Therefore it may be possible that Zoledronate extend lifespan of MSC by inactivation of NF-κB by delaying cellular senescence.

ATM and ATR through their coordinated activities trigger cellular response to DNA DSB or ROS induced damage by activating the DNA repair signalling pathways (Ditch and Paull 2012). More specifically phosphorylation of ATM is central to initiate a number of DNA repair mechanisms including non-homologous end joining or homologous recombination (Shiloh and Ziv 2013). Going forward it will be important to determine which of these DNA repair mechanisms is downstream of ATM as this has implication for the level of fidelity obtained during the repair. Prendergast et al (2011) reported that small molecule inhibition of ATM and DNA-PK in hMSC showed involvement of both these proteins in formation of phosphorylated H2AX foci (Prendergast, Cruet-Hennequart et al. 2011). They suggest that while ATM is required for DNA repair via homologous recombination in association with RAD52, the existence of DNA-PK mediated H2AX phosphorylation in hMSC may prompt the existence of the error prone NHEJ DNA repair pathway which has also been proposed to occur in HSC (White, Choi et al. 2008, Mohrin, Bourke et al. 2010, White, Choi et al. 2010). However absence of ATM may not necessarily mean an error prone NHEJ repair mechanism. Studies in mouse embryonic stem cells have shown that HR repair can occur independent of ATM (Rass, Chandramouly et al. 2013). They used ATM and γH2AX double deficient mice embryonic stem cells and found that of the three possible fractions (ATM, DNA-PK and ATR) generating H2AX in response to DSB, ATM was actually dispensable for HR repair. Therefore both mechanisms of DNA repair are possible in hMSC as ATM is upstream of them two. In this study I have not dissected

out the which repair pathway is triggered following Zol treatment as DNA repair mechanisms downstream of ATM have implication for the level of fidelity obtained during the repair. One way of tracking the repair mechanism is by employing a traffic light reporter (TLR) construct that generates readouts of HR or NHEJ repair at DNA damage breakpoints (generated by restriction enzymes) by either fluorescing green for HR repair or red for NHEJ repair (Certo, Ryu et al. 2011). Similar reporter cassettes have been used by Seluanov et al. in analysing DNA double strand breaks in mammalian cells (Seluanov, Mao et al. 2010). Another possible method would be to detect co-localization of Ku70, Ku80 or Rad51 or 53BP1 (NHEJ machinery) in the irradiation induced DNA damage foci to determine whether at the formation of DNA damage foci is there recruitment of these NHEJ molecules similar to what was done in HSC (Mohrin, Bourke et al. 2010).

In conclusion this chapter has highlighted the mechanism of action of Zol's ability to enhance DNA repair. I have shown that it is mediated via inhibition of mevalonate and mTOR signalling specifically following the mTORC2/AKT/FOXO3a/ATM pathway. DNA repair and maintenance of stem cells is important for overall maintenance of tissue integrity and homeostasis. Tissue regeneration is most important in case of injury/DNA damage; therefore it would be important to investigate whether Zol with such damage repairing ability is able to protect tissue integrity/ tissue regeneration in response to damage, which is what I have investigated in the next chapter.

Chapter 5: Zoledronate enhances Zebrafish tail fin regeneration following radiation induced damage

5.1 Introduction

Tissue regeneration is an important property of stem cells and occurs in response to injury. It is an evolutionarily conserved mechanism. Zebrafish (*Danio rerio*) have become powerful vertebrate models for investigating the mechanisms of regeneration both at cellular and molecular levels (Morrison, Loof et al. 2006, Porrello, Mahmoud et al. 2011, Goldsmith and Jobin 2012, Seifert, Kiama et al. 2012). There are several reasons for this as they can be housed in a laboratory with low husbandry cost and breeding pairs can produce over 200 embryos. The embryos are transparent and embryonic development can be monitored under a light microscope from single cell stage right up to development of distinct tissue and organs (Kimmel, Ballard et al. 1995). Developing organs are visible by 36 hours post fertilization (hpf), and larva move out from chorionic protective layers (process called hatching) by 48-72pf. At this point the embryo is feeding on the yolk sac, and independent feeding starts 5 days post fertilization (dpf). Apart from visible organogenesis, Zebrafish embryo can be used to set up large experiments with multiple treatment groups in just a 96-well plate thus allowing for multiple experimental repeats to be achieved easily. Another important advantage of zebrafish bioassays is the ease with which administration of drugs can be carried out by simply adding compounds in medium in culture dishes/well plates and drugs can be readily absorbed by the embryos. Zebrafish have been used in drug toxicity screening and teratogenicity. For example thalidomide, used in multiple myeloma and leprosy, caused birth developmental

defects when prescribed to pregnant women but the cause of limb malformation was unknown until studies in zebrafish helped decipher the mechanisms of action of thalidomide (Ito, Ando et al. 2010). The most unique feature of zebrafish is their ability to regenerate fins, heart, central nervous system among others and in this chapter I have focussed on the tail fin regeneration assay (Gemberling, Bailey et al. 2013). Post amputation, wound healing (or tail fin regeneration) starts at 0-1 days by formation of actin-purse string structures that causes contraction of the wound. This stump like structure is then sealed by epithelium cells (Kawakami, Fukazawa et al. 2004, Yoshinari, Ishida et al. 2009). This is followed by the formation of blastema which is a mass of highly proliferative lineage restricted mesenchymal-like progenitor cells that is associated with regeneration. The process of regeneration usually takes 3-5 days following amputation in larva (Kintner and Brockes 1984, Echeverri, Clarke et al. 2001, Han, Yang et al. 2005, Stoick-Cooper, Moon et al. 2007, Stoick-Cooper, Weidinger et al. 2007). Zebrafish tail regeneration model has been employed in screening small molecule drug libraries for detecting pathways associated with regeneration such as in the case of Wnt inhibitors or even to determine the roles of immune cells in injury-induced tissue regeneration (Mathew, Sengupta et al. 2007, Stoick-Cooper, Moon et al. 2007, Stoick-Cooper, Weidinger et al. 2007, Chen, Dodge et al. 2009, Lu, Ma et al. 2009, Li, Yan et al. 2012).

Drugs initially screened in zebrafish have been subsequently validated in mice, suggesting that information derived from quick and cheaper assay in zebrafish can yield important information before progressing to more expensive and time consuming experiments in mice. Chemical screening in fish for drug repurposing (identification of a new application of existing drugs) has been most successful and

even validated in mice eventually leading to clinical trials. For example the role of prostaglandin E2 (PGE2) in development of HSC was identified by screening chemicals in zebrafish model (North, Goessling et al. 2007, Durand and Zon 2010). Validation in mice subsequently led to clinical trials suggesting the use of PGE2 in HSC engraftment (Cutler, Multani et al. 2013). This example thus highlights Zebrafish as a good *in vivo* system that can therefore be employed to bridge studies between *in vitro* experiments and higher model organism such as mice which are expensive. Therefore in this chapter I employed Zebrafish as a model to assess the effect of zoledronate on tail fin regeneration ability following injury in the form of amputation alone or accompanied with radiation. I hypothesised that **'Zoledronate enhanced zebrafish tail fin regeneration following amputation and irradiation'.**

5.2 Optimization of tail fin regeneration measurement in the most reproducible way

To optimize the method of measuring tail fin regeneration at 120 hours post fertilization following amputation (48hpf) in Zebrafish embryo, three different measurement strategies were employed. The first measurement included the full length measure of embryo from head to tail (type A), the second method was to measure regeneration from the site of amputation to the end of regenerated portion (Type B), and the third method involved measurement starting from the anal region to the end of the regenerated portion (Type C) (Fig 5.1). Among the three methods, in type C the data points were more clustered closer to the mean suggesting lower standard deviation following amputation (n=45/group; Fig 5.1). This method was therefore observed as most robust and reproducible and hence employed for future experiments.

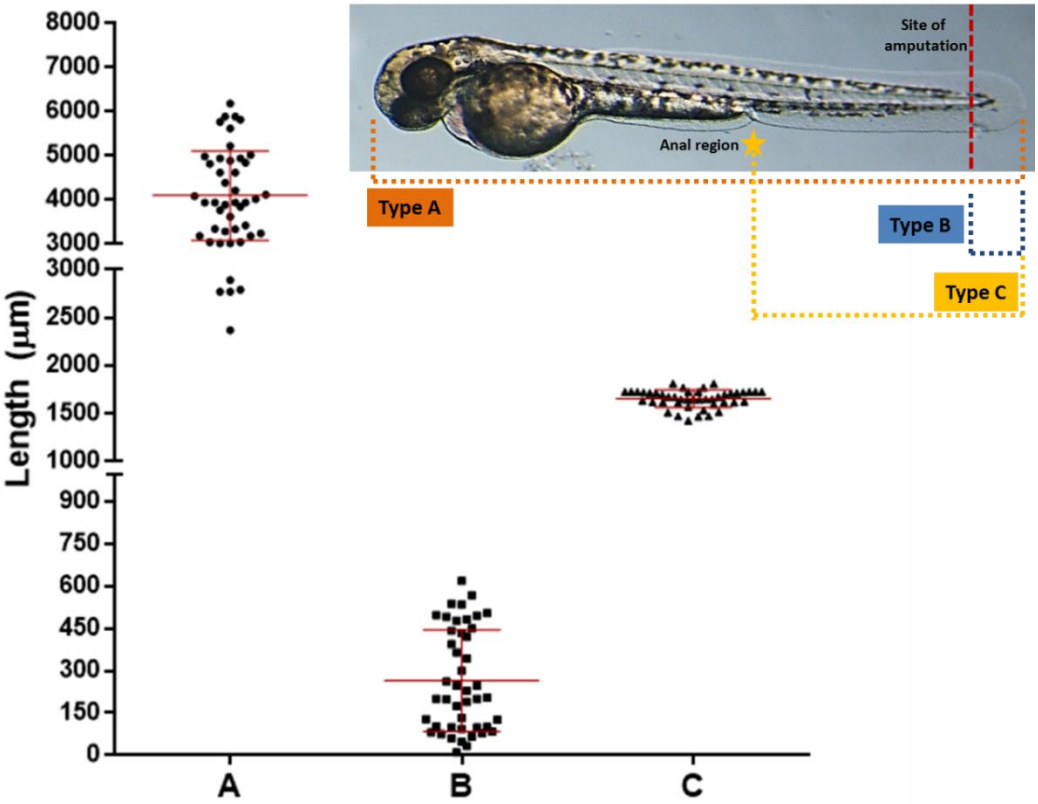


Figure 5.1 Optimization of tail length measurement following amputation in zebrafish embryo

Graph represents the length of zebrafish tail fin regenerated portion following amputation by three different measuring strategies (n=45/group; Type A, B and C) clearly marked on the image of zebrafish in the panel. Data presented as Mean ± SD.

5.2.1 Optimization of a model for tail fin regeneration following irradiation

To determine whether tail fin regeneration following amputation can be abrogated in presence of irradiation, Zebrafish embryos at 48hpf were subjected to irradiation at doses of 1Gy, 3Gy or 5gy respectively. This was followed by tail amputation. At 120hpf (3days post amputation) tail length in zebrafish was measured from the anal region to the end of tail regenerated portion (Fig 5.2 A). Following amputation, significant regeneration was observed although it was not complete. Accompanying irradiation with amputation there was significant inhibition of the regeneration of the amputated portion at all doses of irradiation respectively (n=15/group, repeated 3 times; Fig 5.2 A-B). Together these data suggested that combination of amputation with irradiation significantly reduced the regeneration of the amputated tail fin in a 5day old zebrafish embryo. This model was used further to test the effect of zoledronate in tail fin regeneration.

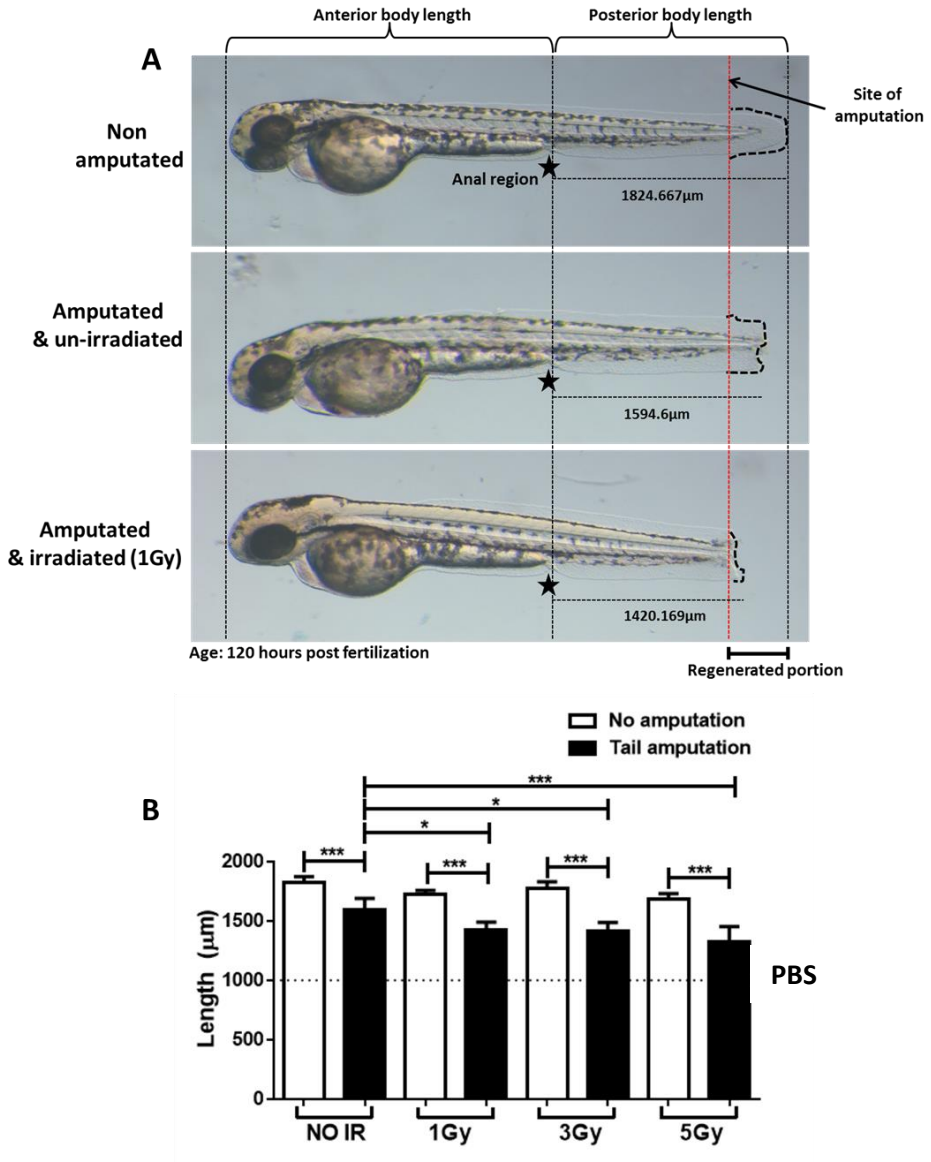


Figure 5.2 Irradiation and amputation inhibited tail regeneration in Zebrafish embryo

*(A) Representative of Zebrafish embryo 120 hours post fertilization (hpf, top panel) either following amputation (middle panel) or amputation combined with irradiation 1Gy (bottom panel). The red dotted line marked the site of amputation and tail length was measured from the anal region (black star) to the end of the regenerated portion. (B) Assessment of tail regeneration by measurement of tail length following either amputation or in combination with irradiation at different doses (1Gy, 3Gy or 5Gy, n=15/group repeated 3 times). Data presented as mean ± SD and analysed by one way ANOVA with Bonferroni multiple comparison post-hoc test *p<0.05, **p<0.01, ***p<0.001, ****p<0.000.*

5.2.2 Optimizing Zoledronate dose and treatment regimen in zebrafish

To determine whether zoledronate enhanced tail fin regeneration in zebrafish model following irradiation, I first established the optimal time point (hpf) when Zoledroante should be administered and the optimal dose of zoledronate that would have no toxic effects on survival of the embryos. To test this, zoledronate was added to E3 medium at concentrations ranging between 0.25-10µM. All embryos were selected at 16 cell-stage and Zol was added at different time points (5hpf, 24hpf, 48hpf) of development. The percentage of live embryos were enumerated (n=45/group; Fig 5.3A-B). In case of drug toxicity the embryos would either be dead or underdeveloped or in an unhealthy condition (Fig 5.3.A) The percentage of viable embryos treated with Zol at doses of 2.5µM onwards was significantly reduced at all three time points (5hpf, 24hpf, 48hpf) of administration when compared to untreated embryos (dotted line; Fig 5.3 B). Administration of doses 250nM, 500nM or 1µM at 5hpf resulted in a significant loss of viable embryos when compared to untreated embryos. Similar results were seen when compared to embryos receiving zol at 24hpf and 48hpf (Fig5.3B). However there was no significant difference in the viable embryo numbers at treatment time points of 24hpf and 48hpf at doses 250nM, 500nM or 1µM when compared to untreated controls. For future experiments I chose to use 1µM as this was the highest dose at which no toxicity was seen and in accordance with our previous experiments the same dose was administered *in vitro* experiments in hMSC as well (Chapter 3 and 4). The earliest time point of 24hpf was selected as time for administration of drugs to the embryos for all future experiments to permit the completion of each assay within 5.2 days age of embryo when feeding of embryo starts and it is considered as a regulated procedure.

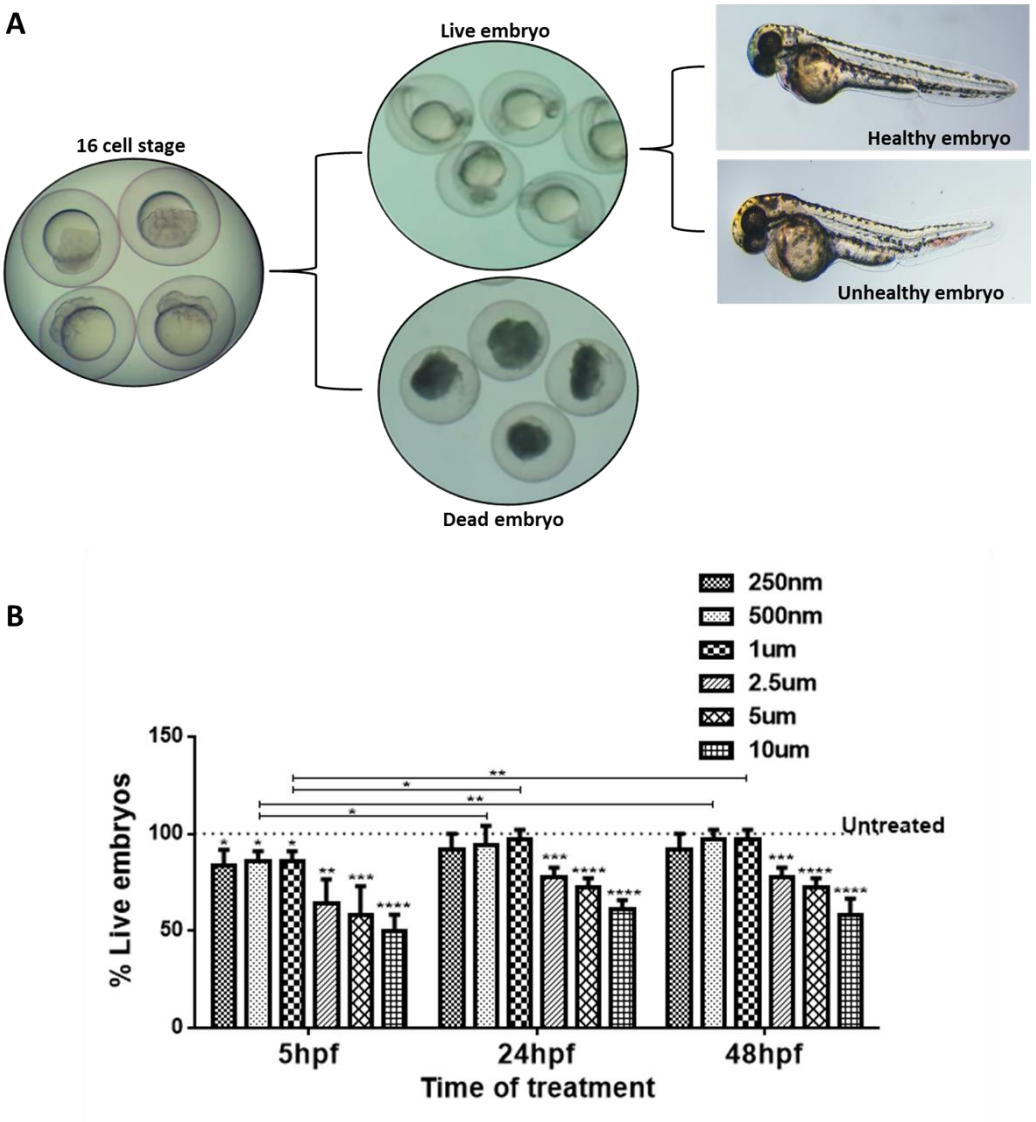


Figure 5.3 Determination of dose regimen for zoledronate administration

*(A) Representative example of Zebrafish embryos in the 16 celled stage. The next two panels represent a live and dead embryo. The panels on the extreme right represent a 120hpf embryo that is healthy (top) or unhealthy (bottom). (B) Percentage of live embryos following zol administration at different times of treatment and different doses (n=45/group). Data presented as mean ± SD and analysed by two way ANOVA with Tukey multiple comparison post-hoc test *p<0.05, **p<0.01, ***p<0.001, ****p<0.000.*

5.3 Zoledronate restores tail regeneration in zebrafish model following irradiation via the mevalonate pathway

To determine whether Zol could restore the tissue regeneration in zebrafish following irradiation, embryos were treated with Zol (1µM) at 24hpf. DNA damage was induced by exposure to ^{137}Cs Gamma source at 48hpf. Following irradiation at 1Gy, 3Gy or 5Gy the embryo tails were amputated and the embryos were then incubated at 28˚C for a further 12 hrs in the presence of different treatments. This 12 hour time point was in accordance with the experimental procedure for human MSC (Chapter 3) where maximum repair of DNA damage following irradiation in presence of Zol treatment was observed at 12 hours post irradiation. After this the drug was washed off and incubated in medium up to 120hf. At 120hpf the tail lengths were measured. In zebrafish subjected to amputation and irradiation in absence of treatment there was a significant loss in tail regeneration. However zebrafish that were treated with zoledronate had complete tail regeneration following irradiation (all doses) and amputation similar to amputated and un-irradiated controls (n=5/group repeated 3 times; Fig 5.4 A-Bi-iii).

To determine whether regeneration by Zol was mediated by inhibition of Mevalonate pathway, farnesol (FOH) and geranyl-geraniol (GGOH) substrates were added to reverse the inhibition of FPPS enzyme. Zebrafish tail regeneration was restored following irradiation in Zol treated embryos compared to PBS treated embryos, however on treatment with FOH or GGOH no restoration of amputated portion was observed. The effect of zoledronate on tail regeneration was thus reversed (n=15/group repeated 3 times Fig 5.4 C). Together these data suggested that zoledronate enhanced tail regeneration via inhibition of mevalonate pathway.

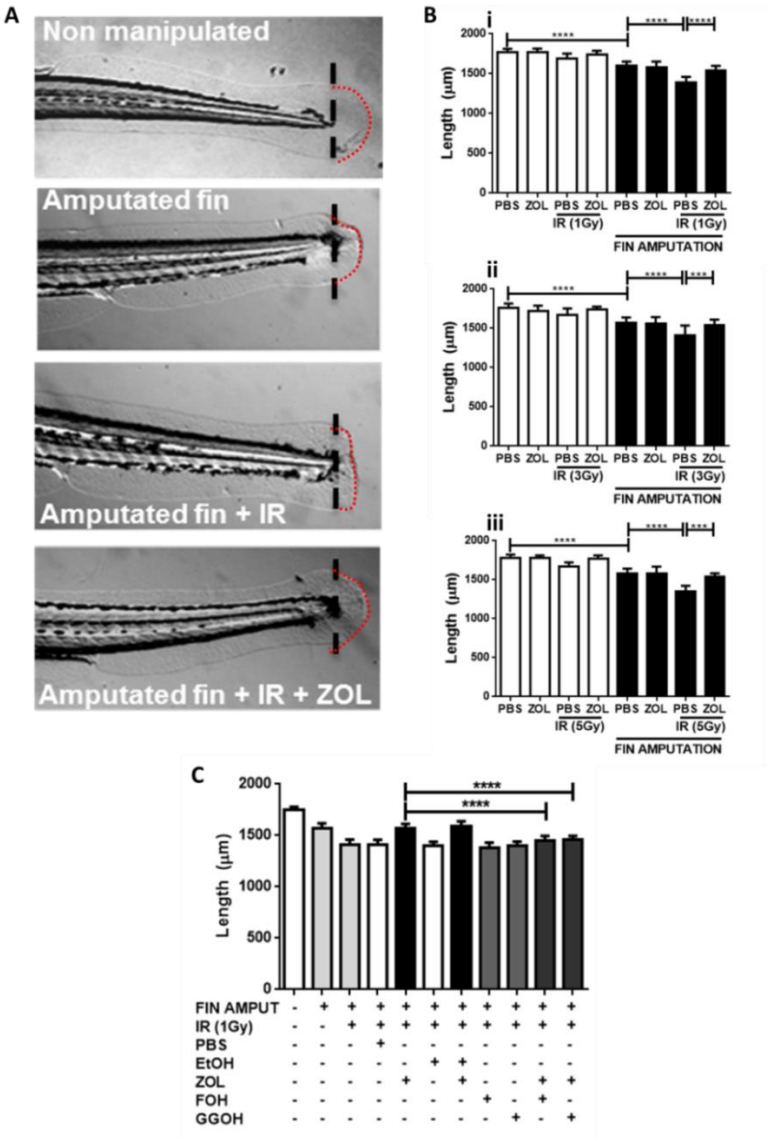


Figure 5.4 Zoledronate enhanced tail regeneration in zebrafish following irradiation and amputation

*(A) Representative examples of Zebrafish embryos 120 hpf without any manipulation (top panel), with amputation (second panel), with amputation and irradiation 1Gy (third panel), amputation and irradiation in presence of Zol (fourth panel) The black dashed line marked the site of amputation and the red dotted line marked the tail regenerated portion. (Bi-iii) Assessment of tail regeneration by measurement of tail length following either amputation or in combination with irradiation at different doses (1Gy, 3Gy or 5Gy, n=15/group). (C) Tail regeneration in zebrafish following irradiation (1Gy) and amputation in presence of Zol and/or FOH and GGOH (n=45/group). Data presented as mean ± SD and analysed by one way ANOVA with Bonferroni multiple comparison post-hoc test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

To determine whether the above was associated with DNA repair, embryos were sacrificed for assessing amount of γH2AX protein expression by western blotting. In absence of irradiation there was no expression of γH2AX however immediately after irradiation there was significant increase in γH2AX expression in both PBS and Zol treated zebrafish embryos (n=3, Fig 5.5 A-B). Twelve hours following irradiation there was elevated expression of γH2AX in PBS treated zebrafish embryos but in case of zoledronate treated zebrafish this was significantly reduced. On addition of FOH and GGOH the levels of γH2AX reverted to those seen in PBS treated zebrafish suggesting a reversal in effect of zoledronate DNA repairing ability.

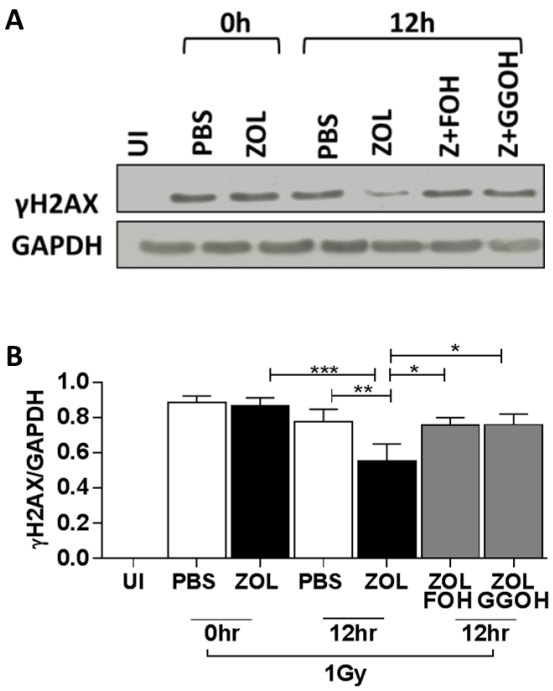


Figure 5.5 Zoledronate reduced incidence of DNA damage following irradiation

*(A) A representative example of a western blot for the expression of γH2AX (top panel) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, bottom panel) at 0 and 12 hrs following irradiation (1 Gy) in the presence or absence of Zol (1µM) and with the addition of FOH or GGOH. (B) Quantification of protein expression of γH2AX normalised to the expression of the 'housekeeping protein', glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using ImageJ (n=3). Data presented as mean ± SD and analysed by one way ANOVA with Bonferroni multiple comparison post-hoc test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

5.4 Zoledronate inhibits mTOR signalling and restores tissue regeneration

To determine whether zoledronate inhibited mTOR signalling in zebrafish as was seen in case of human MSC, zebrafish embryos treated with Zol (1µM) were sacrificed for protein expression analysis of p-mTOR, p-p70S6K, p-AKT and p-FOXO3a and their un-phosphorylated forms. One of the upstream components of mTOR signalling is the phosphatidyl-inositol 3 kinase (PI3K) and its inhibitor, Ly294002 was used as a control to assess the inhibition of mTOR signalling in zebrafish embryos (Fig 5.6).

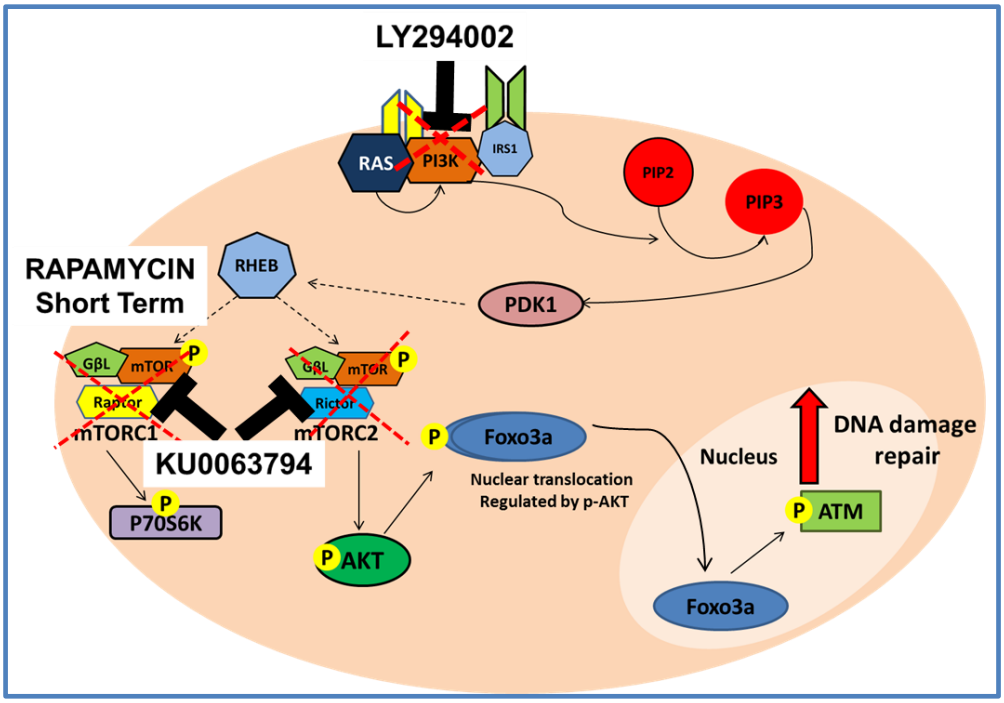


Figure 5.6 Diagrammatic representation of the mTOR pathway

The image shows the different chemical inhibitors of TORC1 and TORC2 signalling that includes the LY294002 (PI3K inhibitor), KU0063794 (inhibitor or mTORC1 and mTORC2) and Rapamycin (inhibitor of mTOR).

In the presence of Ly294002 there was inhibition of p-mTOR, p-p70S6K, p-AKT and p-FOXO3a, whereas the levels of un-phosphorylated forms remained unchanged (n=3; Fig 5.7 A-Bi-vii). In absence of Zol treatment there was no inhibition of these proteins of the mTOR signalling however on zoledronate treatment there was a significant

decrease in levels of the phosphorylated forms of mTOR, p70S6K, AKT and FOXO3a suggesting inhibition of mTOR signalling similar to what was seen in human MSC (n=3; Fig 5.7 A-Bi-vii).

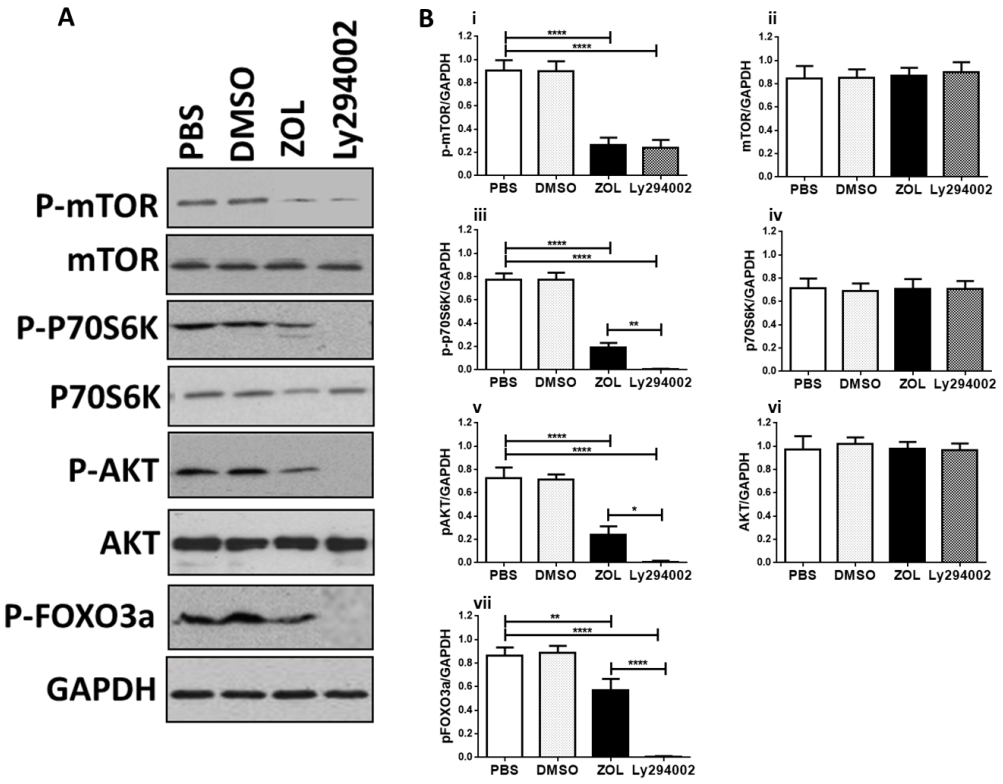


Figure 5.7 Zoledronate inhibited mTOR signalling in zebrafish

*(A) A representative example of western blot to analyse the expression of p-mTOR, mTOR, p-P70S6K, P70S6K, p-AKT, AKT, p-FOXO3a and GAPDH in zebrafish exposed to zoledronate (1µM) or Ly294002 (10µM). PBS was added in the same amount as used for Zol, and di-methylsulfoxide (DMSO) was added in the same amount as used for Ly294002. (B)Quantification of protein expression of (i) p-mTOR, (ii) mTOR, (iii) p-P70S6K, (iv) P70S6K, (v) p-AKT, (vi)AKT and (vii) p-FOXO3a normalised to the expression of the 'housekeeping protein', glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using ImageJ (n=3). Data presented as mean ± SD and analysed by one way ANOVA with Bonferroni multiple comparison post-hoc test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

To determine whether this inhibition of mTOR signaling by other known inhibitors resulted in tail regeneration, Zebrafish embryos were treated with LY294002 (PI3K inhibitor), KU0063794 (inhibitor of both forks of mTOR signaling i.e. mTORC1 and mTORC2) and short exposure to Rapamycin (inhibitor of mTORC1). Following treatment, embryos were subjected to amputation and irradiation and tail regeneration was measure at 120hpf. A significant restoration of tail fin regeneration was observed in embryos treated with either Ly294002 or KU0063794, similar to that observed in Zol treated embryos (n=15/group repeated 3 times Fig 5.8). In contrast and similar to what was seen in hMSC, addition of rapamycin a significant decrease in restoration of tail fin compared to Zol and other mTORC2 specific inhibitors (Fig 5.8). These results suggested that zoledronate induced DNA repair via inhibition of Torc2 specifically and this was associated with restoration of tissue regeneration in a zebrafish model.

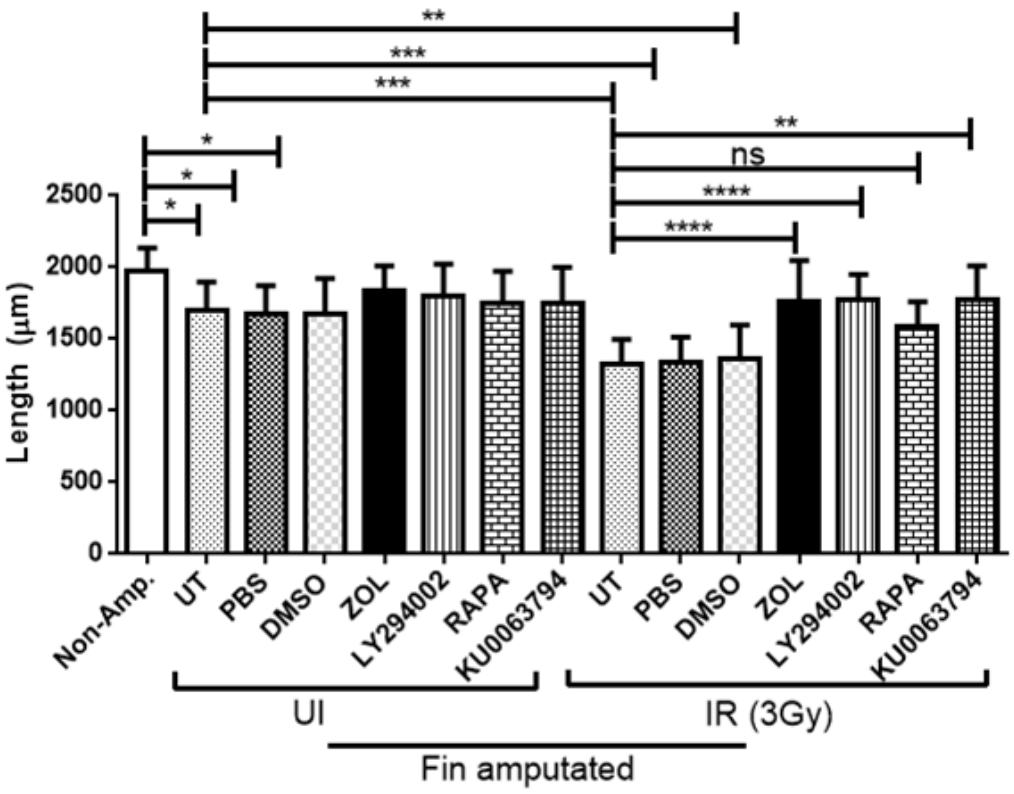


Figure 5.8 Dual inhibitors of mTOR enhanced tail regeneration in zebrafish following irradiation and amputation

*(A) Quantification of caudal fin length at 120hpf. The embryos (n=15/group) were irradiated (IR) at 3Gy and amputated in the presence or absence of Zol (1µM) or inhibitor of Phospho-inositol-3 kinase Ly294002 (10µM), inhibitor of mTOR KU0063794 (10nM), rapamycin (10nM). Data presented as mean ± SD and analysed by one way ANOVA with Bonferroni multiple comparison post-hoc test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

5.5 Discussion

In this chapter I have shown for the first time that irradiation blocks tail fin regeneration ability in 5 day old zebrafish embryo, and that on treatment with zoledronate there is enhanced repair of DNA damage induced by irradiation. I have also shown that tail regeneration is mediated via mevalonate pathway and that inhibition of TORC2 signaling in particular is associated with restoration of tail regeneration.

Studies investigating the effects of genotoxic stress such as radionuclides (uranium, ionizing radiation) on zebrafish have mostly focused on survival and development of embryo (McAleer et al. 2005, (Knowles 1999, Geiger, Parker et al. 2006, Yasuda, Aoki et al. 2006, Bourrachot, Simon et al. 2008, Kuhne, Gersey et al. 2009, Tsyusko, Glenn et al. 2011). However not many studies have reported the effects of ionizing radiations on tail regeneration. Only one study by Tsai et al 2007 has investigated the effect of irradiation at 20Gy on adult 6 month old Zebrafish and found that, following irradiation amputation of tail fin resulted in impaired regeneration 2 weeks following amputation, suggesting that irradiation impaired regeneration ability (Tsai, Tucci et al. 2007). This loss of regeneration ability was also seen with age where cells are known to accumulate DNA damage (Tsai, Tucci et al. 2007). For regeneration to occur there are two steps, one is the immune cells (neutrophils and macrophages) removing the dead cells and second is the formation of the blastema (Li, Yan et al. 2012). It is unknown which of these processes, if not both is compromised. In comparison to Tsai et al 2007, although the experimental set up in my experiments are different but I have shown that when embryos are exposed to irradiation at 48hpf, followed by tail amputation, regeneration is impaired. This could be due to radio sensitivity of cells

associated with regeneration and blastema formation that includes the mesenchymal cells, or due to loss of the immune cells functions or both. Although the origin of blastema cells is still elusive and it is unknown whether blastema form through process of dedifferentiation or through the recruitment of stem cells. Studies on cell fate tracing have shown that Zebrafish caudal fin cells remain lineage committed during regeneration. This means that the cell lineage associated with fin regeneration do not contribute to lineages other than that from which they are derived (Kragl, Knapp et al. 2009, Tu and Johnson 2010, Knopf, Hammond et al. 2011, Tu and Johnson 2011). One key experiment is to determine whether the number of blastema cells is normal following irradiation or is there a loss in these cells. It may be possible that these cells associated with tail regeneration following irradiation and amputation are delayed in producing blastema, because they may undergo cell cycle arrest, senescence or apoptosis which may be prevented in embryos with Zol treatment hence there is restoration of regeneration potential. In the future, experiments on senescence-associated β-galactosidase staining, caspase 3 assays for apoptosis and bromodeoxyuridine (BrdU) incorporation assay (Santamaria, Mari-Beffa et al. 1996, Poleo, Brown et al. 2001) and Hoescht dye labeling (Johnson and Bennet 1999) for proliferation and p16/p21 expression for cell cycle arrest must be incorporated to establish the complete usage of this model to study regeneration ability in presence of irradiation and drug treatment. Moreover employing expression of markers of regeneration such as homeobox transcription factors msxb, msxc (blastema marker), fgf20a, bmp2b and mmp9 (wound healing marker) would be an added benefit to the current method of tail fin regeneration measurement employed in my study to clearly demarcate the effects of radiation on the stem cell compartment if any, based on

what I have seen with Zol treatment in human MSC described in the previous chapters of this thesis (Azevedo, Grotek et al. 2011, Shao, Chen et al. 2011). More recently the involvement of immune cells in tissue regeneration has attained limelight. The existence of neutrophils, macrophages, dendritic cells and B and T cells in Zebrafish, analogous to mammalian immune system have been implicated in tissue regeneration (Renshaw and Trede 2012). Following injury rapid accumulation of neutrophils at site of wounds in larvae results in engulfment of cell debris (Renshaw, Loynes et al. 2006, Mathias, Dodd et al. 2007, Loynes, Martin et al. 2010, Colucci-Guyon, Tinevez et al. 2011, Yoo and Huttenlocher 2011, Li, Yan et al. 2012). Following neutrophil migration there is also appearance of macrophages at site of wound where they exhibit phagocytic behavior in response to bacterial infiltration (Herbomel, Thisse et al. 1999, Lieschke, Oates et al. 2001, Redd, Kelly et al. 2006, Mathias, Dodd et al. 2009, Volkman, Pozos et al. 2010). Thus following irradiation there may be a delay in recruitment of the immune cells to sites of injury which in case of zoledronate may be enhanced and therefore it is a potential question to address. This could be addressed by employing a number of transgenic lines that have been developed that express fluorescent reporters under the control of neutrophil [myeloperoxidase (mpo; mpx – ZFIN); lysozyme C (lyzC)] and macrophage-driven [macrophage expressed 1 (mpeg1)] promoters in order to better characterize the injury response of these cells (Mathias, Perrin et al. 2006, Mathias, Walters et al. 2009) (Ellett, Pase et al. 2011) and these lines could be employed to identify the effect of radiation on tail regeneration in presence or absence of zoledronate.

Reactivation of certain silenced genes associated with development has been characterized for regeneration of tail following amputation injury in zebrafish. If these

effects occur in Zebrafish following irradiation and amputation remains to be determined. These genes contain histone modifications namely a bivalent histone code of trimethyl lysine 4 histone H3 (me^3K4 H3; associated with gene activation) and trimethyl lysine 27 H3 (me^3K27 H3; repressor of gene) which acts as a silencer. However during regeneration, this bivalent histone code loses the me^3K27 H3 by demethylases which has been identified as Kdm6b.1 in zebrafish, and this demethylation of me^3K27 H3 results in activation of genes such distal homeobox gene dlx4a associated with tail fin regeneration by the me^3K4 H3 (Stewart, Tsun et al. 2009) (Schebesta, Lien et al. 2006). Studies in cancer cells have demonstrated by immunofluorescence analysis that there is a loss of me^3K4 H3 and me^2K4 H3 signals following irradiation (Young, McDonald et al. 2013). It would be interesting to determine these epigenetic modifications in tail regeneration following irradiation and amputation on treatment with Zol. Moreover whole gene expression and DNA methylation studies can help determine target genes in play during irradiation and amputation.

Prenylation, that occurs downstream of Mevalonate pathway is associated with development of Zebrafish embryo. Inhibition of the HMGCoA pathway (cholestrol biosynthesis pathway) has been shown to affect development of zebrafish embryo on treatment with statins (inhibitors of HMGCoA)(Thorpe, Doitsidou et al. 2004). In this study I have shown that zoledronate treatment in Zebrafish resulted in enhanced DNA repair. The effects were reversed on addition of FOH and GGOH suggesting that zoledronate inhibited the mevalonate pathway in these embryos. I have reported that treatment with zoledronate at early hours of development (5hpf) resulted in embryo defects and affected viability suggesting the importance of prenylation in

development. However administration of Zol at 24hpf and later did not have detrimental effects at dose of 1µM or lower suggesting that the crucial stages of development that required prenylation proteins may have occurred in the 24hpf time frame. Although later stage defects in development following inhibition of prenylation is not known as in this study all assays lasted 5dpf. In absence of radiation injury treatment with Zol following amputation had no distinct effect different from PBS treated embryos in terms of tail regeneration suggesting that inhibiting prenylation had no detrimental effect on regeneration and developmental growth. In addition, no distinct effects were recorded on behavioral patterns such as swim pattern, movement and reflexes. Although in studies using statins, detrimental effect on primordial germ cell (PGC) migration have been reported but when using specific inhibitors of farnesyl transferase (FTI) whose targets lie downstream of zoledronate and statin activity, no perturbations in PGC migration were noted (Kohl, Omer et al. 1995, Sun, Blaskovich et al. 1999, Crespo, Ohkanda et al. 2001). Studies by Thorpe et al suggest the requirement of only geranylgeranyl transferases over the farnesyl transferases for PGC migrations (Thorpe, Doitsidou et al. 2004)(Thorpe et al 2004). This may mean that effect on PGC may be dependent on only one arm of the prenylation fork. However in my study I find reversal of effect of Zol on addition of both FOH and GGOH suggesting that Zol inhibits protein prenylation in both arms. Having said this, further experiments investigating the different proteins that are unprenylated on Zol treatment is required to understand the multiple effect of blocking prenylation in these embryos.

Conservation of mTOR has been explored in Zebrafish using both pharmacological manipulation and morpholino gene knockdown. Studies have shown that treatment

of zebrafish with rapamycin during early embryogenesis resulted in developmental delay (Makky, Tekiela et al. 2007). mTOR in Zebrafish although expressed ubiquitously becomes more localized to the head and developing gut between 35-57hpf (Sabers, Martin et al. 1995, Long, Spycher et al. 2002, Makky and Mayer 2007, Makky, Tekiela et al. 2007). Moreover morpholino knockdown of mTOR, raptor and S6 kinase which form the TORC1 complex only, have been implicated in loss of intestinal development in Zebrafish. Interestingly there is no developmental defects in brain that undergoes substantial growth and is insensitive to mTOR inhibition. Even in tail regeneration there is differential sensitivity to rapamycin mediated TOR inhibition. Goldsmith et al (2006) showed these mTOR inhibition only selectively abrogated isometric growth in adult caudal fin compared to juvenile zebrafish(Goldsmith, Iovine et al. 2006). Moreover allometric growth in juvenile fish was rapamycin resistant/TOR independent. These data do not necessarily imply that TOR plays a less pivotal role in fin growth but could mean that Rapamycin inhibition of just TORC1 alone may not be associated with juvenile fin growth. Studies by Kujawski et al (2014) showed that rapamycin mediated mTOR inhibition given at concentrations of 50nM only mildly affected caudal fin regeneration when drug was administered after amputation but the growth was not affected following pretreatment (Kujawski, Lin et al. 2014). Interestingly, studies by Hirose et al (2014) state the mTORC1 inhibition mediated by rapamycin (2.4µM) for 12 hrs prior to amputation resulted in inhibited regeneration of tail fin, but when administered immediately after amputation , the inhibitory effect of Rapamycin on regeneration was markedly reduced (Hirose, Shiomi et al. 2014). These two studies are important as they both suggest the importance and effect of mTOR inhibition on regeneration following amputation. In Hirose's study post

treatment of Rapamycin did not affect the regeneration however pretreatment did inhibit regeneration which is in contrast to what I see and one plausible explanation is the dose of Rapamycin used in my study is 10nM which is much less compared to their study (2.4µM). Another point being that in my study Rapamycin was pretreated for 3 days in comparison to the 12 h treatment in their study, suggesting the possibility of involvement of other mechanisms. Indeed the role of TORC2 has not been well investigated in the development and regeneration and in this study I have shown that regeneration following irradiation may be associated with TORC2 inhibition as other inhibitors of TORC2 alone resulted in tail fin regeneration following amputation and irradiation as opposed to Rapamycin that may have only inhibited TORC1.

In conclusion I find that several mechanisms of Zol activity seen in hMSC were conserved in Zebrafish model and this system proved useful to bridge the gap between my *in vitro* data and future experiments *in vivo* in higher model organism such as mice to determine whether similar effects were conserved in them on treatment with Zol.

Chapter 6: Non-skeletal effects of zoledronate in C57Bl/6 mice

6.1 Introduction

Ionizing radiation is often used to kill cancer cells but severely damages DNA in other healthy cells including stem cells. This is especially detrimental to rapidly renewing tissues such as bone, gut and hair follicles that may undergo apoptosis due to DNA damage. As a result there may be decline in normal functions of tissue maintenance and general growth and development eventually affecting quality of life. In case of patients receiving radiotherapy as treatment for cancer, one of the major side effects is damage to the bone marrow (Yarnold and Brotons 2010). Three times higher risk of hip fractures have been reported in women following radiation therapy for cancer in anus, cervix or rectum when compared to those that don't receive radiation (Baxter, Habermann et al. 2005, Baxter, Tepper et al. 2005). In these older patients bone insufficiency due to radiation thus becomes a major issue that causes stress fractures and bone fragmentations which are difficult to treat (Costantino, Friedman et al. 1995, Jegoux, Malard et al. 2010). Even in children with osteosarcoma undergoing radiotherapy, there is risk of developing skeletal defects later in adolescence (Haddy, Mosher et al. 2009, Dorr, Kallfels et al. 2013). Even in cases of bone marrow transplants, radiation therapy results in decreased osteoblasts numbers and damaged osteogenic differentiation associated with osteopenia post-transplant. Damage to the recipient's bone marrow stromal cells is not replaced by donor MSC as the stroma still belongs to the recipient therefore it is important minimise the damage to the host MSC in order to preserve functions of stem cells and their progenitors and mature cells (Galotto, Berisso et al. 1999, Banfi, Bianchi et al. 2001, Lee, Jang et al. 2002,

Tauchmanova, Serio et al. 2002). Studies by Cao et al in mice showed that following irradiation indeed there was loss of mesenchymal stem cells accompanied with a decline in the CFU-F and CFU-O progenitors and the number of osteoblasts and osteoclasts due to increase in free radicals which may affect the survival, self-renewal or differentiation of MSC(Cao, Wu et al. 2011).

The gastrointestinal and oral mucosa is also vulnerable to the debilitating effects of radiation and they undergo deep ulceration and swelling associated with loss of tissue integrity and function (Peterson, Bensadoun et al. 2011). In the intestines at least two cellular locations show properties of intestinal stem cells: the columnar cells at the crypt base (CBCs) and some +4 cells immediately above the Paneth cells. Whole body irradiation model have shown a rapid onset of GI syndrome accompanied with acute intestinal damage due to apoptosis in these cells (Potten, Booth et al. 2003, Potten 2004, Ch'ang, Maj et al. 2005, Qiu, Carson-Walter et al. 2008).These cells are identified as the LGR5 cells that are sensitive to high doses of radiation (Barker and Clevers 2007, Qiu, Carson-Walter et al. 2008, Barker and Clevers 2010, Barker and Clevers 2010, Qiu, Leibowitz et al. 2010, Hua, Thin et al. 2012, Munoz, Stange et al. 2012). Since maintenance of tissue integrity is highly dependent on the stem cells that either regenerate or repair loss of tissue in response to injury, it is important to find ways of protecting these vital masterminds of homeostasis from damage. Thus investigating the effect of Zol *in vivo* in skeletal and non-skeletal tissues is important because enhancing/protecting DNA repair in stem/progenitor/mature cells may have important clinical implications in treatment of old age bone and blood disorders such as osteoporosis and myelodysplastic syndrome and side effects of radiation treatment (Haddy, Mosher et al. 2009, Peterson, Bensadoun et al. 2011, Dorr, Kallfels

et al. 2013, Medyouf, Mossner et al. 2014). Based on the previous work in this thesis that highlights the DNA repairing ability of zoledronate in stem/progenitor and mature cells *in vitro* and their ability to enhance regeneration *in vivo*, I next wanted to determine whether these effects could be translated *in vivo* in higher model organism such as mice. I hypothesised that '**Zol protected stem cells from radiation induced damage *in vivo*'**.

6.2 Zoledronate protected murine bone marrow stem cells from radiation induced damage

To determine whether Zol reduced incidence of DNA damage in bone where the mesenchymal stem cells reside, C57Bl/6 mice pre-treated with either Zol or vehicle (PBS) were subjected to whole body irradiation and incidence of DNA damage foci positive cells (containing more than 5 foci) was enumerated (Fig 6.1 A). In mice that were unexposed to irradiation there was detectable but low levels of DNA damage positive cells in both Zol and vehicle mice however following irradiation vehicle mice accumulated more than double the number of foci positive cells. Zol treatment resulted in significant reduction of number of γH2AX foci positive cells (Fig6.1 B and Ci-ii; n=6). These data suggest that zoledronate's DNA damage repairing ability was retained *in vivo* similar to what I have shown *in vitro.*

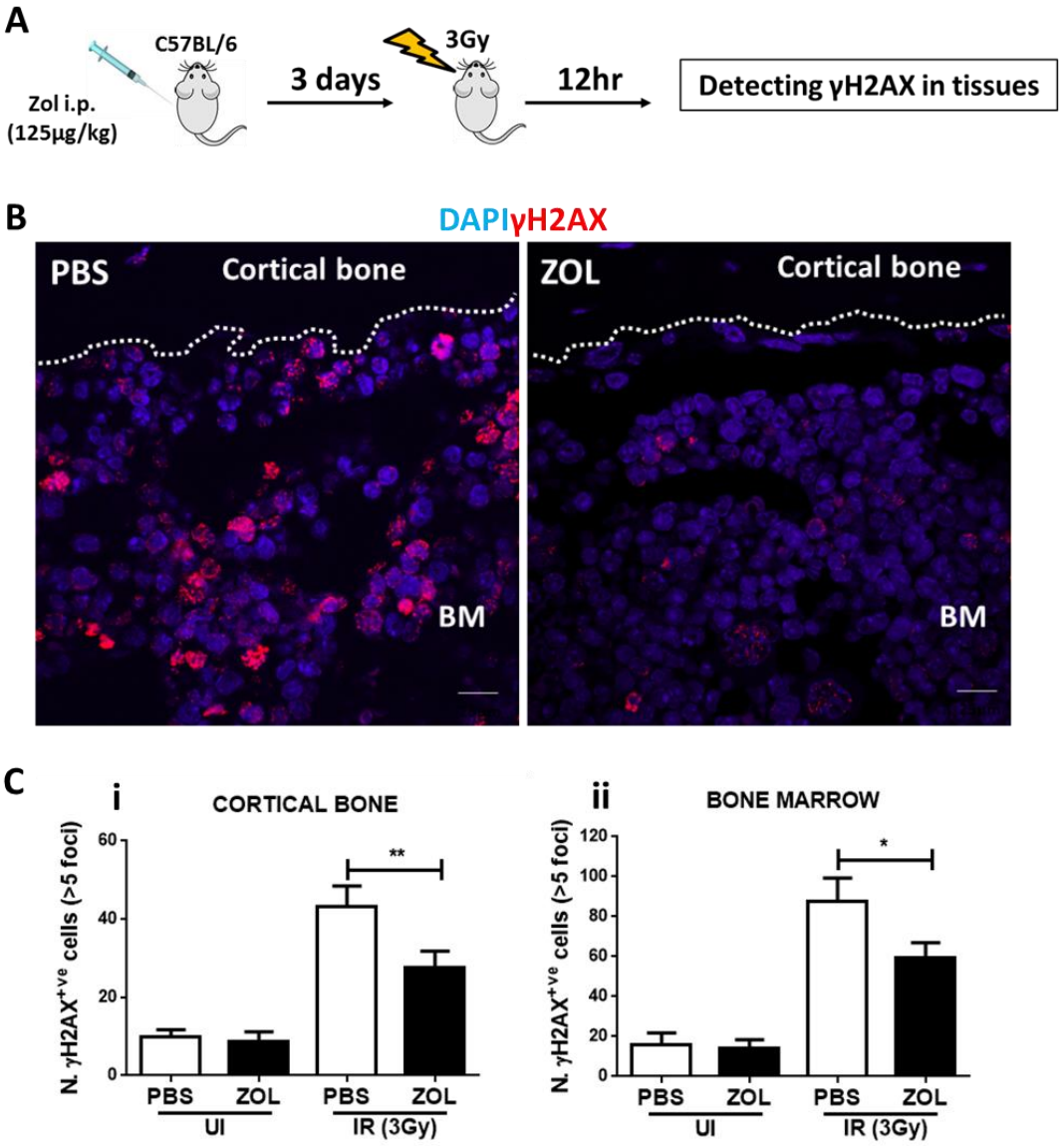


Figure 6.1 Zoledronate reduced incidence of DNA damage foci in cells of cortical bone and bone marrow following exposure to irradiation

*(A) A schematic of the experimental plan to assess the in vivo effect of Zol on irradiation induced DNA damage in cortical bone and bone marrow cells. (B) Representative example of bone tissue sections stained for γH2AX foci in mice pretreated with or without Zol and exposed to irradiation. Dotted lines indicate endosteal surface. (B) Number of cells (with DAPI stained nuclei) consisting of more than 5 γH2AX DNA damage foci adjacent to the (i) cortical bone or in the (ii) bone marrow. Quantification was done using ImageJ (n=6 per group). Data presented as mean±SD were analysed by one way ANOVA with Bonferroni post-test *p<0.05, **p<0.01, ***p<0.001, ****p< 0.0001.*

To determine whether Zol protected bone marrow mesenchymal stem cells from radiation induced damage, C57Bl/6 mice pre-treated with Zol were subjected to whole body irradiation (12Gy) and sacrificed for assessing the clonogenic potential of mesenchymal progenitors (Fig 6.2 A). Radiation reduced the number of surviving bone marrow colony forming unit-fibroblast (CFU-F) and colony forming unit-osteoblast (CFU-O) in comparison to un-irradiated mice, but mice that were exposed IR following pre-treatment with Zol showed a significant increase in survival of these mesenchymal progenitors compared to irradiated vehicle (Fig 6.2 B-C; n=5/group). Together these data suggest that zoledronate protected the murine bone marrow mesenchymal stem cell from radiation induced damage.

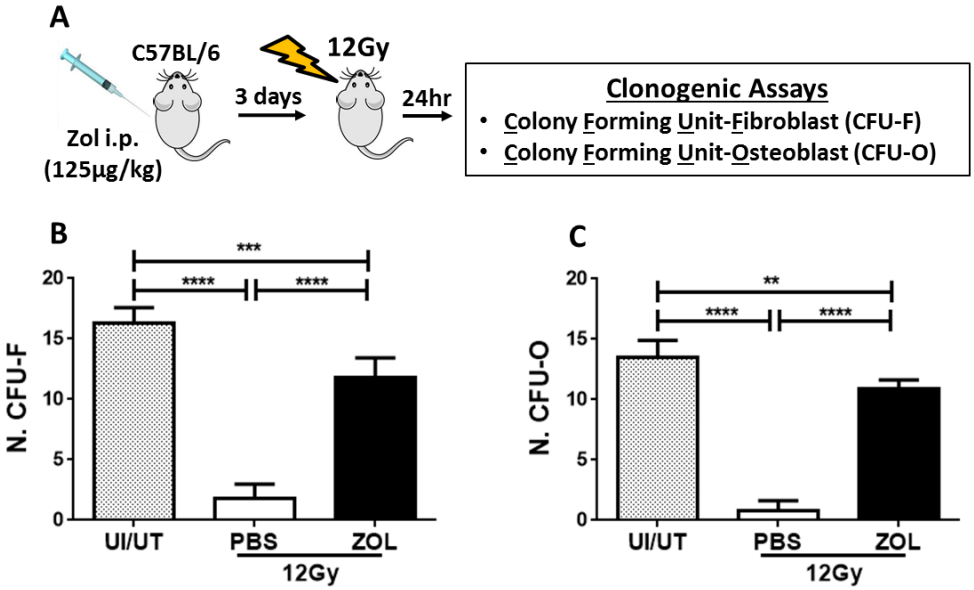


Figure 6.2 Zoledronate protected murine mesenchymal stem cells from radiation induced damage

*(A) A schematic of the experimental plan to assess the in vivo effect of Zol on mesenchymal stem/ progenitor cells of bone. (B) Number of Colony Forming Unit-Fibroblast (CFU-F) and (C) Colony Forming Unit-Osteoblast (CFU-O) in mice pre-treated with Zol or PBS followed by total body irradiation or unexposed to irradiation (UI/UT; n= 5 per group). Data presented as mean±SD were analysed by one way ANOVA with Bonferroni post-test *p<0.05, **p<0.01, ***p<0.001, ****p< 0.0001.*

6.3 Zoledronate unprenylated RAP1A in non-skeletal tissues of C57Bl/6 mice

To determine whether ability of Zol to enhance repair and protect stem cells following exposure to radiation is true in other non-skeletal tissues *in vivo,* I first determined which tissues were exposed to zoledronate *in vivo* since Zol is known to bind to bone. The ability of zoledronate to unprenylate RAP1A through the inhibition of mevalonate pathway (shown in the previous chapters) was employed as a marker to determine the sites of zoledronate activity *in vivo* in C57Bl/6 mice. Normal C57Bl/6 mice injected with Zol (125µg/kg; i.p.) were sacrificed to detect unprenylated RAP1A in the different tissues (Fig 6.3A-C). Mice injected with vehicle (PBS) showed no detectable unprenylation of RAP1A in the different tissues apart from lung and bone marrow where expression was recorded at very low levels. As expected treatment with Zol resulted in unprenylation of RAP1A in bone which is a well-known site of zoledronate activity, but several non-skeletal tissues such as heart, kidney, intestine, liver, spleen, muscle and pancreas also showed varying levels of expression of unprenylated RAP1A (Fig 6.3 B-C; n=6/group). Together these data suggest a wide spread distribution of zoledronate *in vivo* in mice.

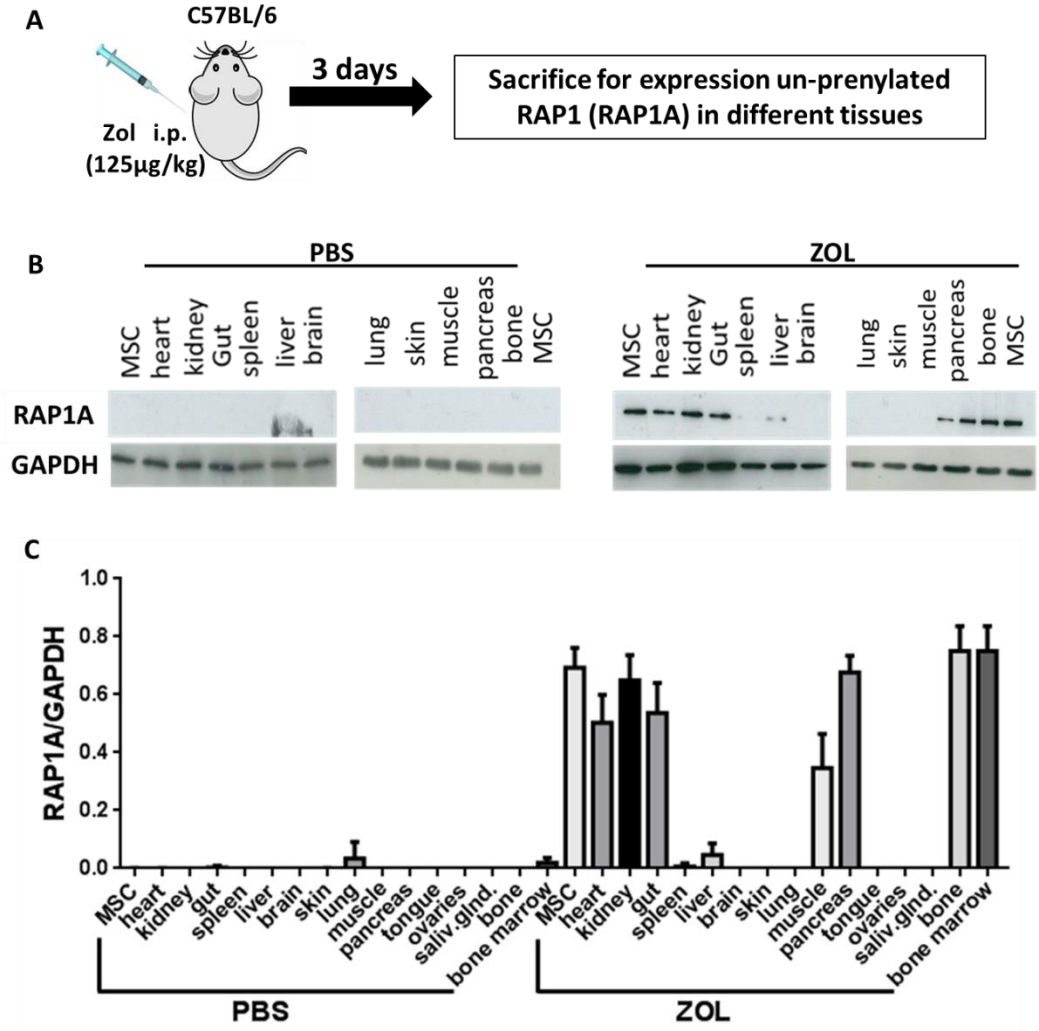


Figure 6.3 Zoledronate unprenylated RAP1A in non-skeletal tissues

*(A) Schematic representation of experimental plan to assess effect of zoledronate in different murine tissues. (B) A representative example of western blot showing expression of unprenylated RAP1A (top panel) and GAPDH (bottom panel) in different murine tissues and human MSC following zoledronate treatment. (C) Quantification of unprenylated RAP1A proteins normalised to GAPDH in different tissues of 6 mice per group. Quantification was done using ImageJ. Data presented as mean±SD were analysed by student t test with Mann Whitney post hoc test *p<0.05, **p<0.01, ***p<0.001, ****p< 0.0001.*

6.4 Zoledronate reduces the incidence of DNA damage in murine tissues following exposure to irradiation

To determine whether zoledronate's ability to repair DNA damage was achievable *in vivo*, C57Bl/6 mice pre-treated with Zol were exposed to total body irradiation (3Gy) and sacrificed for detecting γH2AX foci positive cells (more than 5 foci) in different tissues (Fig 6.4 A). Although un-irradiated mice recorded had detectable numbers of DNA damage foci positive cells but following irradiation vehicle mice had significant increase in number of DNA damage positive cells but mice that were treated with zoledronate had a significant reduction in these DNA damage foci positive cells. zoledronate reduced incidence of γH2AX foci positive cells by as much as 49.57±5.37% in the heart, 68.44±4.20% in the kidney, 60.08±5.05% in the intestine, 27.91±5.03% in the spleen, 33.78±3.86% in the liver, 44.22±7.50% in the muscle, 55.04±4.26% in the pancreas and 40.27±5.37% in the bone compared to vehicle treated mice (Fig 6.4 Bi-viii; n=6/group). These data suggest that zoledronate's ability to enhance DNA repair *in vitro* was also reflected *in vivo* especially in non-skeletal tissues where zoledronate's activity was found.

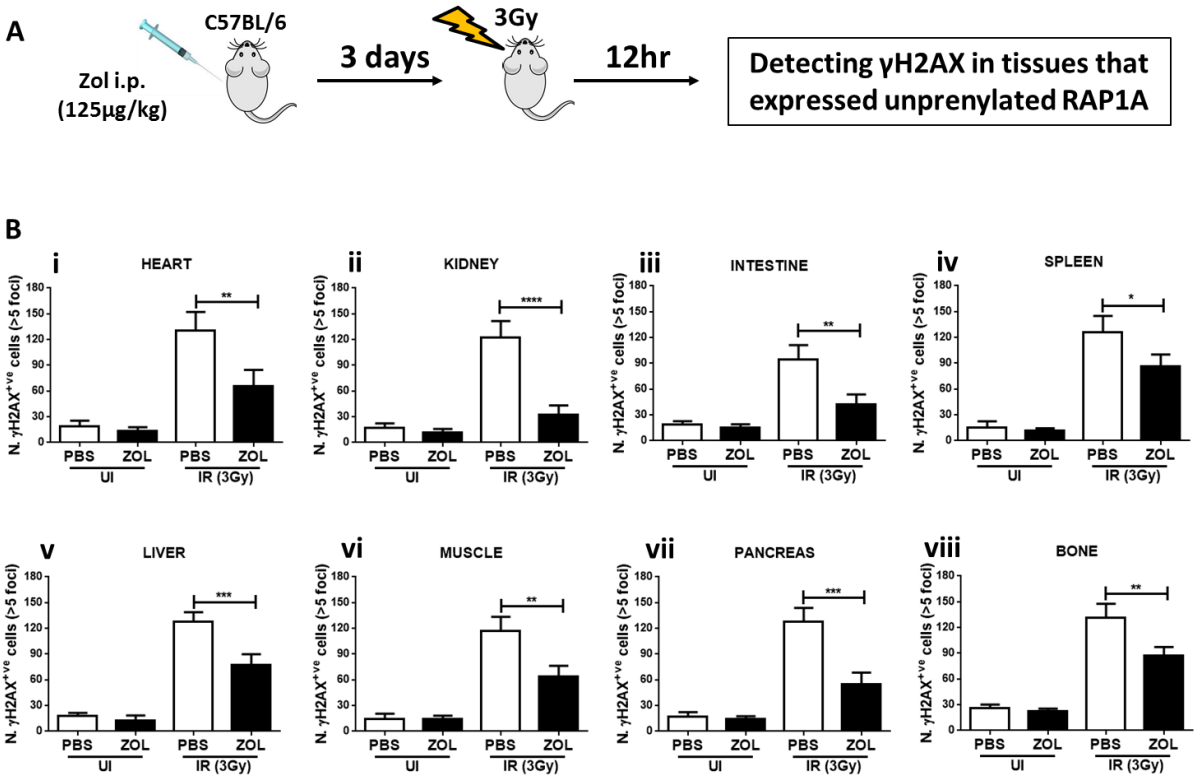


Figure 6.4 Zoledronate reduces incidence of DNA damage in non-skeletal tissues following exposure to irradiation

*(A) Schematic representation of experimental plan to quantify DNA damage in different murine tissues. (B) Number of γH2AX^{+ve} cells having greater than 5 foci in (i) heart, (ii) kidney, (iii) intestine, (iv) spleen, (v) liver, (vi) muscle, (vii) pancreas and (viii) bone tissues in C57Bl/6 mice pre-treated with Zol and subjected to whole body irradiation (3Gy) or unexposed to irradiation (UI). Quantification was done using ImageJ (n=6 per group). Data presented as mean±SD were analysed by one way ANOVA with Bonferroni post-test *p<0.05, **p<0.01, ***p<0.001, ****p< 0.0001.*

6.5 Zoledronate reduced incidence of DNA damage in murine intestinal stem cells

Among the soft tissues where Zol enhanced DNA repair, the intestines were of particular interest. Intestines are a highly regenerative tissue and have stem cells that reside at the base of crypt (Fig 6.5 A). To determine whether Zol protected these intestinal stem cells from radiation induced damage mice pre-treated with Zol or vehicle were exposed to irradiation and sacrificed for detecting DNA damage in the villi and crypt regions of the intestine. In the intestines of C57BL/6 mice pre-treated with Zol, there was a significant reduction in incidence of DNA damage foci positive cells in both the villi and the crypt regions when compared to vehicle mice following exposure to irradiation (Fig 6.5 B-C). The effect was more pronounced in the crypts where the intestinal stem cells reside. To determine whether zoledronate enhanced DNA repair in intestinal crypt stem cells, mice subjected to whole body irradiation and pre-treated with Zol were sacrificed for assessing DNA damage foci in Leucine-rich repeat containing G protein-coupled receptor 5 (Lgr5) expressing cells that were identified by Clevers et al (2007) as crypt stem cells. Twelve hours following irradiation, there was accumulation of DNA damage foci in the $Lgr5^{+ve}$ cells in the vehicle mice but Zol treatment resulted in a significant reduction in incidence of DNA damage foci in these $Lgr5^{+ve}$ intestinal crypt stem cells (Fig 6.5 D-E; n=6/group). To determine whether zoledronate's ability to reduce incidence of DNA damage in intestines was similar to that seen *in vitro* in MSC via inhibition of mTOR signalling, western blotting was performed for phosphorylated proteins of TORC1 and TORC2 signalling (Fig 6.6 A-B). Clearly, there was a significant decrease in the phosphorylated forms of AKT, P70S6K, mTOR and FOXO3a compared to vehicle mice following irradiation. There was also a significant increase in the levels of p-ATM (Fig 6.6 A-B).

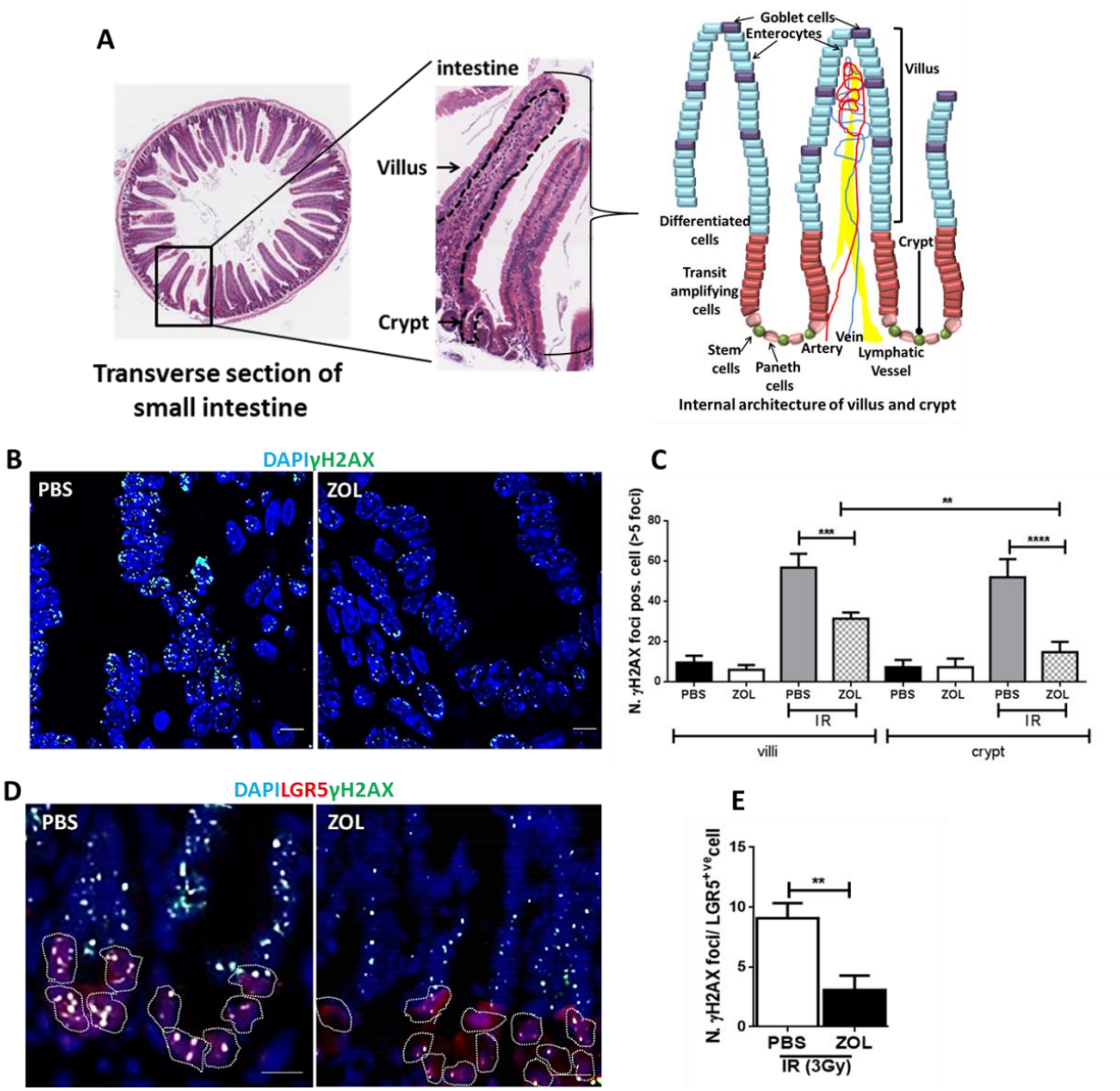


Figure 6.5 Zoledronate reduced incidence of DNA damage in LGR5 expressing intestinal stem cells in mice following exposure to irradiation

*(A) Representative example of transverse section of murine small intestine stained with haematoxylin and eosin showing the villus and crypt regions with a diagrammatic representation of their internal architecture. (B) A representative example of DNA damage γH2AX staining in murine intestine following exposure irradiation in presence or absence of Zol. Blue nuclei are stained with DAPI. (C) Number of cells consisting of more than 5 γH2AX foci in the villi and crypt regions. (D) A representative example of γH2AX staining in LGR5 expressing intestinal stem cells in the crypts. (E) Number of γH2AX foci/LGR5^{+} cells in the crypts of small intestine. Data presented as mean±SD were analysed by one way ANOVA with Bonferroni post-test or by student t test with Mann Whitney post hoc test *p<0.05, **p<0.01, ***p<0.001, ****p< 0.0001.*

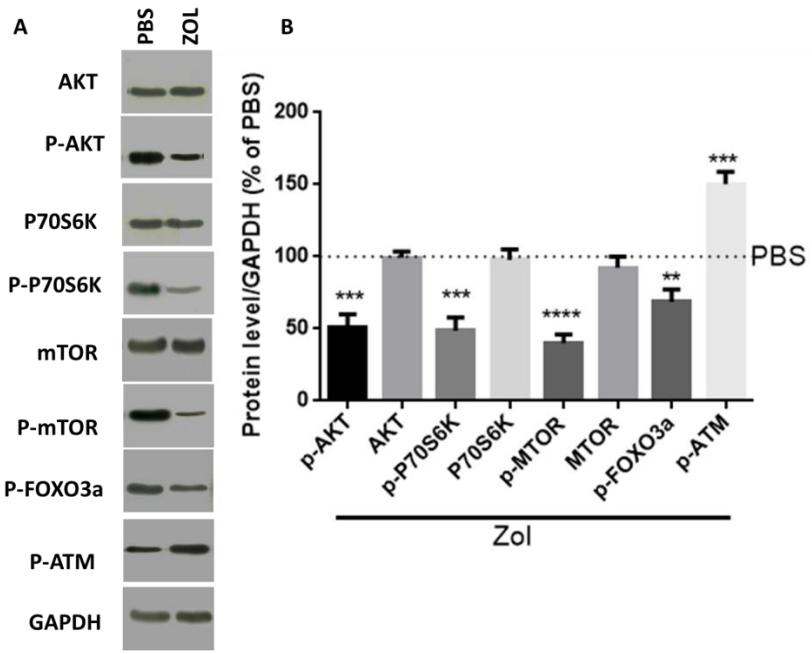


Figure 6.6 Zoledronate inhibited mTOR signalling in murine intestinal tissues.

*(A) A representative example of western blot showing expression of non-phosphorylated and phosphorylated AKT, P70S6K, mTOR FOXO3a and ATM proteins in murine intestinal tissues following zoledronate treatment following irradiation. (B) Quantification of proteins normalised to GAPDH in intestine tissues of 3 mice per group. Quantification was done using ImageJ. Data presented as mean±SD were analysed by student t test with Mann Whitney post hoc test *p<0.05, **p<0.01, ***p<0.001, ****p< 0.0001.*

6.6 Zoledronate protected murine intestines from radiation induced mucositis

High doses of radiation like in radiotherapy cause oral and gastrointestinal mucositis, which is characterised by ulceration, inflammation and loss of villi/crypts thus hampering intestinal functions. To determine whether Zol could protect mice from radiation induced mucositis, mice pre-treated with Zol or vehicle were subjected to whole body irradiation (12Gy) and 4 days later, histological analysis of crypt and villi was performed (Fig 6.7 A). Mice that were unexposed to irradiation had normal crypt and villi morphology. As expected , following irradiation there was a significant loss of the villus length and crypt death in vehicle mice but mice treated with Zol had significantly reduced loss of villus length and crypt death (Fig 6.7 B-D; n=6). There was also significant loss in the number of goblet cells that mediate absorption (Fig 6.7 E). To further dissect out the cause of this loss of morphology I enumerated the number of apoptotic and proliferating cells in the crypts. As expected the un-irradiated mice had less apoptotic cells determined by caspase-3 immuno-staining, but following irradiation vehicle mice had a significant spike in the number of apoptotic cells. Zol treated mice significantly decreased apoptosis of the crypt cells compared to irradiated vehicles (Fig 6.7 F-G). In fact Zol treatment maintained the proliferation of cells in irradiated mice similar to un-irradiated controls whereas in the vehicle mice there was a dramatic loss of proliferating cells (Fig 6.7 H-J; n=6). Together these data suggest that zoledronate protected the loss of intestine tissue integrity from radiation induced damage.

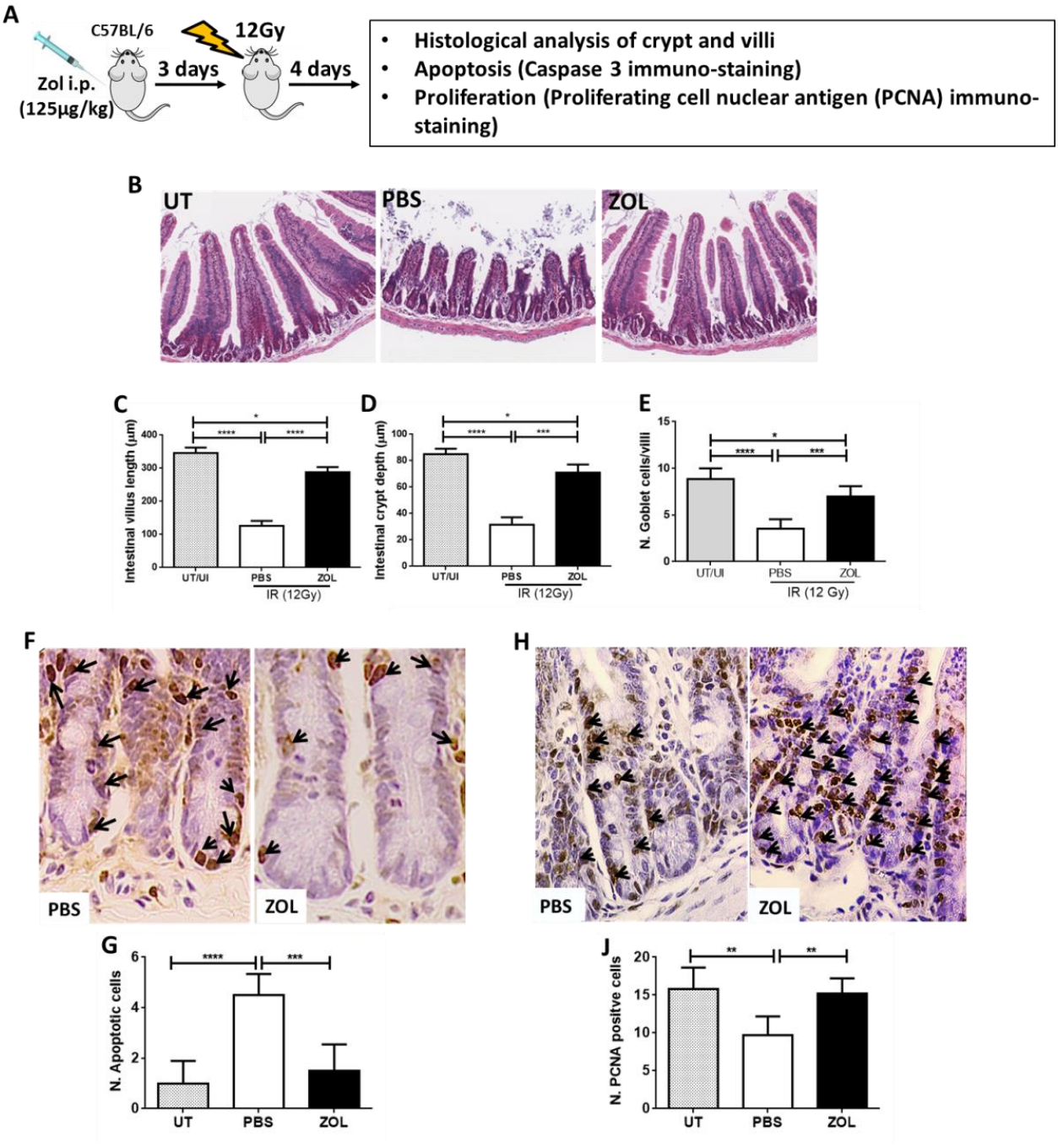


Figure 6.7 Zoledronate protected intestine from radiation induced damage

*(A) A schematic of the experimental plan to assess the in vivo effect of zoledronate on murine intestines. (B) Representative example of transverse section of murine small intestine stained with haematoxylin and eosin showing the villus and crypt regions in mice that were either un-irradiated (UI) or irradiated (12Gy) in presence or absence of Zol. Quantification of (C) villi length and (D)crypt depth (E) number of goblet cells in mice following exposure to irradiation. (F) Representative example of apoptotic cell stained for caspase 3 marker of apoptosis in murine intestine. (G) Number of apoptotic cells. (H) Representative example of proliferating cell stained for proliferating cell nuclear antigen (PCNA) marker in murine intestine. (J) Number of cells stained positive for PCNA marker. Data presented as mean±SD were analysed by one way ANOVA with Bonferroni post-test *p<0.05, **p<0.01, ***p<0.001, ****p< 0.0001.*

To confirm whether this effect of Zol was attributed to protection of intestinal stem cells, mice pre-treated with Zol were subjected to total body irradiation (12Gy) and sacrificed at 1 day and 4 days post irradiation to determine the fate of Lgr5 expressing crypt stem cells (Fig 6.8 A). On day 1 following irradiation, the vehicle mice had a significant loss in the number of $Lgr5^{+ve}$ cells per crypt compared to un-irradiated mice (Fig 6.8 B-C; n=6). In fact there was a significant reduction in the number of $Lgr5^{+ve}$ crypts as well (Fig 6.8 D). Even by day 4, vehicle mice subjected to radiation damage had not recovered the loss in these $Lgr5^{+ve}$ crypt stem cells. However mice that were treated with zoledronate had no significant loss in numbers of the $Lgr5^{+ve}$ cells per crypt at both time points suggesting zoledronate protected these stem cells. In fact even the number of $Lgr5^{+ve}$ crypt were similar to those seen in the un-irradiated controls (Fig 6.8 B-D; n=6). Together these data suggest that zoledronate protected the Lgr5 expressing crypt stem cells from radiation induced damage and this may be a vital contributing factor to the reduced loss of intestinal tissue integrity in mice subjected to extensive radiations.

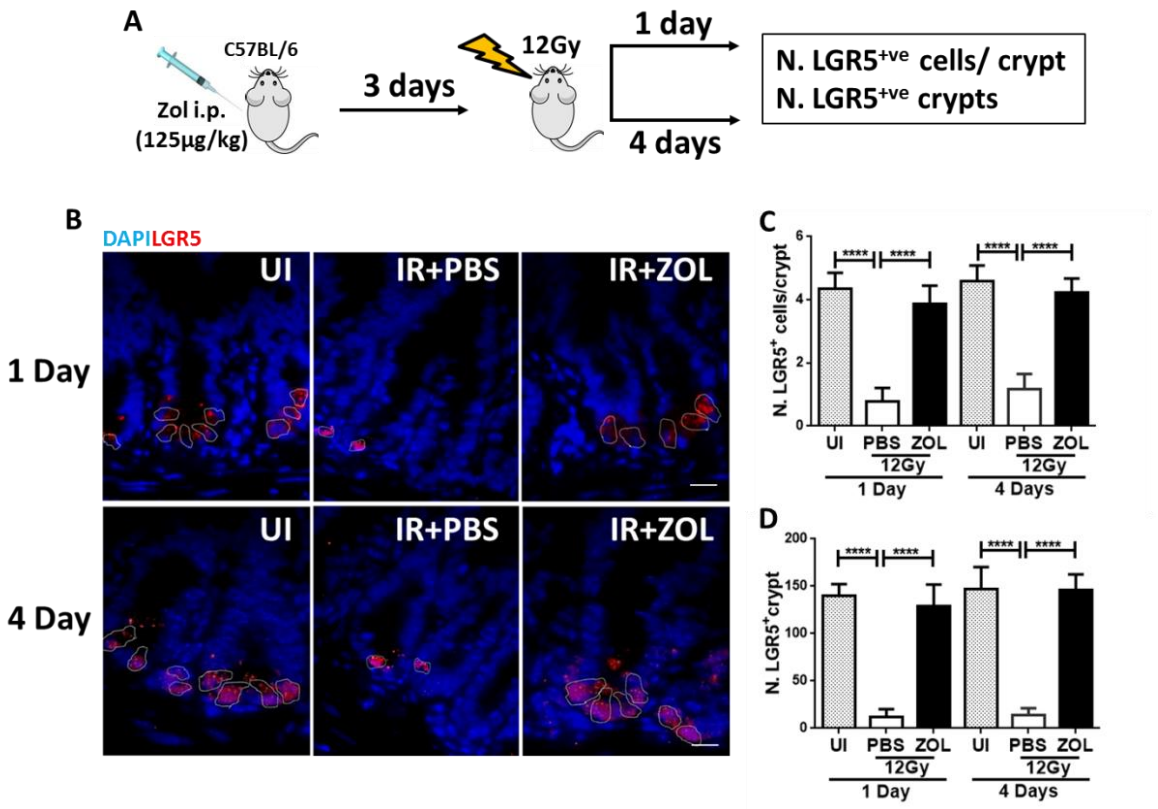


Figure 6.8 Zoledronate protected LGR5 expressing crypt stem cells from radiation induced damage

*(A) A schematic of the experimental plan to assess the in vivo effect of zoledronate on murine LGR5 expressing intestinal stem cells. (B) Representative example of LGR5 expressing cells of the intestinal crypts at day 1 and day 4 post irradiation in mice which were un-irradiated (UI) or irradiated (12Gy) following pre-treatment with Zol or vehicle. (C) Number of $LGR5^+$ cells/crypt in mice following irradiation and sacrificed at day 1 or day 4 post irradiation. (D) Number of $LGR5^+$ crypts in murine small intestine. Data presented as mean±SD were analysed by one way ANOVA with Bonferroni post-test *p<0.05, **p<0.01, ***p<0.001, ****p< 0.0001.*

6.7 Zoledronate did not enhance repair of DNA damage in cancer cells

To determine whether zoledronate with its DNA damage repairing abilities was able to enhance repair in cancer cells following exposure to irradiation different cancer lines of myeloma, breast and prostate were employed. Following treatment with Zol or PBS these cells were subjected to irradiation (1Gy) and at different time points (0h, 4h, 12h, 24h and 48h) post irradiation the number of γH2AX foci was enumerated. Immediately after irradiation (0h) a significant increase in DNA damage was seen compared to cells prior to irradiation and no significant difference was seen the cancer cells treated with PBS or Zol. In fact there was no progressive decrease in DNA damage foci in any of the cancer cells with increasing time which was in contrast to what was seen in case of hMSC suggesting that Zol had no DNA damage repairing ability in these difference myeloma, prostate and breast cancer cell lines (Fig 6.9 A-E; n=3; Appendix 3). This was further confirmed by the single-cell gel electrophoresis alkaline comet assay, wherein hMSC and the different cancer cells pre-treated with Zol or PBS were subjected to 3Gy dose of irradiation and 12 hours later sacrificed to assess the DNA damage by measuring the length of the comet tails to assess the extent of total DNA damage. As expected hMSC following irradiation had significantly increased percentage of cells in the comet categories CC3 and CC4 signifying long comet tails and high amount of DNA damage, in contrast to Zol treated hMSC where the percentage of cells in the CC1 category was significantly high suggesting significantly low levels of DNA damage (Fig 6.10 A-B; n =3). However the different cancer lines of the myeloma, prostate and breast cancer had no significant difference in any of the recorded comet categories (CC1-CC5) irrespective of treatment administered to these cells (Fig 6.10 A-B). Together these data suggest a differential

effect of zoledronate on normal stem cells and cancer cells in enhancing repair of DNA damage following exposure to radiation.

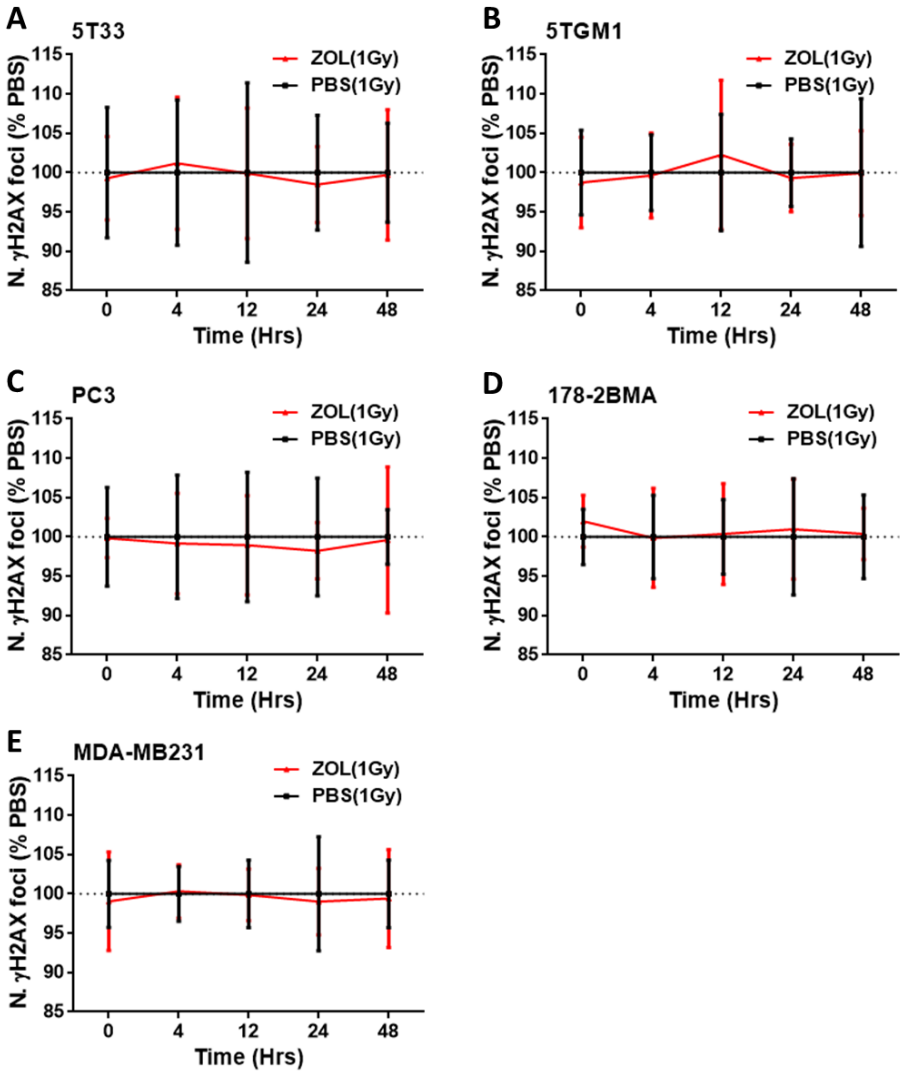
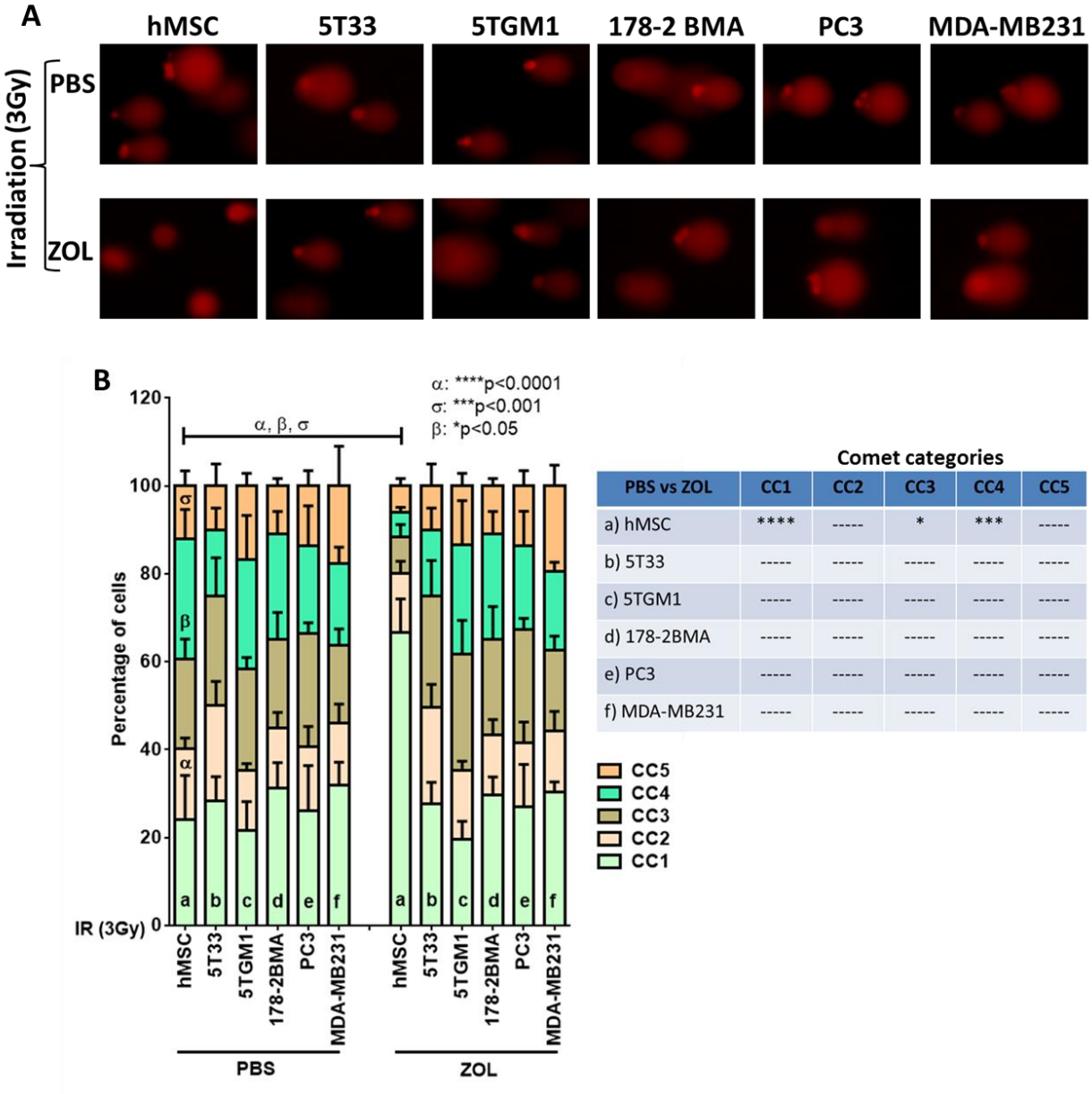


Figure 6.9 Zoledronate did not enhance repair of γH2AX foci in different cancer lines

*(A-E) Number of γH2AX foci enumerated in (A)5T33 and (B)5TGM1 myeloma cancer, (C)PC3 and (D) 178-2BMA prostate cancer and (E) MDA-MB231 breast cancer lines cultured in the presence or absence of Zol (1μM) for 3 days and stained immediately or 4, 12, 24, and 48h post irradiation at 1Gy (n=3). Data presented as mean±SD were analysed by one way ANOVA with Bonferroni post-test *p<0.05, **p<0.01, ***p<0.001, ****p< 0.0001*



Comet categories

PBS vs ZOL	CC1	CC2	CC3	CC4	CC5
a) hMSC	****	-----	*	***	-----
b) 5T33	-----	-----	-----	-----	-----
c) 5TGM1	-----	-----	-----	-----	-----
d) 178-2BMA	-----	-----	-----	-----	-----
e) PC3	-----	-----	-----	-----	-----
f) MDA-MB231	-----	-----	-----	-----	-----

Figure 6.10 Zoledronate enhanced DNA repair in hMSC but not cancer cells

*(A) Representative example of comet tails generated following irradiation (3Gy) in hMSC and different cancer lines in presence (bottom panels) or absence (top panels) of Zol. (B) Quantification of percentage of cells in the different comet categories CC1-CC5 (CC1 representing small or no tail signifying no DNA damage and CC5 representing longest DNA tail length signifying intense DNA damage, n=3). Data presented as mean±SD were analysed by two way ANOVA with Tukey's multiple comparison post-test *p<0.05, **p<0.01, ***p<0.001, ****p< 0.0001.*

To determine inability of Zol to enhance DNA repair in cancer cells and rule out the possibility that cancer cells did not take up Zol, I employed the surrogate marker of zol uptake ie unprenylated RAP1A. Zol at 1µM concentration was administered to the 5T33 and 5TGM1 myeloma cancer, PC3 and 178-2BMA prostate cancer and MDA-MB231 breast cancer lines. Three days post treatment cells were sacrificed to obtain protein and a western blot was performed to assess the unprenylation of RAP1A in these different lines. Cells in PBS had no detectable expression of RAP1A but Zol treatment resulted in significant expression of unprenylated protein suggesting the uptake of zoledronate and inhibition of mevalonate pathway in these cancer cells (Fig 6.11 A-B; n=3).

6.8 Zoledronate did not protect cancer cell clonogenicity following exposure to irradiation

To determine the effect of zoledronate on cancer cell functionality, I assessed the clonogenic ability of these different cancer cells following irradiation and pre-treatment with Zol or PBS. Following treatment with zoledronate there was a significant reduction in the number of colonies of the myeloma, prostate and breast cancer lines and this effect was further pronounced with accompaniment of irradiation, thus suggesting the negative impact of Zol and radiation on cancer cells clonogenicity (Fig 6.12 A-E; n=3).

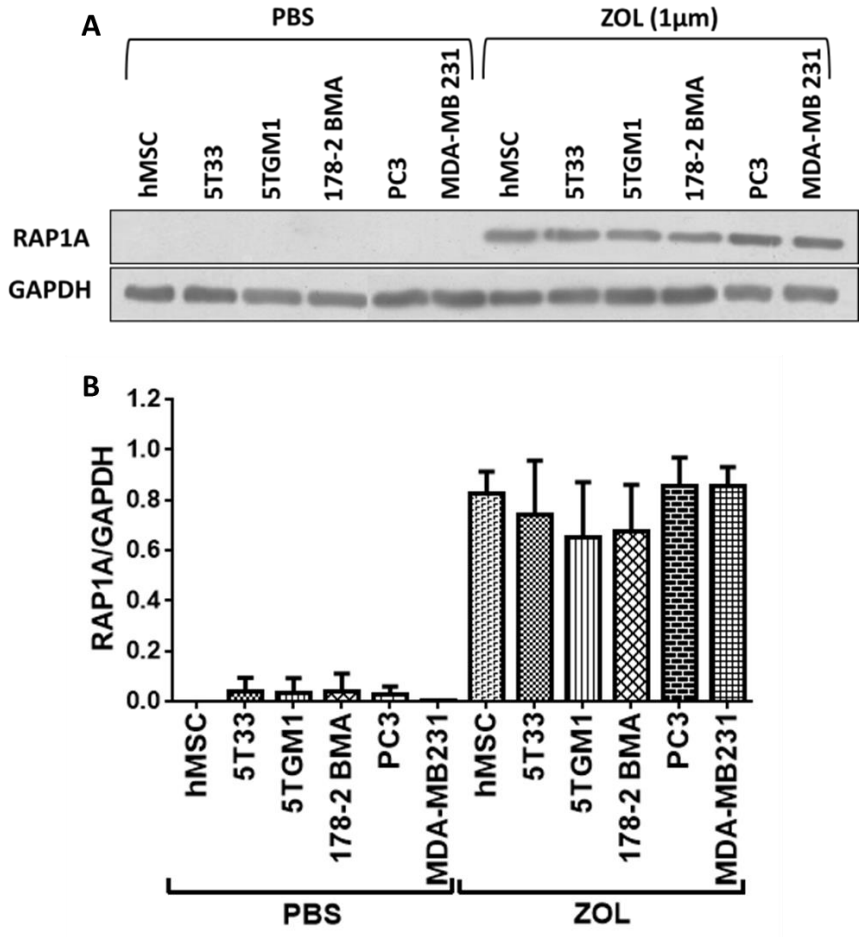


Figure 6.11 Zoledronate unprenylated RAP1A in cancer lines

*(A) A representative example of expression of unprenylated RAP1A by western blot analysis in human MSC and different myeloma, prostate and breast cancer lines cultured in the presence or absence of Zol (n=3). (B) Quantification of unprenylated RAP1A in hMSC and cancer lines normalised to GAPDH (n=3). Data presented as mean±SD were analysed by one way ANOVA with Bonferroni post-test *p<0.05, **p<0.01, ***p<0.001, ****p< 0.0001.*

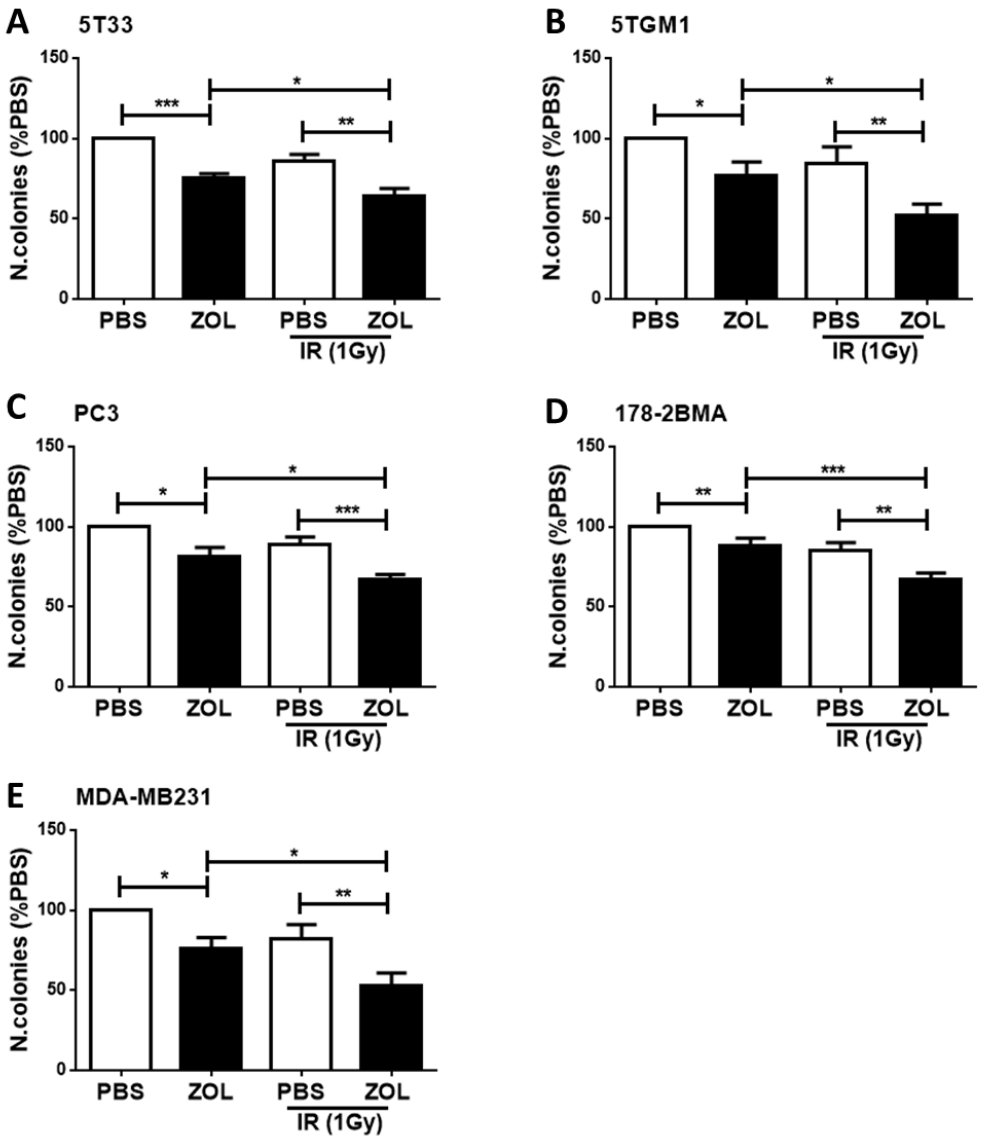


Figure 6.12 Zoledronate did not protect cancer cell clonogenicity

*(A-E) Number of colonies obtained from (A)5T33 and (B)5TGM1 myeloma cancer, (C)PC3 and (D) 178-2BMA prostate cancer and (E) MDA-MB231 breast cancer lines cultured in the presence or absence of Zol (1µM). Following 3 days of treatment, cells were exposed to irradiation (1Gy), the drug was washed of 12 hours post irradiation and cells incubated for 14 days before colonies were enumerated (n=3). Data presented as mean±SD were analysed by one way ANOVA with Bonferroni post-test *p<0.05, **p<0.01, ***p<0.001, ****p< 0.0001.*

6.9 Discussion

In this chapter I have shown that zoledronate protected murine mesenchymal stem cells of the bone marrow and the intestinal stem cells in the crypts from harmful radiation induced damage. In fact zoledronate reduced the incidence of DNA damage following irradiation in several other non-skeletal tissues in mice. Moreover Zol had differential effects on cancer cells of myeloma, prostate and breast cancer wherein it did not enhance repair of damaged DNA following irradiation, instead further hampered their functionality.

Radiation sensitivity in different stem cells populations is variable. In case of mouse melanocytes, irradiation resulted in premature greying in mice due to differentiation of these stem cells to mature melanocytes as a response to radiation (Inomata, Aoto et al. 2009). Haematopoietic stem cells undergo only lymphoid differentiation in response to radiation (Wang, Sun et al. 2012). However other stem cells like hair follicle bulge and keratinocyte stem cells are unaffected by irradiation (Tiberio, Marconi et al. 2002, Sotiropoulou, Candi et al. 2010). Now MSC are a heterogeneous population and studies on radiation effects in MSC *in vitro* have indicated the existence of heterogeneity in radiation sensitivity. Human MSC isolated from different anatomical sites display varying levels of radio-sensitivity for example MSC isolated from the orofacial regions are more resistant than those derived from the iliac crest (Damek-Poprawa, Stefanik et al. 2010). Even Carbonneau et al demonstrated a higher level of radio-sensitivity in MSC isolated from flushed bone marrow compared to collagenase digested bone suggesting the heterogeneity in radiation responses in the these two populations of MSC (Carbonneau, Despars et al. 2012). Therefore there is uncertainty whether the MSC population surviving within

the bone marrow following irradiation is true mesenchymal stem cells or a progenitor cells would require further investigation employing mice with identifiable MSC sub-populations such as the Nestin+ MSC or CAR cells or Leptin receptor expressing cells (Mendez-Ferrer, Michurina et al. 2010, Park, Spencer et al. 2012, Zhou, Yue et al. 2014). Despite the absence of *in vivo* identifiable MSC, analysis of CFU-F and CFU-O assay using bone marrow of mice subjected to 12 Gy total body irradiation resulted in loss of clonogenic potential of MSC precursors *in vivo,* and in presence of Zol treatment this was recovered. These data are in accordance with other studies where following total body irradiation of 4Gy a significant decrease in CFU-F was observed at 3 days (Poncin, Beaulieu et al. 2012). This was also the case in another study the by Ma et al (2007) where CFU-F decreased with IR (Ma, Chen et al. 2007). Even studies by Cao et al (2011) demonstrated no colony formation of MSC in CFU-F and CFU-ob assay (Cao, Wu et al. 2011). All these together confirm that there is a loss in clonogenic potential that could be attributed to DNA damage double strand breaks induced by irradiation. However in presence of zoledronate there is repair of DNA damage and reduced incidence of DSB that results in enhanced CFU-F and CFU-O potential. Cao et al proposed that irradiation injures the vasculatures and microenvironment which may affect the MSC functions. It may be possible that Zol has a protective effect on the microenvironment as well which may result in retaining MSC function in the niche.

DNA damage following irradiation was observed in a lot of other murine tissues and the damage foci were significantly reduced by Zol treatment in most tissues raising the question of bio-distribution of Zol. The pharmacokinetic profiles of nitrogen containing bisphosphonates such as Zol point at a major limitation of these drugs to

have direct effects *in vivo* on soft tissues including cancer other than bone. Intravenous administration of Zol at clinically relevant doses had increased peripheral blood concentration with maximum plasma concentrations of Zol being about 1µM. *In vivo* however these concentration levels may have limited anticancer activity and may not have unfavourable effects on non-skeletal tissues like cancerous tumour when compared to *in vitro* doses that are 10-100 folds higher for inducing apoptosis and growth inhibition. It is important to note that the positive effects of these concentrations on non-skeletal tissues, if any, have not been investigated. Although several studies on BPs have shown that their concentration in non-calcified tissues declines rapidly, whereas concentration in bone continuously increases, there is some amount of BP that is released back in to circulation thus resulting in a terminal half-life of about 7 days as reported in case of Zol or 200 days in case of Alendronate (Lin 1996, Chen, Berenson et al. 2002, Ibrahim, Scher et al. 2003, Skerjanec, Berenson et al. 2003, Barrett, Worth et al. 2004). Considering the high affinity that zoledronate has for bone mineral and the mounting evidences of its ability to inhibit osteoclast activity that directly implicates treatment of bone loss diseases and may contribute to reduction in tumour burden in some animal models, not much investigation have been made on the possibility of an existing non-skeletal effect in different soft tissues. Although available evidences from some studies have suggested non-skeletal related antitumor effect of bisphosphonates in soft tissues of breast, melanoma and cervical cancers, there is no available information on the non-cancer soft tissues where zoledronate activity is recorded (Giraudo, Inoue et al. 2004, Hiraga, Williams et al. 2004, Yamagishi, Abe et al. 2004). The only available study is on bio-distribution of zoledronate by Weiss et al 2008, wherein, using ^{14}C labelled zol administered in rats

and dogs, the varying levels of drug retention was recorded in the plasma, bone and other soft tissues such as the kidney, liver, small intestine and spleen (Weiss, Pfaar et al. 2008). Studies on bio-distribution of Zol *in vivo* by Weiss et al also show that levels of ^{14}C Zol rapidly decrease from non-calcified tissue but persist in bone and slowly diminish with half-life of 240 days. Together these studies suggest that disposition in blood and non-calcified tissue maybe governed by uptake and slow release. In this study I administered Zol doses equivalent to those already used in patients affected by cancer bone loss and found that un-prenylation of RAP1A used as a surrogate marker for Zol uptake occurred in several soft tissues most of which were identified as sites of radioactive Zol (Weiss, Pfaar et al. 2008, Coleman, Cameron et al. 2014). This is probably the first time that the bio-distribution of Zol in these different tissues *in vivo* has been based on the ability of Zol to inhibit FPPS enzyme. Studies on pharmacokinetics of bisphosphonates with fluorescently labelled bisphosphonates have shown that macrophages and osteoclasts internalize them by endocytosis into membrane bound vesicles, suggesting that BPs enter intracellular vesicles but very small amounts enter the cytosol, however what is important to note is that nitrogen containing BPs such as Zol have extremely high potency for inhibition of farnesyl pyrophosphate synthase, hence even if they are up taken relatively poorly, they may still inhibit the mevalonate pathway to prevent prenylation of GTPases such as RAP1A and this may explain how tissues such as intestine where Zol is known to have minimal absorption show un-prenylation (Dunford, Thompson et al. 2001, Rogers 2004, Thompson, Rogers et al. 2006).

Radiation is known to cause acute gastrointestinal damage. Mice receiving 14 Gy die within 7-12 days of irradiation damage due to high sensitivity on the intestinal

mucosa. Radiation toxicity in can occur at low doses but crypt sterilization occurs when the stem cells are depleted which generally occurs above 8Gy (Potten and Grant 1998, Potten 2004, Barker and Clevers 2007). Following irradiation, the Lgr5 expressing stem cells accumulated damage (3Gy) which was significantly reduced in Zol treatment, however at 12 Gy dose which should ideally cause sterilization of the intestines, I found that there was significant loss of intestinal crypt villus architecture accompanied with significant reduction in Lgr5 positive cells and Lgr5 positive crypt similar to what was reported by Metcalfe et al, (2014)(Metcalfe, Kljavin et al. 2014). Interestingly Zol treatment resulted in recovering the loss of the Lgr5 cells following irradiation, which we find was associated with DNA repair, reduced apoptosis and increased proliferation compared to vehicle mice where the stem cells were highly sensitised and succumbed to apoptosis that may be triggered by pro-apoptotic protein accumulation implicating tumour suppressor p53 and ATM and pro apoptotic PUMA proteins pathways (Ch'ang, Maj et al. 2005, Qiu, Carson-Walter et al. 2008).

mTOR signalling has been implicated in intestinal stem cells proliferation and function. Calorie restriction that has effect on mTORC1 signalling has shown to restrict intestinal stem cells in drosophila however in case of murine intestines this has resulted in preservation of intestinal stem cells (O'Brien, Gorentla et al. 2011, Yilmaz, Katajisto et al. 2012). It enhances ISC proliferation but reduces the proliferation of more differentiated transit amplifying cells. Moreover the paneth cells niche may also play a role in enhancing ISC. Investigations on reduced mTOR activity in paneth cells showed enhanced ISC function. In fact *in vivo* models of Rheb2 overexpressing mice resulted in inhibiting the clonogenicity of ISC. Together this results support our finding wherein in inhibition of TOR signalling results in no loss of

ISC. Whether persistent inhibition of TOR signalling results in intestinal atrophy requires further investigation hence further time points are required in this study.

DNA damage induced by irradiation leading to cancer risk is presumably lower in small intestine than large intestine despite the fact that the small intestine have large division potential, 24h cell cycling time, and this suggests/supports the altruistic loss of damaged cells through apoptosis. Following irradiation there was increase in apoptosis in vehicle mice compared to Zol treated mice. Therefore the DNA repair/protective effect that Zol renders to intestinal stem cells then warrants further investigation to study cancerous transformations of these Lgr5 cells that are protected from loss by radiation. In fact recent developments in long term crypt cells cultures where single sorted lgr5 cells can initiate crypt villus organoids will be more useful in assessing malignant transformation of these cells (Sato, Vries et al. 2009, Sato, van Es et al. 2011).

The fact that the enhanced DNA repair activity in response to Zol does not occur in cancer cells and that the combination of Zol and irradiation inhibits the clonogenic activity of cancer cells is an important advantage. Reduced mTOR/AKT signalling has been shown to mediate differential protection of normal cells from cancer cells (Lee, Safdie et al. 2010) thus supporting these findings. Indeed mTOR signalling pathways have been shown to be hyper-active in certain cancer types such as breast, prostate, and haematological malignancies rendering cancer cells insensitive to such inhibitory signals (Cully, You et al. 2006). Clinical trials with analogues of rapamycin (rapalogs), inhibiting the TORC1 complex of the mTOR pathway, have shown efficacy in glioblastomas and breast cancer, although to a limited extent (Gibbons, Abraham et al. 2009). The limited effect is thought to be due to the compensatory mechanisms

via TORC2 and reduced negative feedback of the S6K-PI3K loop that normally acts to modulate the mTOR pathway (Bracho-Valdes, Moreno-Alvarez et al. 2011). Considerable effort has been devoted to developing dual inhibitors of TORC1 and TORC2 in the hope of reducing the feedback activation of PI3K signalling, and to stop the proliferation of cancer cells (Xue, Hopkins et al. 2008, Wallin, Edgar et al. 2011). The fact that zoledronate has shown to inhibit both forks of TOR signalling in stem cells needs to be confirmed in cancer cells as well. As different cancer cells also express different levels of PTEN which is a natural inhibitor of PI3/AKT signalling pathway known to inhibit proliferation; it is imperative to determine the effect of Zol on PTEN. Previous studies on MG63 cells have shown that Zoledronte in combination with RAD001 potentiates inhibition of mTOR signalling and this result in cytotoxic effect on these osteosarcoma lines suggesting a pivotal role of prenylation in cancer cell survival (Moriceau, Ory et al. 2010). Whether these effects are seen in breast, prostate and myeloma lines is important to address as in this study I have already shown inhibition of the Mevalonate pathway in these cells but what happens downstream warrants further investigation.

In conclusion I have shown in this chapter that zoledronate was able to reduce the incidence of DNA damage in murine tissue. Zol also protected intestinal stem cells from radiation induced damage and rescued the intestinal morphology by reducing the incidence of apoptosis. Interestingly zoledronate had differential effects in cancer cells and was unable to repair DNA damage in them; however it further sensitised these cells to radiation damage and reduced their clonogenic potential.

Chapter 7: General Discussion

Tissue integrity is highly dependent on the resident self-renewing stem cells which are vulnerable to endogenous and exogenous insults thus compromising their function. These events eventually cause disruption of normal functions of the tissues and organs, age related diseases and reduced health span in individuals. Clinical approaches involve the use of stem cells as regenerative therapeutics to address some of these issues. Human mesenchymal stem cells are important tools in these regenerative approaches in a clinical setting, and are being exploited for use in treating orthopaedic injuries, graft versus host disease following bone marrow transplant, gastrointestinal tracts, autoimmune diseases and cardiovascular diseases among others (Brooke et al, 2007). Thanks to their unique characteristics to differentiate to multi-lineages, secrete soluble factors to induce immuno-modulatory environment and migrate to sites of injury, these stem cells have received immense attention (Caplan 2009; Kim and Cho 2013, (Chanda, Kumar et al. 2010, De Miguel, Fuentes-Julian et al. 2012). To facilitate these uses, *in vitro* expansion of these cells is a necessary step in order to generate sufficient numbers as the yield of these prospective hMSCs is very low. Unfortunately following *in vitro* expansion, these cells undergo cellular ageing associated with senescence that can be triggered by accumulation of DNA damage, telomere shortening, proto-oncogene activation leading to loss of proliferative and differentiation capacity (Mendes, Tibbe et al. 2002, Stenderup, Justesen et al. 2003, Campisi and d'Adda di Fagagna 2007, Sharpless and DePinho 2007, Cichowski and Hahn 2008). For their expansion ideally low oxygen levels are better as they allow faster proliferation of stem cells as opposed to the 20% oxygen levels that are traditionally used, but culture in these hypoxic conditions has

problems of its own. Hypoxic conditions tend to favour one differentiation pathway in impairment to others and even attenuate differentiation of hMSC in low levels of oxygen (Csete, Walikonis et al. 2001, Fehrer, Brunauer et al. 2007, Potier, Ferreira et al. 2007, Potier, Ferreira et al. 2007). Secondly maintenance of hypoxic conditions requires specialised equipment that is commercially/clinically a labour intensive and expensive concern. Under these circumstances, chemical interventions that can delay cellular ageing are most sought after and in this thesis I have identified zoledronate as a potential chemical intervention that extended hMSC life span and delayed hMSC cellular ageing by enhancing DNA repair either with time in culture or following exposure to radiation. Repair by zoledronate was mediated by inhibition of mevalonate and mTOR signalling pathways and its usage was extendible to intestinal stem cells as well. Interestingly DNA damage repairing abilities were not extended to cancer cells.

Accumulation of DNA damage, a key driver of cellular ageing in stem cells has been evidenced in this study. Results from this study suggest that zoledronate's ability to reduce incidence of DNA DSB has potential uses in alleviating DNA damage related loss of stem cell functions by targeting DNA repair pathways and delaying cellular ageing. This may potentially help to postpone tissue degeneration and development of age related cancers by maintaining tissue homeostasis. Indeed previous literature suggests that age related decline in tissue homeostasis causes loss of tissue function and decrease in lifespan leading to major age related disorders such as cancer, cardiovascular disorders, neurodegenerative diseases and musculoskeletal damage (Lopez-Otin, Blasco et al. 2013). Insufficient cell replacement accompanied with increased cellular loss due to decline in stem cell function and regenerative capacity

has been observed with age in several tissues (Nishimura, Granter et al. 2005, Rossi, Bryder et al. 2005, Molofsky, Slutsky et al. 2006, Gago, Perez-Lopez et al. 2009). Even studies in adult haematopieitc stem cells have established a link between age related accumulation of DNA damage and functional decline of stem cells' regenerative capacity (Nijnik, Woodbine et al. 2007, Rossi, Bryder et al. 2007, Rube, Fricke et al. 2011, Wang, Sun et al. 2012). In fact the effect of accumulation of DNA damage is also seen in other stem cell types with different responses depending on the extent of damage and strength of DNA damage response (Mandal, Blanpain et al. 2011, Sperka, Wang et al. 2012). Therefore through this study I have identified zoledronate and other nitrogen containing bisphosphonates as potential therapeutics that can target repair pathways and delay cellular ageing not only in human mesenchymal stem cells but also mature dermal fibroblasts. Since bisphosphonates are FDA approved drugs with relatively safe clinical profiles, their potential applications to test these novel functions of DNA repair in clinical settings should be easy to achieve. In fact retrospective and meta-analysis of data from patients already administered with bisphosphonates for treatment of fragility fractures and osteoporosis have impacted the mortality and lifespan of patients (Lyles, Colon-Emeric et al. 2007, Lyles, Colon-Emeric et al. 2007, Bolland, Grey et al. 2010, Colon-Emeric, Mesenbrink et al. 2010, Beaupre, Morrish et al. 2011, Center, Bliuc et al. 2011, Sambrook, Cameron et al. 2011). Reduced mortality benefits in these patients was only minimally explained by prevention of secondary fractures whereas a vast majority was attributed to reduced occurrence of infectious diseases like pneumonia, cancer and cardiovascular diseases. One hypothesis is that Zol's ability to extend lifespan and delay cellular ageing by enhancing DNA repair in my current research may provide a mechanistic explanation

to the robust reductions in mortality in these patients by actually extending health span of patient's stem cells. Having proposed this of course further work needs to be done with samples from these patient groups to dissect out the exact mechanism of action. A quick readout could also be obtained from assessing the blood samples in these patients for DNA damage marker or even muscle biopsies since Zol has shown effects on this tissue.

The AKT - FOXO3A - pATM signalling downstream of TORC2 has been shown to be central to the survival of several stem cell types such as the HSC and neural stem cells (Miyamoto, Miyamoto et al. 2008, Paik, Ding et al. 2009, Renault, Rafalski et al. 2009). Similar effects have been reported in melanocyte stem cells (Inomata, Aoto et al. 2009). Interaction of FOXO3a and ATM with ROS and DNA repair mechanism leading to cell survival has been reported as well (Tsai, Chung et al. 2008, Yalcin, Zhang et al. 2008, Chung, Park et al. 2012). This study further adds to the literature that chemical interventions (zoledronate) modulate hMSC cell survival and lifespan extension and this was mediated via DNA damage repair through mTORC2/ AKT/ FOXO3a/ ATM signalling. This finding has wider relevance as a similar protective effect of zoledronate on radiation-induced damage in intestinal stem/progenitor cells as also found in MSC *in vivo*. These results suggest that even other stem cell types, such as HSC may also benefit from the DNA damage repair activity of Zol. Indeed loss of the DNA damage sensors ATM and FOXO3a has been shown to deplete HSC (Ito, Hirao et al. 2004, Miyamoto, Miyamoto et al. 2008). As phosphorylation of ATM is central to initiate a number of DNA repair mechanisms including non-homologous end joining or homologous recombination (Shiloh and Ziv 2013) going forward it will be important to determine which of these DNA repair mechanisms is downstream of

ATM as both of these mechanisms have different levels of fidelity obtained during repair, the NHEJ being more error-prone. This is particularly important since error prone mechanism can lead to cancerous phenotypes. Moreover the mTOR signalling is central to longevity and cancer survival therefore it is therefore important to determine whether treatment with Zol in hMSC on short and long term can stimulate cancer formation in cells that are already predisposed to develop cancer or whether Zol exposure can even prevent this possibility of developing cancer. Careful evaluations are needed to test whether these cultured cells treated with zoledronate for short and long periods show signs of cancer phenotype when transplanted *in vivo* in syngeneic animal models.

An important strategy to highlight in this study was the use of zebrafish as model to bridge the *in vitro* work to *in vivo* in higher model organisms like mice. Zebrafish have been previously employed for drug screening and due to their multifarious advantages I used the same model but for developing a quick assay to test regeneration ability of zoledronate in 5 day old embryos following injury. One important finding of this study, reported for the first time was that irradiation impaired tail regeneration ability of zebrafish in embryos following amputation. Despite the lack of clarity on which mechanisms actually cause regeneration to occur; whether it is the immune cells (Renshaw and Trede 2012) or the formation of blastema (Azevedo, Grotek et al. 2011), this model provides a good readout for assessing the ability of drugs that can regenerate tail fin or not. Moreover I found that mechanism of action of Zol on hMSC was actually conserved in Zebrafish models. For example mTOR has been previously explored in Zebrafish in studies associated with embryogenesis and development (Makky, Tekiela et al. 2007). Moreover studies

associated with tail regeneration following amputation in juvenile and adult zebrafish have shown association with TOR signalling where they have focussed only on TORC1 but not TORC2 (Goldsmith, Iovine et al. 2006, Hirose, Shiomi et al. 2014, Kujawski, Lin et al. 2014). Interestingly in my study in absence of irradiation tail regeneration occurs to similar levels in both rapamycin and other dual TORC1 and TORC2 inhibitors (Ly294002, Ku00634; zoledronate) but following irradiation only the dual inhibitors could regenerate tail following amputation suggesting that implication of TORC2 in tail regeneration may actually be associated specifically with the irradiation damage in Zebrafish. These results throw light on how the mechanism of action of Zol is conserved from hMSC to zebrafish in light of enhancing DNA repair following radiation injury. Although further establishment is required on the identifying which cells are associated with tail regeneration (blastema or immune cells) following amputation and irradiation, however this model can potentially be used to test radioprotective drugs and immunosuppressive drugs to determine effects on tissue regeneration following injury *in vivo* prior to testing them in mice, where regeneration studies are limited and expensive.

Zoledronate is known to have high affinity for bone but its effects on other tissues *in vivo* is limited as previous studies have only established the presence or absence of the fluorescent drugs in skeletal system. This study highlights the non-skeletal effects of zoledronate on repair of DNA damage following whole body irradiation in tissues such as the intestine which is a highly regenerative tissue. Effects on intestine suggest a potential clinical application of zoledronate in patients receiving radiotherapy which is one of the most widely used cancer treatments. Radiation has severe side effects that our debilitating both to bone, haematopoietic system and gastrointestinal

mucosa (Mauch, Constine et al. 1995, Yarnold and Brotons 2010, Peterson, Bensadoun et al. 2011, Yu 2013). Loss of cancer cells is accompanied with loss of normal healthy cells including stem cells resulting in loss of tissue homeostasis and functional decline. Radiation induced gastrointestinal mucositis compromises the absorptive and barrier function of the mucosa by killing the crypt stem cells, impairing normal regeneration and at present there is no effective treatment for gastrointestinal mucositis (Andreyev 2007, Andreyev 2007). Results from this study are suggestive of zoledronate as a potential drug for use in treating the side effects of radiation therapy. Cytopenia is one more example, where Zol could exert its effects through direct protection of HSC or via protection of MSC, which is an important component of the HSC niche (Sacchetti, Funari et al. 2007, Mendez-Ferrer, Michurina et al. 2010). Whether Zol has effects on oxidative stress has not been explored in this study, but due to its direct role in DNA repair ability, zoledronate may be potentially used in patients with myelodysplastic syndrome (MDS) which occurs in older people or cancer patients following irradiation and chemotherapy. In MDS patients, damage to bone marrow is characterised by impaired haematopoiesis. This is followed by acute myelopoiesis and increased risk of leukemic cancer (Mufti 2004).

Since DNA damage occurs due to irradiation, pre-treatment with Zol prior to radiotherapy may mediate repair mechanisms that help prevent damage or enhance repair and delay development of cancer or other side effects or radiotherapy. In this study only short single doses of drug have been studied and in order to establish use of Zol in the aforementioned clinical settings, further titration of doses is required to establish proper dose regimens that can have DNA repairing ability for longer. Pre-treatment of zoledronate is logical in cases where radiotherapy is administered to

prevent accumulation of damage and repair any pre-existing damage, but in cases where damage is un-announced like in case of a heart-attacks or radiation spills, it is important to establish what doses of zoledronate that would be sufficient to enhance repair if at all they can. I have established in this study that apart from pre-treatment, administration of Zol at 1µM after induction of damage by irradiation resulted in repair of damage although delayed by 3-4 days. This delay could be associated with the dose or in reality the minimum time necessary to initiate a repair response by Zol but further investigations on dose regimen is definitely needed. One possible approach is to administer single clinically relevant dose Zol *in vivo* in mice and determine the levels of tissue unprenylation at different time points (in days). This should provide an idea of how soon the effect of Zol wears out in different tissues and help estimate when a second dose would be needed. Once established the effects of pre and post treatment should be determined *in vivo* in mice following irradiation. Again zoledronate is ideally given to treat bone loss and whether this formulation would be ideal for non-skeletal tissues for multiple dosing is another key question that needs further investigation. Ideally there is a need for a bisphosphonate of same potency and functionality but reduced bone affinity that can also be taken orally as opposed to injecting in case of a need for multiple dosing regimens. This is especially useful based on my findings of the effect of Zol on intestines. An oral formulation if possible would be ideally suited in cases where patients receive multiple doses of radiotherapy especially in cases of pelvic cancers where treatment is spread over 5-6weeks and amounting to nearly 60Gy at the end of radiation therapy. One of the limitations of this project was that irradiation was administered whole body in one go, however from a clinical perspective it is important to identify

whether these effects of Zol hold true in case of multiple titrated doses of radiation. Future experiments would surely be needed on titrating doses of radiation that are site specific to determine whether radiation effects are isolated to radiated tissue or affect non-radiated areas as well and whether bisphosphonates can alleviate these effects. All these experiment should finally help identify a complimentary dose regimen of Zol treatment along with titrated radiation dosing that would have beneficial effects of radioprotection on normal cells but allow termination of cancer cells.

Zoledronate had effects on several tissues such as the heart and muscle among others but my focus has been primarily on the intestines in particular the small intestines. Depending on the types of cancer radiation effects can be isolated to the head and neck region where damage affects the oral mucosa, tongue and oesophagus, or the pelvic region where effects are not isolated to small intestines but a major effect being on the colon region that is susceptible to colitis. Therefore further in depth analysis of the GI tract is required both at a morphological and molecular level. It is important to note that radiation effects *in vivo* were assessed at the 4 days post irradiation, time point that was ideal for assessing changes in small intestine due to measurable morphological changes in the villi and crypt, and this could be similar to the colonic regions where there is only crypts, but in case of oesophagus and tongue definitely longer time points may be needed to assess damage and repair. In fact even site specific radiations would be more clinically relevant in those cases.

The lack of DNA repair activity in cancer cells by Zol and the inhibition of their clonogenic activity, when zol treated cells were exposed to irradiation, are important

findings. Differential effects of reduced mTOR/AKT have been shown to mediate protection to normal cells and not cancer cells (Lee, Safdie et al. 2010). In fact studies by Iglesias-Bartolome et al have shown that mTOR inhibition by Rapamycin, radio-sensitized head and neck squamous cell carcinomas (HNSCC) but had protective effect on normal oral epithelial cells from ionizing-radiation-induced epithelial stem cell depletion (Iglesias-Bartolome, Patel et al. 2012). Indeed my data are consistent with these results wherein Zol has been shown to mediate differential protection of normal cells from cancer cells. *In vitro* studies have suggested that Zol inhibits Ras prenylation resulting in radio-sensitization of cancer cells (Bernhard, Stanbridge et al. 2000, Brunner, Gupta et al. 2003). Ras mediates both ERK and PI3/AKT pathways and inhibitions of these have been associated with radio-sensitization more importantly being tissue type and cancer cell line dependent (Hagan, Wang et al. 2000, Gupta, Bakanauskas et al. 2001). Cancer cells sensitivity by zoledronate may also be induced by other signalling pathways such as the signal transducer and activator of transcription 1 (STAT1) which has recently been implicated in sensitizing renal cell carcinoma cells and hepatocellular carcinoma cells to radiation (Inamura, Matsuzaki et al. 2005, Kijima, Koga et al. 2013). Whereas certain other cancer cells types have shown hyperactive mTOR signalling specially the breast, prostate and blood malignancies, which may render these cells insensitive to inhibitory signals (Cully, You et al. 2006). Clinical trials with analogues of rapamycin, capable of inhibiting the TORC1 complex of the mTOR pathway, have been shown to have some efficacy in glioblastomas and breast cancer (Gibbons, Abraham et al. 2009). Their limited effect is thought to be due to the compensatory mechanisms via TORC2 and reduced negative feedback of the S6K-PI3K loop that normally acts to modulate the pathway

(Bracho-Valdes, Moreno-Alvarez et al. 2011). Investigation on developing dual inhibitors of TORC1 and TORC2 in the hope of reducing the feedback activation of PI3K signalling, and to stop the proliferation of cancer cells has been ongoing (Xue, Hopkins et al. 2008, Wallin, Edgar et al. 2011) and in this study therefore provides a potential drug that can target both components of the mTOR signalling pathway in stem cells and has cytostatic effects in cancer cells. However further investigations on signalling pathways downstream of Ras are required for a clearer understanding of the differential effects in normal and cancer cells *in vitro*. More importantly *in vivo* work on different cancer mouse models of radiation is needed to determine whether Zol has these differential effects on normal and cancer cells *in vivo*. In fact another important future work is to determine whether these differential effects of Zol are specific only to radiation induced injuries or whether similar effects are seen in response to chemotherapy. Detailed *in vitro* analysis would be necessary in case of chemotherapy depending on the type of treatment used as mode of DNA damage induction in different chemotherapies is different and may not necessarily be similar to ionizing radiations.

Chapter 8: Conclusion

Loss of stem cells function due to age, diseases, accumulation of damage at DNA and protein levels due to age or damaging agents such as radiation, epigenetic changes, changes in environment and several other factors leads to dysfunctional tissue repair and loss of tissue homeostasis. As these stem cells are important both to maintain in situ harmony as well as have therapeutic usages, therefore understanding loss of their function and finding ways to prevent or reverse this has potentially important impacts in a variety of areas such as healthy ageing, age related diseases and more importantly in exploiting these cells for therapeutic applications. Whilst studies like the one conducted by Jaskelioff et al (2011) have used genetic approaches to demonstrate that it is possible to develop a therapeutic intervention to target age related diseases by reversing stem cells ageing, the same effect could also be achieved through chemical interventions (Jaskelioff, Muller et al. 2011). Chemical interventions could directly have an effect on stem cells by increasing the stem cell pool, redirecting their differentiation process, protecting them from damage, promoting rejuvenation, or even re-programming them. Evidence of reprogramming cells *in vivo* has been published recently (Abad, Mosteiro et al. 2013). In fact even modulation of the microenvironment in which these cells reside can improve their function as well (Conboy, Conboy et al. 2013). Moreover interventions that involve transplantations have also been extensively proposed especially in targeting a number of age related diseases (Harding and Mirochnitchenko 2014). However there are several advantages of chemical interventions over these transplants or even tedious genetic approaches. They would be substantially cheaper, could overcome the limiting factors of transplantation that includes the engraftment levels and

integration time of the newly formed tissue with the existing one, reduce the extensive issues with manufacturing and regulatory affairs and more importantly, may require only short intermittent dosing strategies that may be sufficient to obtain significant effects. The work presented in this thesis highlights the use of one such chemical intervention, zoledronate, nitrogen containing bisphosphonate. The evidences from experiments in this thesis support the hypothesis that 'Bisphosphonates extend stem cell lifespan by protecting them from incidence of DNA damage and enhance tissue regeneration *in vivo* in models of zebrafish and mice'. Chapter 3 clearly concludes the repurposing of zoledronate in enhancing DNA repair and rescuing the declining function of adult mesenchymal stem cells due to cellular ageing or exposure to radiation. Zoledronate not only enhances DNA repair but also extends the cellular lifespan in culture while retaining stem cells functions suggesting it as a potential intervention in ex vivo expansion of these cells required for transplants in a clinical setting. Chapter 4 highlights the mechanism of action of zoledronate. Zoledronate acts via inhibition of mevalonate and mTOR signaling pathway, a key pathway associated with extension of lifespan and cancer progression. Although zoledronate enhances repair of DNA damage, it is still not known whether zoledronate promotes the choice of using homologous recombination which is an error free repair pathway, or non-homologous end joining repair which is an error prone repair pathway, or whether there is a completely different repair mechanism involved, and this is one of the key questions to be addressed in future investigations. Since regeneration is an important aspect of stem cell function, and it is difficult to achieve this through transplants assay that have limitations of engraftment potential or even transplantation strategies, Chapter 5

investigated the use of an *in vivo* regeneration model of zebrafish. This chapter concludes that radiation damage inhibits fin fold regeneration in zebrafish embryo following amputation in an mTOR dependent manner and zoledronate enhances regeneration by inhibiting mTOR signalling. These findings point at a very highly conserved mechanism of action of zoledronate in fish. Moreover the optimized regeneration assay last only for 5 days thus making it a very useful, feasible and cheap assay to bridge studies on regeneration following *in vitro* studies before moving to more expensive and long term *in vivo* experiments in higher models like mice. In chapter 6, the use of mice models to test the hypothesis of DNA damage repair with zoledronate unraveled several important findings. The first and most important finding was that zoledronate which was historically known to have higher affinity for only skeletal tissues was shown to have effects in a number of other non-skeletal tissues such as the heart, kidney, intestines and muscle. This is a very crucial and important finding that immensely changes the old adage of zoledronate's action being restricted to bone alone. Zoledronate was able to reduce the incidence of DNA damage in several tissues thus suggesting the conservation of its mechanism of action. More importantly zoledronate was able to protect the intestinal stem cells from radiation damage by protecting them from apoptosis. As a result zoledronate was able to protect intestinal morphology as well, thus making it a useful intervention in radiation induced mucositis condition which currently has no effective treatment. Chapter 6 also highlights the differential role of zoledronate in cancer cells where the effects of zoledronate to enhance DNA damage repair are not observed and zoledronate only further sensitizes the cancer cell clonogenicity following radiation. Further investigations are needed to understand what mechanism of actions is

involved in cancer cells following zoledronate treatment that result in this differential effect.

This thesis has primarily focused on the novel effects of zoledronate on enhancing repair following damage induced due to radiation or cellular ageing *in vitro*, however further investigations are needed to determine whether these effects can be translated to ageing scenarios in young versus old mice and also following other sources of external damage such as chemotherapy. Through this work, a series of exciting new potential applications for the protection of stem cells from the side effects of radiotherapy in cancer patients and during ageing have evolved. More importantly this study has effectively demonstrated the repurposing of an already existing, extensively used drug. Thus the novel effect of bisphosphonates on stem cells and regeneration can be readily exploited for potential therapeutic opportunities.

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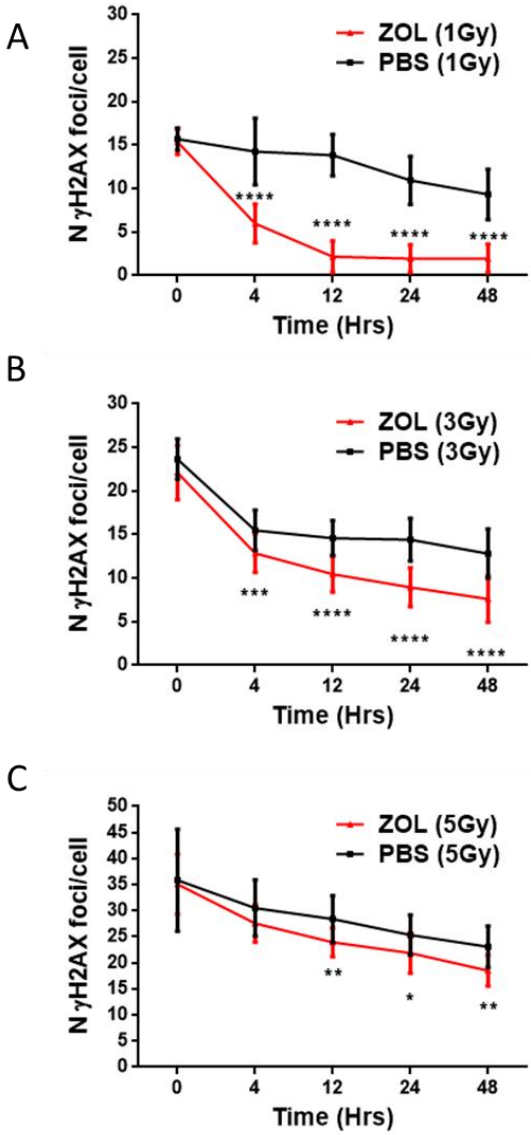
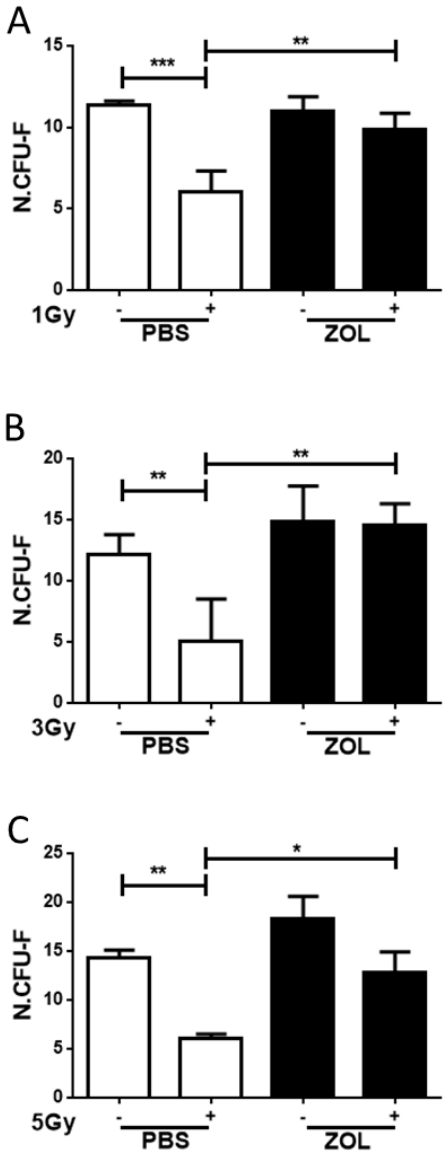
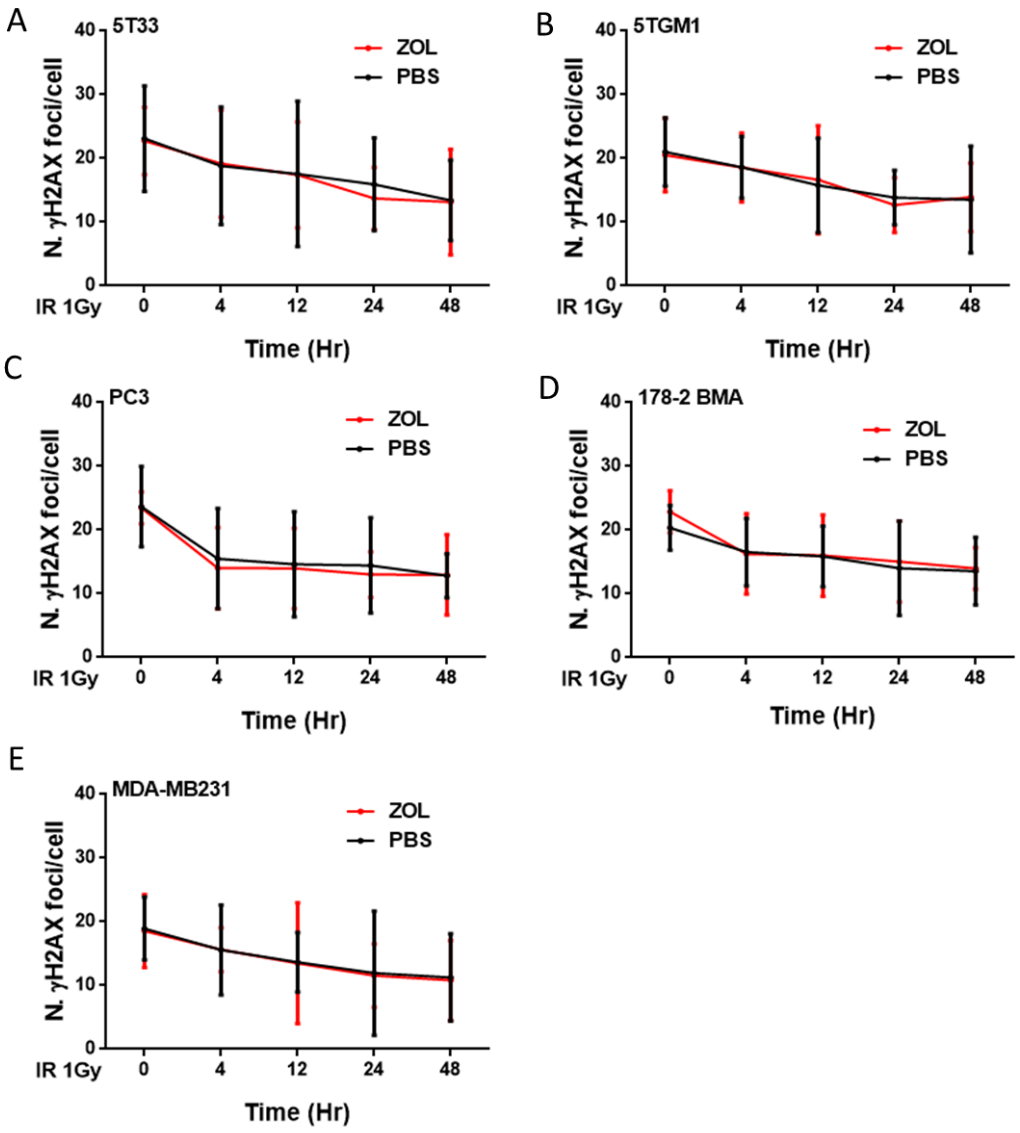
Appendix



*Appendix Figure 1 (A-C) Number of γH2AX foci enumerated in hMSC (n=3) cultured for 3 days in the presence or absence of Zol (1μM). Cells were irradiated at (A) 1Gy; (B) 3Gy or (C) 5Gy and assessed at time points 0, 4, 12, 24 and 48h post irradiation at any of the doses. Data presented as mean ± SD and analysed by one way ANOVA with Bonferroni multiple comparison post-hoc test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*



*Appendix Figure 2 (A-C) Number of CFU-F obtained from hMSC (n=3) seeded at low density and exposed to irradiation at either (A) 1Gy; (B) 3Gy or (C) 5Gy in the presence or absence of Zol (1µM) and left to grow for 14 days at 37˚C, 5% CO_2 in air. Data presented as mean ± SD and analysed by one way ANOVA with Bonferroni multiple comparison post-hoc test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*



*Appendix Figure 3 (A-E) Number of γH2AX foci enumerated in (A)5T33 and (B)5TGM1 myeloma cancer, (C)PC3 and (D) 178-2BMA prostate cancer and (E) MDA-MB231 breast cancer lines cultured in the presence or absence of Zol (1μM) for 3 days and stained immediately or 4, 12, 24, and 48h post irradiation at 1Gy (n=3). Data presented as mean±SD were analysed by one way ANOVA with Bonferroni post-test *p<0.05, **p<0.01, ***p<0.001, ****p< 0.0001*

Abbreviations

%	Percentage
-/- or KO	Knock-out
+ve/+	Positive
137Cs	137- Caesium
4-OHT	4 hydroxytamoxifen
A	Absorbance
AA	Amino acid
ACVR1	Activin A receptor, type I
AD	Adipocyte
AGE	advanced glycation end products
AKT	protein kinase B
Ale/Aln	Alendronate
ALP	Alkaline phosphatase
APC	allophycocyanin
Apppl	triphosphoric acid 1-adenosine-5'-yl ester 3-(3-methyl-but-3-enyl) ester
APS	Ammonium-per-sulphate
ARF	Alternate-reading frame
ASC	Adult stem cells
ATM	Ataxia-telangiectasia mutated
ATM	ataxia-telangiectasia mutated
ATP	adenosine triphosphate
ATR	Ataxia-telangiectasia Rad3 related
ATR	ataxia-telangiectasia Rad3 related
B cells	B cells are called as B lymphocytes and are produced in the bone marrow
Bak	Bcl-2 antagonist killer
BAX	Bcl2 associated X
BBC3	Bcl-2-binding component3
BCA	Bicinchonic acid
BER	Base excision repair
BER	base excision repair
BH2	Bcl-2 homology domain
BM	Bone marrow
BMP-2	Bone morphogenetic protein-2
bp	Base pair
BP	bisphosphonates
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride

cAMP/PKA	Cyclic adenosine monophosphate /protein kinase A
Cbfa1	Core binding factor alpha-1
CC	comet category
CD	Cluster differentiation
CDKI	Cyclin-dependent kinase inhibitor
cDNA	Complementary Deoxy-ribonucleic acid
CFU	Colony Forming Unit
CFU-A	Colony forming unit adipocyte
CFU-F	Colony forming unit fibroblast
CFU-O	Colony forming unit osteoblast
cm^2	Centimetre square
CO2	Carbon dioxide
COSHH	Control of Substances Hazardous to Health
CT	Cycle Threshold
Cu-Zn	Copper-Zinc
CXCR4	C-X-C chemokine receptor type-4
D7-FIB	Fibroblast/epithelial cell marker
DAPI	4', 6-diamidino-2-phenylindole
Dcx+	Doublecortin
DDR	DNA-damage response
DDT	Dichloro-diphenyl-trichlo-roethane
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulphoxide
DNA	Deoxy-ribonucleic acid
DNA-PK	DNA-damage sensing protein kinases
DNA-PKs	DNA-damage sensing protein kinase
dNTP	deoxynucleotide triphosphates
DSB/dsb	Double strand break
E	efficiency
E-Cadherin	Epithelial cadherin
ECL	Electro-chemilluminescence
ECM	Extra-cellular matrix
EDTA	Ethylene diamine tetra acetic acid
EDTA	ethylene diamine tetra-acetic acid
eGFP	Enhanced green fluroscent protein
ERK	Extra-cellular signal related kinase
ESC	Embryonic stem cell
ESTR	expanded simple tandem repeats
EtOH	Ethanol
FACS	Fluorescent activated cell sorting
FBS	Foetal bovine serum

FCS	Fetal Calf serum
FGF	fibroblast growth factor
FISH	Fluorescence in situ hybridisation
FITC	Fluorescein-isothiocyanate conjugated
FOH	farnesol
FOXO	forkhead box O
FPP	farnesyl pyrophosphate
FPPS	farnesyl pyrophosphate synthase
FSC	Forward scatter
g	Gravitational acceleration
G0	Gap 0 checkpoint of cell cycle where the cell is in resting phase and has stopped cell division
G1	Gap 1 checkpoint of cell cycle to ensure all parameters is ready for DNA synthesis
G2	Gap 2 checkpoint of cell cycle to ensure cell enters the mitosis phase and divides
GAG	Glycosaminoglycans
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green Fluorescent protein
GFP+	Green Fluorescent protein positive
GGOH	geranylgeraniol
GGPP	geranyl-geranyl pyrophosphate
GSK-3	Glycogen synthase kinase
Gy	Gray (unit of radiation)
H2O2	Hydrogen peroxide
HA/TCP	Hydroxyapatite/tricalcium phosphate
HC	Hydrophobic core region
HCl	Hydrogen chloride
HDM2	E3 ubiquitin protein ligase (MDM2 in mice)
HEK	Human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hESC	Human ESC
HGPS	Hutchinson-Gilford progeria syndrome
HIV-1	Human immunodeficiency virus
HLA	Human leukocyte antigen
HLA-DR	Human leukocyte antigen-D related that are polymorphic glycoproteins on lymphoid cells
hMSC	Human mesenchymal stem cells
Hpf	hour post fertilization
hr	Hours
HR	homologous recombination

HRP	Horse radish peroxidase
HS	Heparin sulphate
HSC	Haematopoietic stem cell
Hsp	Heat shock protein
HSPC	Haematopoietic stem and progenitor cell
hTERT	Human telomerase reverse transcriptase
i.p.	intraperitoneal
i.v.	intravenous
IBMX	3-isobutyl-1-methylxanthine
IDO	Indoleamine 2,3-deoxygenase
iDTR	inducible diphtheria toxin receptor
IFN-γ	Interferon-γ
IgG1	Immunoglobulin G1
IL	Interleukin
iNOS	Inducible nitric oxide synthase
iPSC	Induced pluripotent stem cells
IR	Ionising radiation
IR	Irradiation
ISC	Intestinal stem cells
K	Kruppel-like factor (Klf-4)
kDa	Kilo Dalton
Klf-4	Kruppel-like factor
KO	Knock out
LGR5	Leucine-rich G protein coupled receptor 5
LIF	Leukaemia inhibitory factor
Lig4	ligase 4
Lin	Lineage
LM	Laminin
LNGFR	Low affinity growth factor receptor
Log	Logarithm
LPL	Lipoprotein lipase
LT	Long term
LT-HSC	Long term-HSC
M	Molar
mAB	Monoclonal antibody
MAPK	Mitogen activated protein kinase
MCAM	Melanoma associated adhesion molecule
M-CSF	Macrophage colony stimulating factor
M-CSF	macrophage colony stimulating factor
MHC	Major Histocompatibility complex
miR	Micro-RNA
ml	Millilitre

mm	Millimetre
mM	Millimolar
MMP	Matrix metalloproteinase
MMR	Mismatch repair
MMR	mismatch repair
mMSC-CM	Murine-mesenchymal stem cell complete medium
MNC	Mononuclear cells
mRNA	Messenger RNA
MSC	Mesenchymal stem cell
MTOR	Mammalian target of rapamycin
mTRF	Mean telomere restriction fragment
N	NANOG
Na2PO4	Sodium phosphate
NaCl	Sodium chloride
N-BP	nitrogen containing bisphosphonate
N-cadherin	Neural cadherin
NER	Nucleotide excision repair
NER	nucleotide excision repair
ng	Nanogram
ng	Nanogram
NHEJ	Non- homologous end joining
NHEJ	non homologous end joining
nM	Nano molar
NMR	Nuclear magnetic resonance
nNOS	Neuronal nitric oxide synthase
NOD/SCID	Non-obese diabetic/ severe combined immunodeficiency
NSC	Neuronal stem cell
OB	Osteoblast
^{o}C	Degree Celsius
OC	osteocalcin
OH	hydroxyl group
OI	Osteogenesis Imperfecta
Oligo2+	Oligodendrocyte populations in the corpus callosum
ONFH	Osteo necrosis of femoral head
OPN	osteopontin
OSX	osterix
p	Passage number
p10	Late passage
p3	Early passage

p53/p16/p21	Phosphoprotein 53/16 are involved in tumor suppressor pathway
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline-Tween 20
PCR	Polymerase chain reaction
PCR	polymerase chain reaction
PD	Population doubling
PD	progressive disease
PD/t	Population doubling time
PDGFβ	
PE	phycoerythrin
PEG2	Prostaglandin E2
PFA	paraformaldehyde
PFS	progression free survival
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PMSF	phenylmethanesulfonylfluoride
PPARγ	Peroxisome proliferator-activated receptor gamma
Ppi	inorganic pyrophosphate
pRB	Phospho-retinoblastoma protein
PTHrP	Parathyroid hormone related protein
PUMA	p53 upregulated modulator of apoptosis
rhBMP-2	recombinant bone morphogenetic protein
Ris	Risedronate
RNA	Ribonucleic acid
ROS	Reactive Oxygen species
rpm	Rotations per minute
RPMI	Roswell Park Memorial Institute
RT	reverse transcriptase
RT-PCR	Reverse transcriptase-PCR
RT-qPCR	Reverse transcriptase-quantitative PCR
S	Synthesis phase of cell cycle where DNA replication occurs
SAHF	Senescence associated heterochromatin foci
SASP	Senescence associated secretory phenotype
SDF	Senescence associated DNA-damage foci
SDF-1	Stromal-derived factor 1
SDS	Sodium dodecyel sulphate
SDS-PAGE	Sodium dodecyel sulphate-poly acrylamide gel electrophoresis
Ser	serine
SH2	Src homology 2

siRNA	small interfering RNA
SKY	Spectral karyotyping
SMA	Smooth muscle active
SMAD-8	Small mother against Decapentaplegic transcription factor-8
SMR	skeletal morbidity rates
SOD	Superoxide dismutase
Sox2+	Early neural progenitor cells
SRE	skeletal related events
ss	single stranded
SSC	Side scatter
STAT1	signal transducer and activator of transcription 1
T cells	T cells are called T lymphocytes. The B cells leave BM and mature in thymus
TBBMC	Total body bone mineral content
TDD	Trichothiodystrophy
TEMED	(N,N,N,N)-tetramethly-ethlyenediamine
Terc	Telomerase Ribonucleic acid
Tert	Telomerase reverse transcriptase
Tert-ER	Telomerase reverse transcriptase-oestrogen receptor
TGF-β	Transforming growth factor-β
TH	Tissue homeostasis
Thr	threonine
TNFα	Tumour necrosis factor alpha
TORC1	TOR complex 1
TORC2	TOR complex 2
TR-/-	Telomerase knock-out
UD	Undifferentiated control
UI	Un-irradiated
UT	Untreated
UV	Ultra-violet
V	voltage
-ve/-	Negative
WT	Wild type
Z	Zoledronate
Zol	Zoledronate
α	Alpha
α4β1 integrin	Alpha-4 Beta-1 integrin
B	Beta
γ	Gamma

γH2AX	Gamma-variant of histone 2A protein family
µCT	Micro-computerised tomography
µg	Microgram
µl	Micro litre
µM	Micro molar