UNIVERSITAT ROVIRA i VIRGILI

Acute radiation effects in bone vasculature

Treball de final de grau

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Abstract

Research into the bone vascular system has been a scientific field in which interest has increased greatly in recent years. The complexity of the blood vessels together with the endothelium creates a microenvironment in the bone marrow that affects the health of the bones. In recent years, two types of blood vessels have been discovered that coexist in the bone marrow: L and H type vessels. Angiogenesis and vasculogenesis play a key role since these vessels have their own characteristics and their distribution is a determining factor in the maintenance of the bone marrow's homeostasis. On the other hand, radiotherapy is a common treatment for people with cancer and it is highly effective but leads to various side effects such as hypoxia and cellular stress. The aim of this thesis is to study the effect of acute radiotherapy treatment on the bone marrow. To determine this effect, we used mice genetically modified mice that have been exposed to radiotherapy treatment. They have been used to obtain the bone and various staining tests have been carried out to compare untreated mice with treated mice. The results show a marked loss of bone marrow stability in the treated mice; there is an increase in type H blood vessels (inflammation and loss of homeostasis). There is a decrease in the synthesis of collagen and an increase in the number of adipocytes in the medulla of the samples that received radiation. This study concludes that radiation has a direct effect on the bone marrow, disrupts homeostasis, and creates a stressful situation that involves an increase in the vascularisation of type H vessels. Furthermore, osteogenesis was altered, affecting the density of the bones and a displacement of microenvironment cells by adipogenic cells. This study, therefore, determines a new secondary effect of treatment with radiation and therapeutic target.

1. Introduction

1.1 Blood vessels and endothelium

The delivery of the nutrients, immune cells, blood and molecules to all our body is done by blood vessels. To provide the supplement, soluble factors and oxygen to all the organs and tissues, blood vessels are organized in hierarchical fashion, precise and regulated way. This is a part of a complex organization which involves mural cells, matrix and the connection between vascular and nervous system. [1–3]. Blood vessels are composed of a diversity of cells; the inner layer is composed of endothelial cells, which form the endothelium and also covered by mural (perivascular) cells. These are subject to classification depending on the markers they express and their morphology; they can be either pericytes or vascular smooth muscle cells. Pericytes are found in the basement membrane and have cell-cell contact with endothelial cell capillaries, this are related to the Mesenchymal stem cells (MSCs) and although few experimental tests exist, it is believed that these have the ability to differentiate into different cell types [4] . Vascular smooth muscle cells, on the other hand, are found around major blood vessels such as arteries and veins but without contact with endothelial cells.[5]

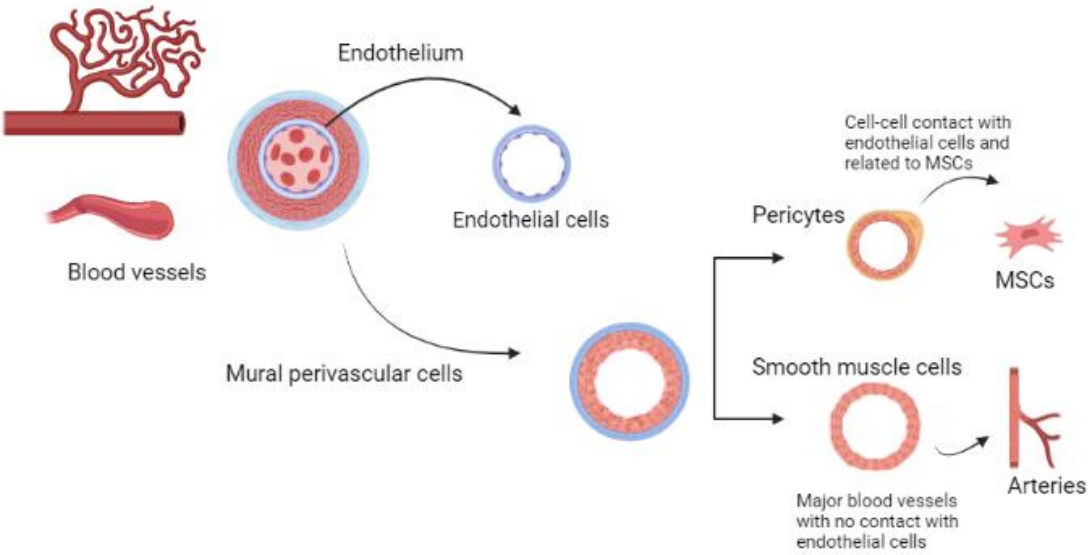

Figure 1: Blood vessels cells

The vascular network is formed by two processes: vasculogenesis and angiogenesis ; these two processes are crucial. [1,2,6,7] Vasculogenesis takes place in an early stage of embryogenesis in which mesodermal cells differentiate into haemangioblasts, that are precursors of endothelial cells and blood cells which are specifically positioned to form vessels. On the other hand, angiogenesis, is a process that usually occurs in new blood vessels. The process consists of an expansion of existing vessels that involves the various types of vascular cells to ensure that they remain functional. Vascular remodelling, stabilisation and maturation are all part of the angiogenesis process and the cells involved in this process are the smooth muscle cells and pericytes.[8]

The endothelium is one of the most important components of the vasculature. For a long time the endothelium was considered as a cellophane envelope of the vascular system with the only function of acting as a specific barrier for water and electrolytes. Later it was discovered that it played a much more important role. Endothelial cells have a key role in the entire circulatory system, ranging from the central organ (the heart) to the smallest and most remote capillary. [9] The endothelium enables the metabolic exchange between blood and tissues because of its permeability and proximity to all cells, which contributes to the endothelial cell communication with the adjacent cells. [3] The functions in which it participates are crucial to vascular biology; many examples include: fluid filtration, maintenance of vessel structure, haemostasis (a process that occurs during haemorrhage to prevent blood from leaking out of vessels), neutrophil selection and hormone regulation. The endothelium, through these actions, maintains homeostatic functions.[10]The signals that endothelium provides affect to organs cell differentiation. The communication that endothelium provides is very rich and it is because of the heterogeneity of the endothelial cells and their vessel size-specific, tissue-specific and age-specific differences. This communication between the endothelium and the tissue or surrounding cells is suggested by the variety of humoral growth factors, cytokines and cells surface molecules. [3]. Focusing on vascularity, endothelial cells are actively involved in regulating blood flow. This regulation occurs when, for example, in a resting state, the cells act to prevent

thrombus formation and facilitate the transit of plasma throughout the body. In certain situations, however, the homeostasis achieved can be disrupted by inflammation, which promotes the development of prothrombotic microenvironments. Alternatively, it can regulate by the secretion or uptake of vasodilator or vasoconstrictor molecules that have a paracrine action in response to a stimulus; an example would be in the presence of endotoxin. Endotoxin is also known as lipopolysaccharide, it is a toxin found in the outer membrane of gram-negative bacteria, which, among other responses, stimulates adhesion molecules in the endothelium.[10]

The creation of new blood vessels requires the co-operation and co-ordination of the endothelium with the rest of the vascular cells. Signalling between cells will allow complex changes to take place to enable vascularisation. Vascular endothelial growth factor (VEGF) is a key signalling molecule involved in these processes, as it is specific to the endothelium for the creation of new vessels. VEGF is critical for vascularisation in both angiogenesis and vasculogenesis. VEGF acts with Angiopoietin1 and Ephrin2 to promote the remodelling and maturation of immature vessels that subsequently require smooth muscle cells and pericytes.[11]

The vasculature has a key role in the skeletal growth, development, bone modelling and remodelling processes. However, this relationship between the vasculature and bone is still not entirely clear. In recent years, new hypotheses and publications have emerged that propose different vessels that were not known yet. The vasculature must maintain homeostasis. The biochemical interactions between vasculature and bone cells may be altered causing different pathologies like a defect or an excess of bone vasculature or endothelial metabolism; these can happen because of trauma, or metabolic and genetic diseases. The fact that some bones have a terminal vascularization give them a high risk and this alteration can cause them dramatic effects like decreasing bone formation .[3]

1.2 Bone: vasculature and inflammation

1.2.1 Bone, bonemarrow and vasculature

Bones are a fundamental part of the human body. They are usually considered to have little vasculature and have been studied less; recently research about this topic has grown and great discoveries have been made. To study vascularisation properly, one must look at the bone marrow. The bone marrow has bone marrow stem cells that are self-renewing and they can differentiate into various cell types. One of this cell types is haematopoietic stem cells. Haematopoietic stem cells reside in a niche in the bone marrow which has a specific microenvironment. Haematopoietic stem cells can have myeloid and lymphoid progenitor cells to maintain haematopoiesis. These cells in a homeostatic situation remain stationary as the niche sends signals to maintain it.[12] The niche, specifically the microenvironment, is maintained by endothelial cells together with perivascular cells that send paracrine signals (stem cell factor and CXC12 chemokines) that maintain homeostasis. [12,13]

The endothelium again plays a crucial role modulating bone marrow functions. Bone marrow endothelial cells are involved in osteogenesis (bone synthesis) and angiogenesis; they are also involved in the regulation of the relationship between the lumen of the blood vessels and the bone marrow. Endothelial cells can express heterogeneous proteins.[14] The blood vessels of the bone marrow are mostly sinusoidal capillaries, also called L-type vessels, which express low levels of endomucin and CD31.[15] L-type vessels are characterised by branching and are mainly found in the diaphyseal bone marrow. On the other hand, we have the H-type endothelium, a novel type that was recently discovered. H-type vessels show elevated expression of endomucin and CD31 and are usually found near the growth plate. H-type vessels form tubules at the distal end..[15] They are associated with osteoprogenitors and are organised in a columnar fashion; they can be associated to malfunction . They are connected to arterioles so that they are more oxygenated than L-type arterioles. They are also less permeable than the L-type, so that the microenvironment is lower in ROS levels.[16] The functional and shape differences of the vessels create bone marrow niches

with different functions that regulate the differentiation of osteoprogenitors and blood cells, due to oxygen tension gradients.

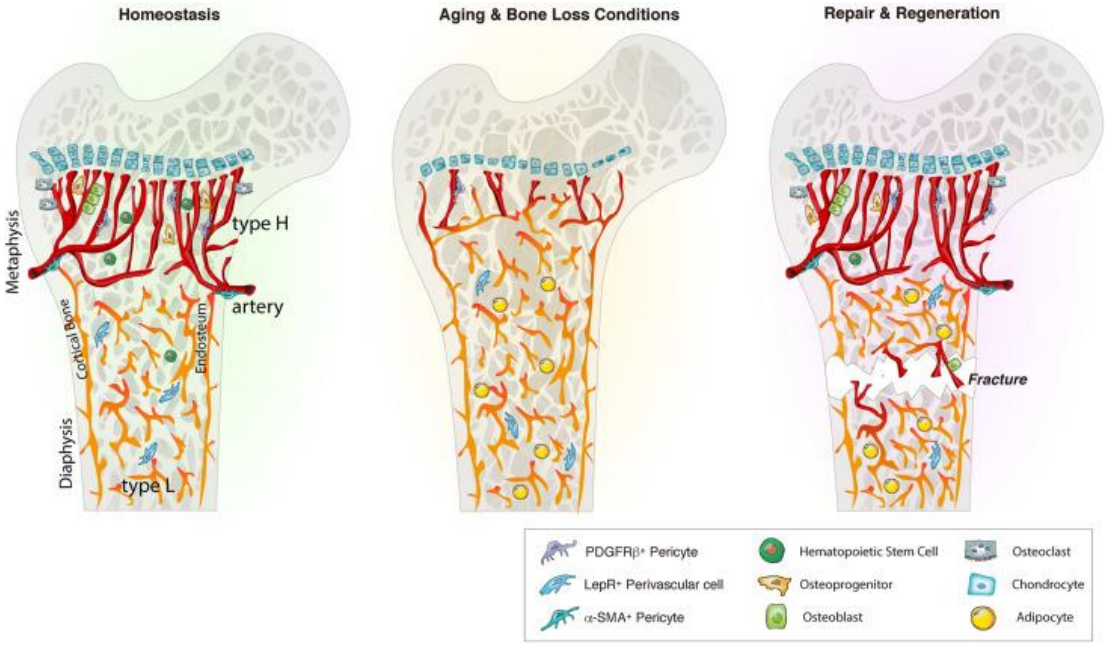

***Figure 2**: Blood vessels in bone marrow, organitzation and niche microenivroment in three situations: homeostasis, aging and regeneration.Adapted from Stucker, et at(2020)* [16]

Figure 2 shows three different bone marrow situations: homeostasis, aging and regeneration. When the situation is homeostasis a large number of H-type vessels in the metaphyseal zone are present. The endothelium of the H-type vessels is associated with the stimulation of angiogenesis and osteogenesis. When the signs of ageing begin to appear, they lead to a loss of H-type vessel density and thus to a loss of bone mass. Lastly, in the case of fracture, H-type vessels appear in the distal zone, as an increase in osteogenesis is necessary at that time.

The survival, stability and function of endothelial cells depend on perivascular cells; perivascular cells play an important role in angiogenesis and tissue repair. Perivascular cells are very heterogeneous in a way that they form distinctive associations with bone marrow vessels, thus contributing to specialised niches. H-type vessels are usually found near arterioles, with pericytes expressing PDGFR (B) and NG2 are found. Moreover, type H vessels are associated with glioma-associated oncogene homolog 1 (Gli1) expressing mesenchymal stem cells.(MSCs)[17]. MSCs are multipotent and possess the ability to differentiate into multiple lineages, including osteoblasts

chondrocytes and adipocytes. On the other hand, L-type vessels are enveloped by perivascular LepR+ cells and reticular cells (CAR) which are abundant in CXCL12.[17,18]

1.2.2 Inflammation in bone marrow

When inflammation occurs (infection, bone injury or radiation) it can change the morphology and function of the niche. It has been shown that the inflammatory situation eliminates and inhibits osteoblasts and thus bone formation (decrease of the collagen).[19,20] Many studies have shown that acute inflammation leads to a proliferation of haematopoietic stem cells. Inflammatory cytokines induce the proliferation, differentiation and mobilisation of haematopoietic stem cells in order to adapt to the inflammatory response as the immune cells that act during the response must be restored.[21,20]

A new challenge in inflammation has been a treatment with INF-α which stimulates endothelial proliferation and increases vascularisation of the bone marrow niche. On the other hand, a treatment with lipopolysaccharide (LPS) resulted in an increase in lumen dilatation due to induced stress. Other studies have shown that inflammation increases vascular permeability and filtration by opening gap junctions. Gap junctions open due to trans-endothelial migration of immune cells.[22] Many models have been studied and are advancing in resolution and approach to study the effects of inflammation on the molecular components of the bone marrow niche; although progress has been remarkable, the function of the bone marrow niche and the change brought about by inflammation has yet to be fully described.[12,22,23]

1.3 Radiation

Cancer is a disease that constantly challenges science and involves major alterations. Many advances have been made in the treatment of this disease mainly: surgery radiotherapy, chemotherapy and immunotherapy... One of the most common treatments for this pathology is radiotherapy and this can cause significant damage due to the inflammatory response and the effect it has on the bone marrow. Radiation treatment is given in moderate doses but still

causes cellular stress, cell death, hypoxia, DNA damage and may even lead to bone marrow failure. Some research concludes that therapeutic radiation leads to cell loss in the bone marrow. [24,25] Although it is known the effects of this treatment on the tumour have been widely evaluated, it's effect on the bone marrow and its microenvironment are still under study.[24,26]

The study of radiation and inflammatory changes affecting the bone marrow niche may be a key to understanding mechanisms that are not yet fully understood. Moreover, progress in this direction may lead to improved radiation efficiency and increasing survival.[26,25] If we focus on the effects it has on the microenvironment, we can use targeted therapies to avoid these effects.

This thesis continues the study of the effects of radiation [23]; studies the integrity of the endothelial cells when they are treated with radiation and focus on the bone and bone marrow niche. The hypothesis proposed in this thesis is that radiation (in this case single dose) causes inflammation and inflammation leads to a loss blood vessels homeostasis in the bone marrow. The loss of the homeostasis would be responsible for the loss of bone density , secondary effects of the radiation treatment for cancer and disruption of bone marrow niche.

This study aims to compare and visualise the effects of radiation on the bone marrow; focusing on the blood vessels and cells that create the bone marrow niche and osteogenesis. In order to study this we will use immunohistochemical staining and high-resolution imaging.

2 Material and Methods

2.1 Experimental animal procedures

All experiments involving animals were approved by the local University of Oxford animal ethics committee and the UK Government Home Office and were performed in accordance with institutional laws and guidelines. This thesis has been made based on the results obtained in External practices at Anjali Kusumbe group of the University of Oxford under the supervision of Anjali Kusumbe.

All mice used in this study (Gli1-CreERT2 (JAX #007913), Pdgfrb-CreERT2 (JAX #030201) and R26tdTomato mice (JAX #007909)) were purchased from the Jackson Laboratory. For genetic lineage tracing of perivascular MSCs, tamoxifen-inducible Gli1-CreERT2 mice were crossed to a tdTomato reporter strain to generate Gli1-CreERT2; R26tdTomato mice. Genotyping of all animals was done by PCR. At 6 weeks of age, Gli1-CreERT2; R26tdTomato mice were intraperitoneally injected with tamoxifen (Sigma) on three consecutive days to induce Cre recombinase activity and genetic labelling. For genetic lineage tracing of perivascular Pdgfrb+ cells, Pdgfrb-CreERT2; R26tdTomato mice were generated by crossing Pdgfrb-CreERT2 mice to R26tdTomato mice. Genetic labelling was induced as previously described

for Gli1-CreERT2;R26tdTomato mice. Mice were sacrificed and perfused with 2% paraformaldehyde solution. After perfusion and fixation, bones were collected for histological preparation and analysis.

2.2 Radiation.

Mice were treated with a single dose of 900 rad whole-body irradiation at 7 weeks of age and sacrificed 7 days post-irradiation treatment.

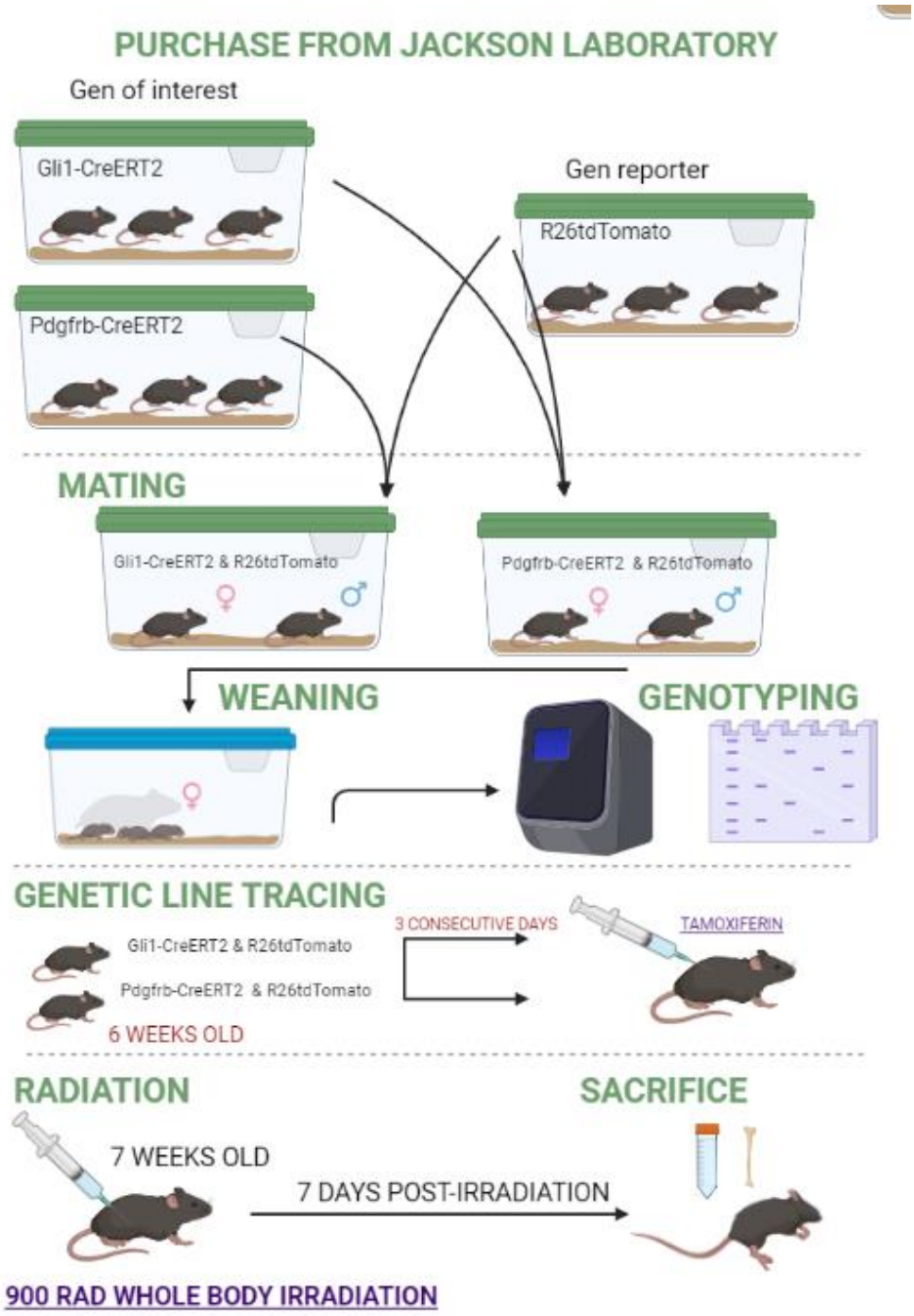

Figure 3: *Mice treatment*

2.3 Bone decalcification and sectioning.

Bone decalcification and sectioning was performed as previously described by Singh *et al* [27]. Briefly, freshly harvested bones were fixed in ice-cold 4% paraformaldehyde solution for 4hours. Bones were decalcified by incubation with 0.5 M EDTA at 4°C on a shaker. Decalcified bones were cryoprotected via incubation in 20% sucrose (Sigma-Aldrich, S9378) and 2% polyvinylpyrrolidone (PVP) (Sigma-Aldrich, PVP360) solution at 4°C overnight. The next day, bones were embedded and frozen in 8% gelatin (Sigma-Aldrich, G2625) embedding medium supplemented with 20% sucrose and 2% PVP. Frozen bones were cut into sections of 100µm using a Leica CM3050 cryostat (low-profile blades, Leica, 14035838382) and were directly transferred onto microscope slides. After air-drying for 3 hours, sections were frozen and stored at -20°C until further use.

Bone immunohistochemistry. Immunostainings were performed following the protocol by Singh *et al* [27]. Bone sections were thawed at room temperature and washed with 150µl PBS per section. Sections were permeabilized in 0.3% Triton X-100 diluted in PBS for 10 minutes and blocked in blocking buffer (5% donkey serum in PBS) at room temperature for 5 minutes. Sections were incubated with primary antibodies diluted in blocking buffer (1:150) for 4 hours at room temperature or overnight at 4°C. The following primary antibodies were used (dilution 1:150): Endomucin (sc-65495, Santa Cruz), PDGFRβ (ab32570, Abcam), collagen (AB765P, Millipore), perilipin antibody. Sections were washed in PBS for seven times and incubated with Alexa Fluor-conjugated secondary antibodies diluted in blocking buffer (1:300) at RT in the dark for 3.5 hours. All secondary antibodies (dilution 1:300) were purchased from Thermo Fisher Scientific (donkey anti-goat IgG Alexa Fluor 488 (A11055), donkey anti-goat IgG Alexa Fluor 647 (A21447), donkey anti-rabbit IgG Alexa Fluor 488 (A21206), donkey anti-Rat IgG Alexa Fluor 594 (A21209), donkey anti-rabbit Alexa Fluor 546 (A10040), goat anti-rabbit Alexa Fluor 546 (A11035), donkey anti-goat Alexa Fluor 546 (A11056)). Nuclei were counterstained with DAPI or TO-PRO-3. After secondary antibodies incubation, sections were washed with PBS for seven times and mounted with coverslips using Fluoromount-G mounting medium

(Invitrogen, 00-4958-02). Stained sections were stored at 4°C until imaging. All sections were imaged within 14 days of staining.

2.4 Image acquisition. Z-stacks

(Frame size: 1024 x 1024 pixels; pixel size: 0.69 µm) of immunofluorescence stainings were acquired on a Zeiss laser scanning confocal microscope (Zeiss, LSM-880, Germany) with 7 lasers (405, 453, 488, 514, 561, 594, and 633nm). Whole sections were imaged with 10x Plan Apo 0.45 WD=2.0 M27 and 20x Plan Apo/0.8 dry lenses using the tile scan function with the appropriate number of tile scans depending on the size of the region of interest. Images were stitched with 10% overlap in Zen Black software (version 3.1, Zeiss) and saved as czi-files. Acquired z-stacks were imported into Zen Blue software (version 3.1, Zeiss) for 3D reconstruction, processing, and analysis. Adobe Photoshop, Adobe Illustrator and Microsoft Powerpoint were used for image processing and figure compilation.

3 Results

As we aimed to evaluate the effect of radiation on bone marrow niche cells, it was treated Gli1-CreERT2;R26tdTomato mice and Pdgfrb-CreERT2;R26tdTomato mice with tamoxiferin. Tamoxiferin injection was done to get genetic labelling of gli1+ MSC and Pdgfrb+ perivascular cells when the mice were 6 weeks old. The next week (week 7) they were exposed to radiation treatment and sacrificed the following week (week 8). From these mice, both control and irradiated tibia were obtained and stained for tomato, endomucin and the other vascular and perivascular markers. After the staining we got the following images:

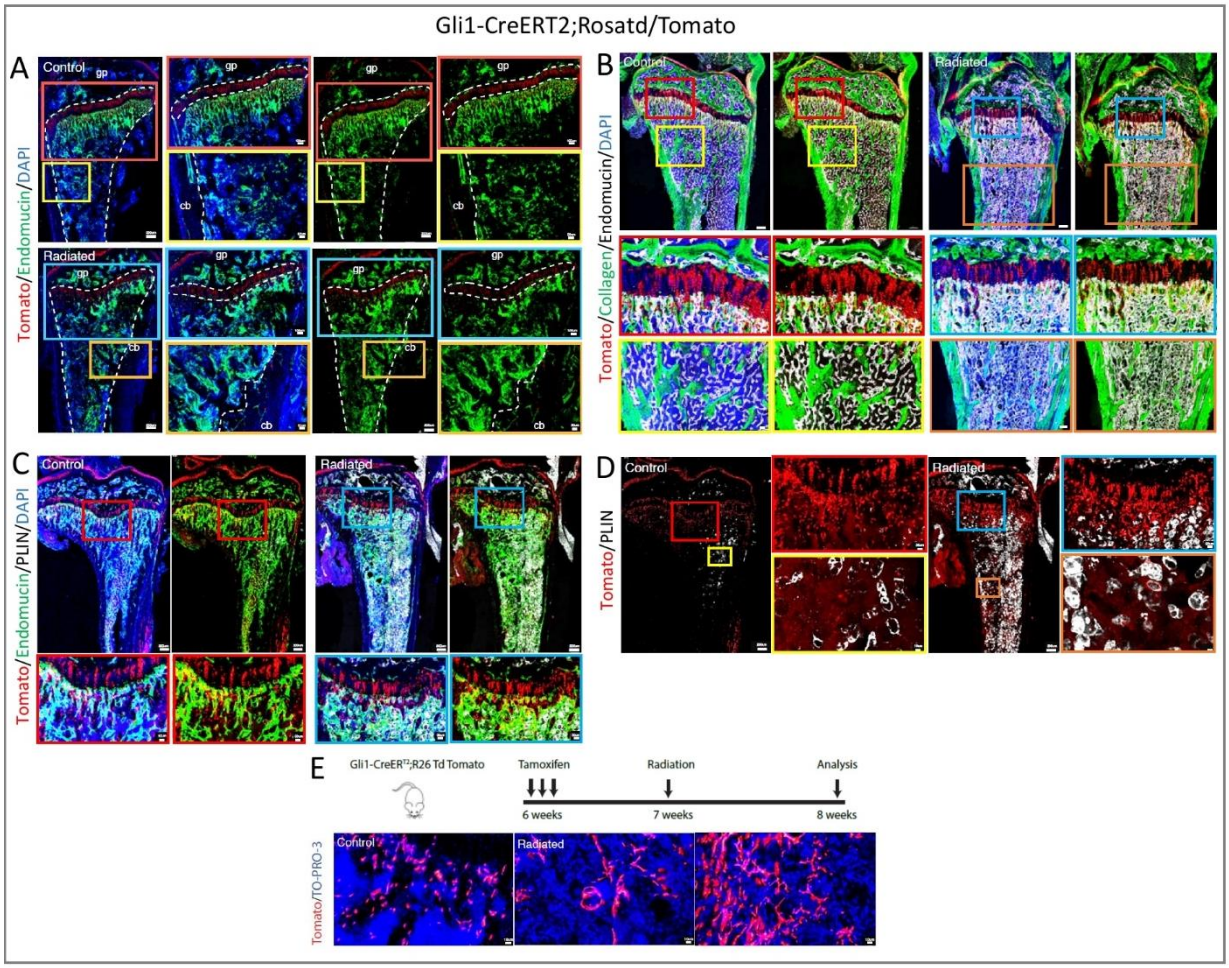

Figure 4 : Tibial expression of the diferents vascular and perivascular markers in the mice control and irradiated. A. Tomato, Endomucin and DAPI immunostaining in control and irradiated tibia. B. Images of Tomato, Collagen, Endomucin and To-PRO-3 expression in control and irradiated tibial. C. Tomato, Endomucin, perilipin and DAPI expression in control and irradiated tibia. D. Tomato and perilipin in control and irradiated tibia. E (above) illustration of the procedure/timeline; (Low) tamoxiferin treatment and tomato expression in control and irradiated tibia.

Figures 1A-C and 2A-B show the effects caused by inflammation due to radiotherapy treatment on the rat's body. One of the effects that can be seen is an increase in the expression of endomucin in the long part of the bone, the metaphyseal zone. An increase of endomucin in the irradiated bone indicates an increase in H-type vessels. Thus can be seen that the irradiated bone shows an alteration of the vessels and vascularisation compared to the control. Apart from that, figures 1A-C and 2A-B the loss of homeostasis in the radiated bones, from a hierarchical fashion structure in the controls respect the radiated samples.

However, focusing on the Pdgfrβ+ staining, figure 2A, which shows the irradiated samples compared to the control, there is a strong increase in the Pdgfrβ+ expression in the samples that have been irradiated. This increase in

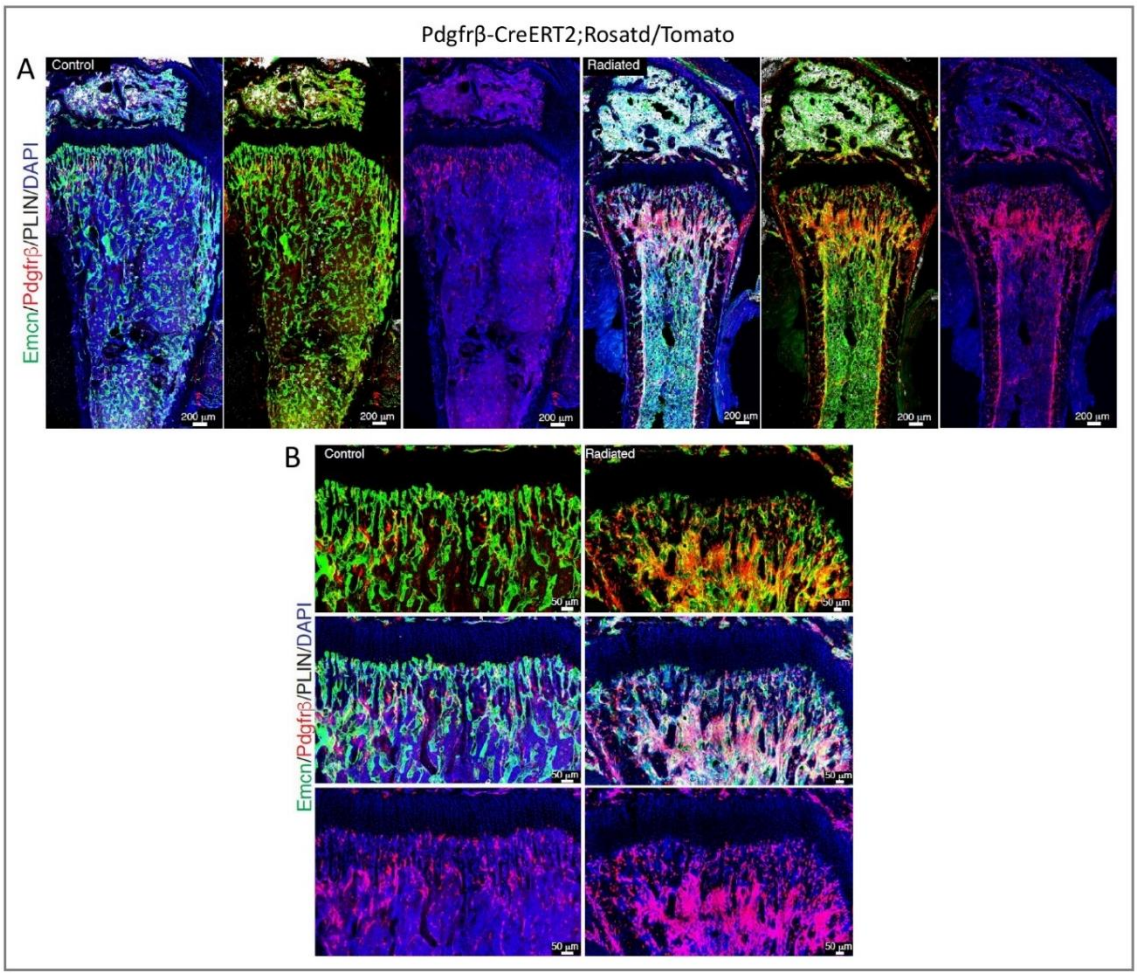

Figure 5: Tibial expression of various vascular and perivascular markers in control and irradiated. A. Endomucin, Pdgfrβ, perilipin and DAPI immunostaining in control and irradiated tibia. B. Magnified images of tile scans in A.

expression is found in the diaphyseal zone of the bone, as can be seen in figure 2B, which informs us of the increase in the H type associated with the Pdgfrβ+ pericytes in response to irradiation.

Focusing on Figure 1B the expression of collagen between the control and the irradiated sample it can be seen that the collagen expression was slightly decreased in irradiated tibia, particularly in the bone marrow, suggesting that a radiation-induced degradation of collagen.

On the other hand, the expression of perilipin is strongly increased in the bone marrow of irradiated bears, as can be seen in figure 1C-D and 2B. Perilipin is a protein associated with the lipid membrane. This confirms the accumulation

of adipocytes in response to radiation treatment. If you look at figure 1E you can see that the expression of the Tomato stain increases, in this case but with the Gli1-CreERT2;R26tdTomato mice, which showed a proliferation of perivascular MSCs.

4 Discussion

The purpose of this research was based on the premise that radiation would have an impact on the mouse's bone marrow and focus on the tibia. With this in mind, the genetic lineage is used, immunohistochemistry stains and a high-generation image to be able to observe the differences it shows due to treatment. Previous studies[23] shows the integrity of the endothelial cells when they are treated with radiation; An haemorrhagic phenotype appeared but did not get a big picture of angiogenesis and bone marrow. This loss of integrity and perturbation has a reconstitution mediated by VEGF2 signaling to achieve normal hematopoiesis by giving a key role for radiation and chemotherapy.. In our research we focus on the angiogenesis and bone marrow microenviroment. In our results we can see a clear increase in endomucin in the bone marrow of the irradiated sample; this shows an increase in the vascularization of H-type vessels above all in the growth plate, proving that there is a loss of vascularization due to radiation. This increase and expansion of H-type vessels is usually given as a response to malformations and bone defects or in case of various types of fractures or bone damage; this is how the negative effect of radioactive treatment of the bone marrow is demonstrated.

Our results suggest that there is a response to radiation to specific bone tissue and it's shown by increase in PDGFRβ. This increase in the expression of PDGFRβ reflects an expansion of pericytes; pericytes are a new line of research that is quite unknown, but in the future experimental evidence will be done to determine the relationship with MSCs and the differentiation capacity of different cellular types. The increase in both the H-type vessels and the expression PDGFRβ and suggest an interrelation between the

formation of H-type regenerative blood vessels and the expansion of the pericites by the secretion of PDGFRβ.

The increase in the vasculature and proliferation of radiation-response vessels are a pharmacological challenge because it is a therapeutic target that has taken very little account historically but in recent years has shown many advances. This increase in the vasculature of H-type vessels in an inflammatory situation is accompanied by a better permeability, allowing the transendothelial migration to the bone marrow, which is much higher to be able to maintain homeostasis and may be a line of research to be able to reduce the effects of radiation in the future.

Another result is the decrease in the expression of collagen in the bone, while on the other hand, there is a drastic increase in the expression of perilipin which shows an increase in adipogenesis in irradiated bone: the treatment of the radiation results in an increase in adipocytes in the bone marrow. Breaking of homeostasis and disruption of the bone marrow leads to the creation of hollow which is occupied by adipocyte. These results allow for new hypothesis of the breakdown of osteogenic and adipogenic differentiation, thus causing the mass to be lost from the bone and the medullae to increase with the number of adipocytes. Radiation further degrades the structure of collagen by causing loss of the mechanical strength and may increase the risk of fracture. Our results indicate that perilipin and tomato staining do not show superposition, thus confirming that the increased adipogenesis of the bone marrow does not come from the MSCs Gli1+. This result suggests us that there is a high heterogeneity between MSCs and that there are many sub-populations that have not yet been characterised. The MSCs within a single tissue, in this case the bone marrow, may have different molecular identification, function, and response to inflammation, playing a key and still an unknown role in adipogenesi in irradiated bone.

Therefore, this study concludes by determining what the effects of inflammation caused by irradiation in the bone medulla niche caused only by acute radiation treatment. The results obtained are immediately as they are only a few weeks after treatment but they give us an image of what the effect is. An increase in angiogenesis and the vascularization of H-type vessels is determined as an immediate response with an increase in the expansion of

pericytes (PDGFRB+), resulting in disruption and loss of the niche homeostasis of the bone marrow. On the other hand, disruption allows for the enhanced of adipogenic cells that increase the occupied site to bone marrow. In the last place there is a disruption in osteogenesis along with the degradation of the bone (collagen). This research, however, does not solve the effects that it would cause to maintain sustained or repeated inflammatory treatments for microenvironment; the Kusumbe laboratory will continue to investigate the effects of the sustained inflammatory effects treatment and in comparison of the various vascular and perivascular markers in response to both treatments. Finally, the effect of high irradiation in the long term will be studied in the next studies, as we see momentary disruption, but it is unclear whether the bone marrow returns completely to the pre-inflammatory homeostasis.

The research that has been done nowadays has allowed for an improvement in protocols that have made it easier to obtain a better image and with greater resolution; the research group Kusumbe has made optimized protocols that have brought great advances in research. This study and the subsequent ones will allow the development of specific therapies in the bone marrow to improve efficiency, reduce side effects and increase the patient's survival; the development of new treatments, specific drugs, and the ability to return or not to the homeostasis in the bone marrow after radiation are new study lines that will be studied in the next years and will play a critical point.

5 Acknowledgments

I would like to thank all those who have supported me throughout my curricular and extracurricular practices in a both professional and personal manner. First and foremost, I would like to thank Dr. Kusumbe for her continuous support and supervision throughout this experience and for the opportunity to study such a fascinating area or research. I would also like to thank Dr. Lincoln Biswas and Dr. Unnikrishnan Sivan for their support and guidance throughout all the project, for their patience and for his words of encouragement and acting as my secondary supervisors. I am also grateful to Zhi Yi Wong for his support and training from the start making my experience so positive.

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Lastly, I would like to thank Kennedy Institute of Rheumatology and University of Oxford for welcoming me.

6 Annex

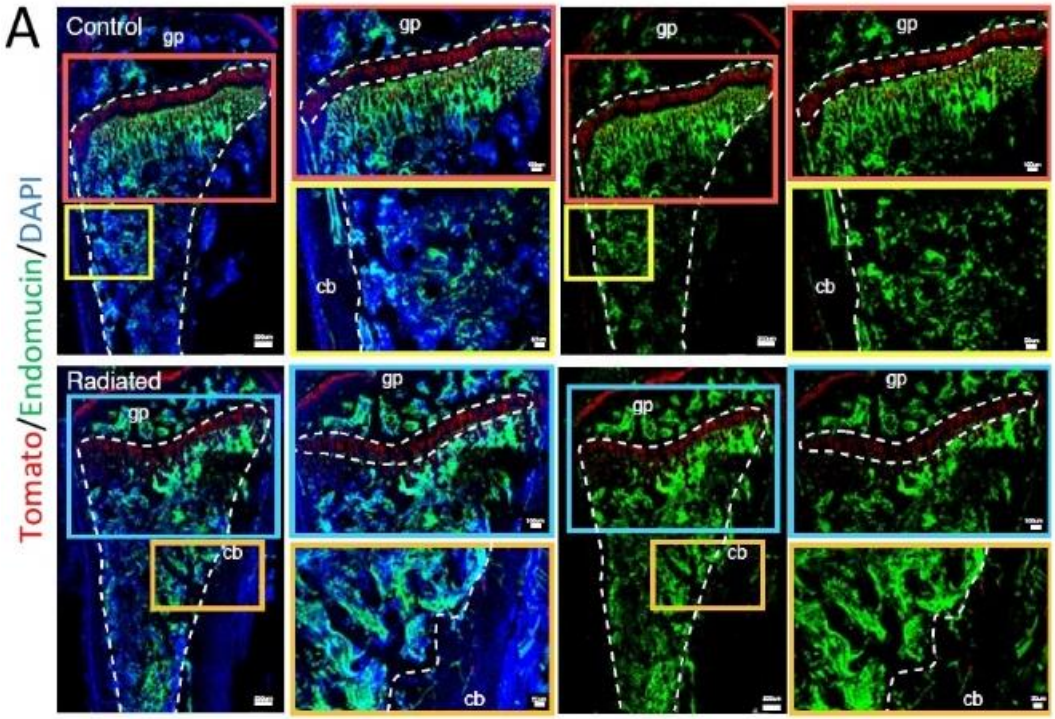

Figure 6: Representative confocal images of Tomato (red), Endomucin (green) and DAPI (blue) immunostaining in control and irradiated tibia of Gli1-CreERT2;Rosatd/Tomato mice. Scale bars: 200µm; 100µm (red and blue panel); 50µm (yellow and orange panel).

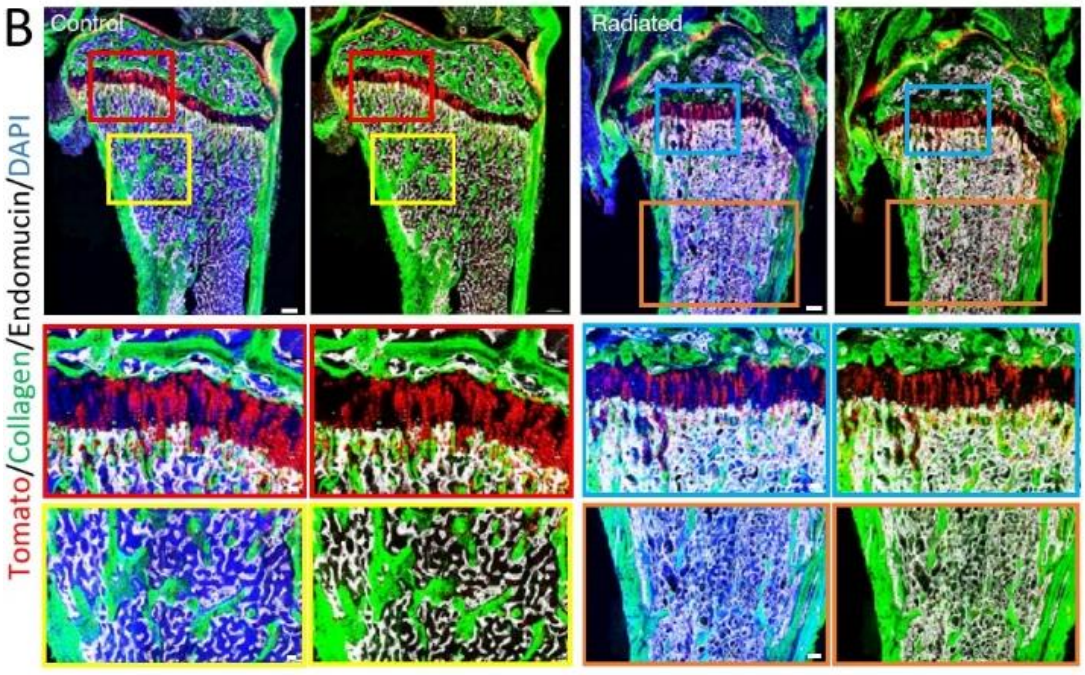

Figure 7: Representative confocal images of Tomato (red), collagen (green), Endomucin (white) and TO-PRO-3 (blue) expression in control and irradiated tibia. Scale bars: 200µm; 50µm.

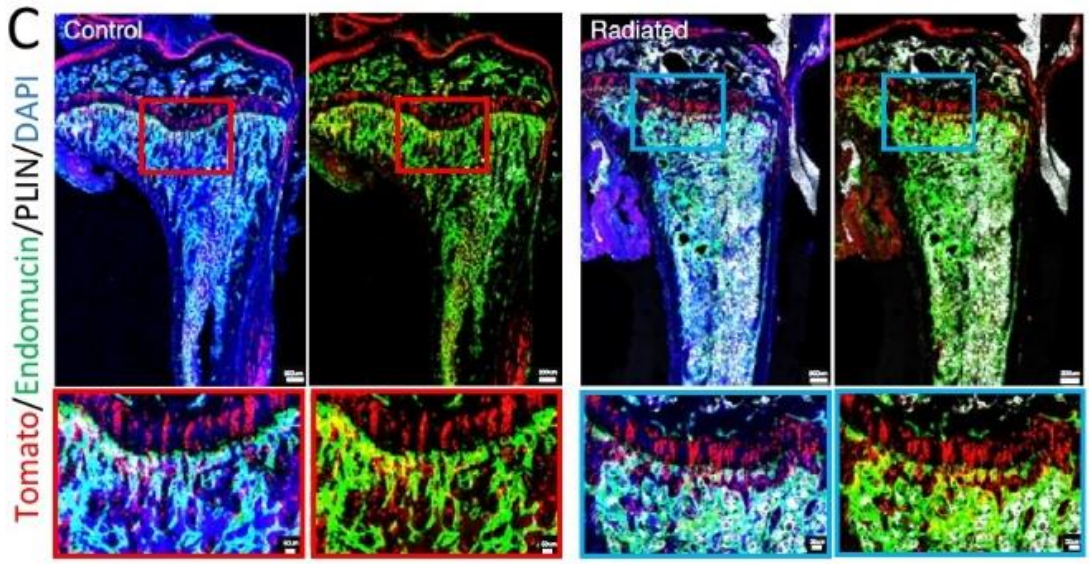

Figure 8: Representative confocal images **t**omato (red), Endomucin (green), perilipin (white) and DAPI (blue) expression in control and irradiated tibia. Scale bars: 50µm (red and blue panel)

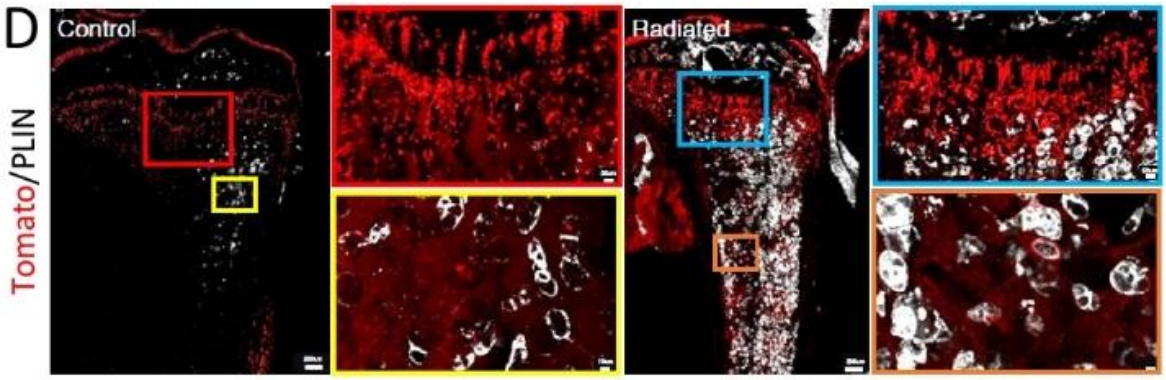

Figure 9: Representative confocal images tomato (red) and perilipin (white) staining in control and irradiated tibia. Scale bars: 200µm; 50µm (red and blue panel), 10µm (yellow and orange panel).

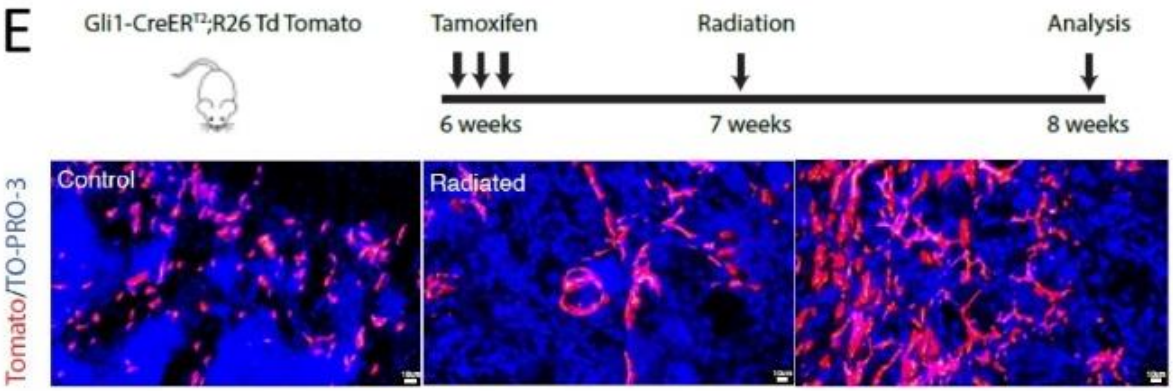

Figure 10: The upper graph shows a schematic illustration of the experimental procedure/timeline/Experimental scheme of tamoxifen administration and radiation treatment. The lower panel shows tamoxifen-induced tomato (red) expression in control and irradiated tibia of Gli1-CreERT2;Rosatd/Tomato mice. Scale bar: 10µm.

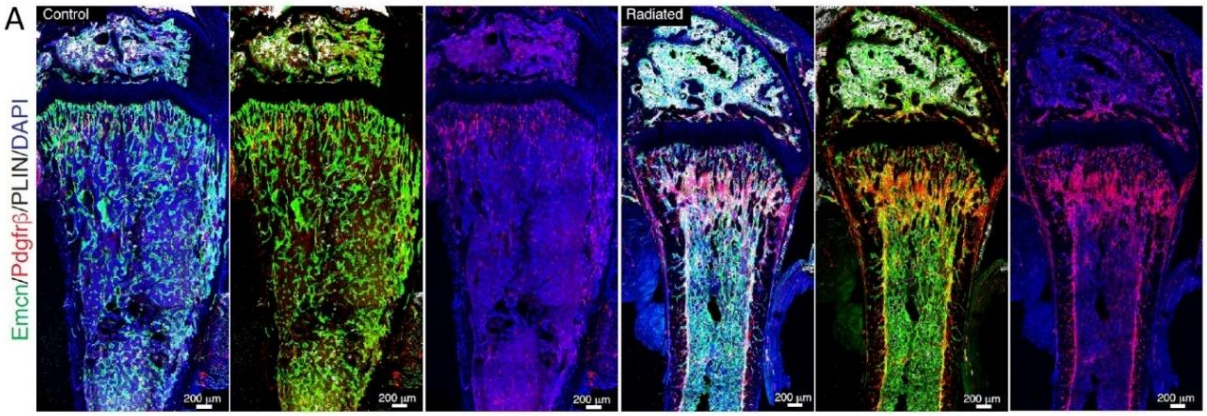

Figure 11: Representative confocal images of Endomucin (green), Pdfgrβ (red), perilipin (white) and DAPI (blue) immunostaining in control and irradiated tibia. Scale bar: 200µm

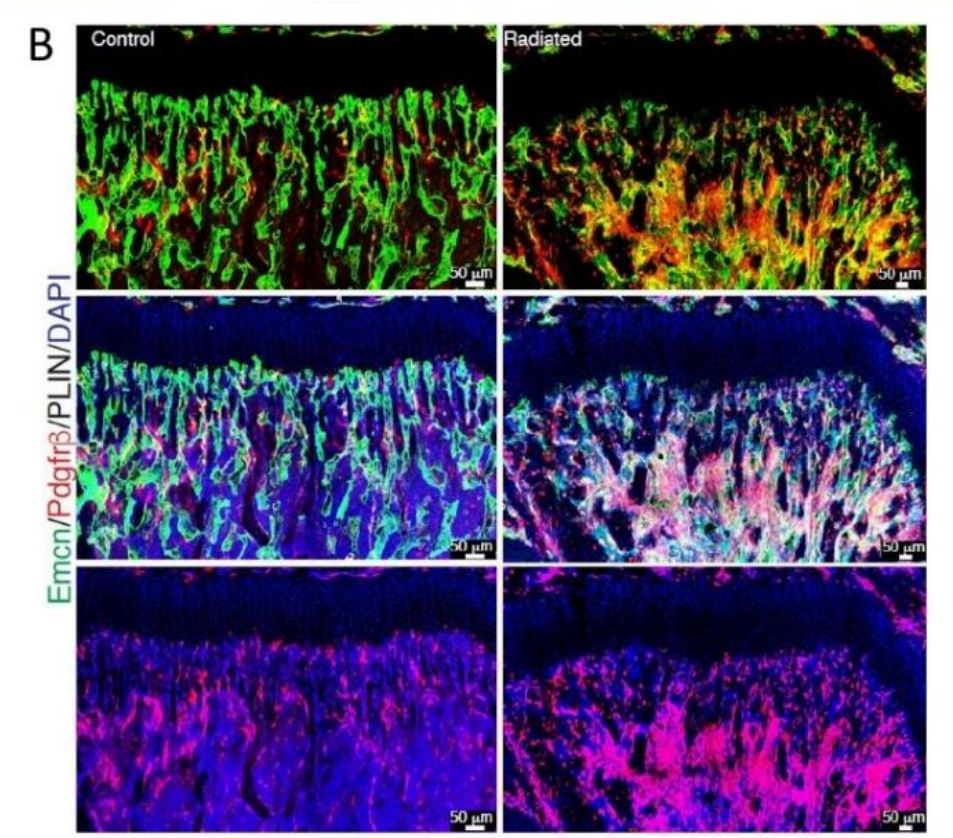

Figure 12 : Magnified images of tile scans in A (figure 10). Scale bar: 50µm. PLIN: perilipin. Emcn: Endomucin.

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