for TRO-BMD, *IGSF11*). We also replicated two loci 3p21 (*rs148725943*, discovery p=6.61x10-7, replication p=5.22x10-4 for TRO-BMD, *CTNNB1*) and 8q24 (*rs7839059*, discovery p=2.28x10-7, replication p=1.55x10-3 for TRO-BMD, *TNFRSF11B*) that were reported previously.
Conclusion: Our findings provide useful insights that enhance our understanding of bone development, osteoporosis, and fracture pathogenesis.
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PHOTODYNAMIC THERAPY (PDT) TO ENHANCE HEALING OF FEMUR FRACTURES WITH A CRITICALLY SIZED DEFECT

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Introduction: The majority of long bone fractures heal successfully without complications; however fractures resulting from high impact trauma can result in delayed healing or non-union. Early intervention could decrease patient morbidity and reduce health care system costs. Photodynamic therapy (PDT) is a minimally invasive local treatment involving administration of a photosensitizer, which is activated by laser light leading to the production of singlet oxygen, which can induce apoptosis and/or necrosis of targeted cells and tissue and also influence immune responses. PDT treatment of metastatically involved vertebrae resulted in improved vertebral bone strength, stiffness and architecture, motivating studying PDT as an approach to augment bone healing. The aim of this study was to evaluate the ability of PDT treatment to enhance healing in fractures exhibiting critically size defects.
Materials and Methods: Femoral fractures with critically sized defects (6 mm) were generated in 30 adult female Sprague-Dawley rats (7 or 15 week survival). Under general anaesthesia an 8-hole PEEK plate was attached laterally to the femur. Using a Gigly saw, a bone piece was removed followed by closure of musculature and skin. Rats were randomly allocated to three groups: control, PDT applied either 1 day, or 7 days post fracture. A photosensitizer (Visudyne, Novartis, Canada) was injected (1mg/kg) followed 15 minutes later by light delivery (75J; 690 nm) using a 1 cm diffuser fibre placed parallel to the fracture. The rats were euthanized 7 or 15 weeks after induction of the fracture. μCT images of the femur at an isotropic 13.3 μm/voxel resolution (Inveon MicroCT, Siemens, Germany) were acquired and analysed (AmiraDev 5.2, FEI Visualization Science Group, USA). Thereafter, the bone was decalcified and processed for histology. Statistical analysis was performed using a 1-way ANOVA.
Results: All rats recovered well; however five animals were euthanized early due to plate displacement. The total bone volume (TV) evaluated from μCT images within the fracture gap did not show significant differences. In contrast, BMD (gHA/cm^2) trended toward higher values in the PDT treated groups compared to controls. The fracture gap measured on μCT images of the 7 week group demonstrated a trend toward smaller gaps in the PDT treated groups ($p = 0.0535$). A statistically significant ($p = 0.0085$) smaller gap is present in the PDT treated groups after 15 weeks. Histology of the control group showed more cartilage and woven bone formation in contrast to the PDT treated groups which exhibited more structured and mature bone.
Discussion: PDT treatment of rat femur fractures led to lower overall formation of bone, but the bone had higher density with a decrease in the size of the fracture gap. The increase in bone density in the PDT treated groups may suggest formation of better quality bone (vs. quantity of bone). Histologically, with more cartilage and woven bone present in the control group in contrast to more mature bone and in the PDT group, the fracture healing seems to follow a different pattern, which requires further investigation.
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PATHOPHYSIOLOGY OF CHEMOTHERAPY-INDUCED DAMAGE OF BONE MARROW MICRO-VASCULATURE AND POTENTIAL PROTECTIVE EFFECTS OF FLAVONOIDS IN RATS

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Introduction: It has been widely shown that bone formation and remodelling would not occur unless there is already a properly established micro-vasculature. However, blood vessels can be damaged by extrinsic causes such as chemotherapeutic agents. Methotrexate (MTX) is an anti-metabolite chemo-agent widely used in treatment of many diseases including childhood leukaemia and inflammatory disorders. While previous studies showed that MTX can cause long-term skeletal side effects, whether and how it damages bone marrow micro-vasculature remains unclear. Using a rat model and endothelial cell-culture models, we addressed these questions. In addition, since we recently showed that the osteogenic, anti-oxidant, and anti-inflammatory flavonoid genistein can protect bone in MTX-treated rats, here we also investigated effects of genistein in the recovery of damaged blood vessels in rats treated with MTX. Furthermore, we also examined potential treatment effects of genistein and a related flavonoid, icariin, on viability and tube-formation ability of endothelial cells treated with MTX *in vitro*.
Methods: Animal studies: To study the effect of MTX on blood vessels, groups of male (6-week-old) Sprague—Dawley rats were subcutaneously injected with MTX (0.75mg/kg) once daily for 5 days and were sacrificed on day 1, 3, 6, 9, 11, and 14. To study the protective effects of genistein, in some MTX-treated rats, genistein was administrated by oral gavage (2 mg/100 g BW) for the whole period starting from day 0 until one day before kill (day 9). Treatment effects on number and sizes of bone marrow micro blood vessels were examined histologically in tibiae. MTT viability assay: Concentration-/time- dependent treatment effects of MTX (10nM-10μM) were examined on viability of cultured rat sinusoid endothelial cells (SECs) and effects of 24 hour treatment with MTX plus icariin/genistein (10nM-10μM) were also studied. Apoptosis detection by flow cytometry: SECs were treated with MTX (1μM/mL) for 24 and 48 hours and apoptosis was detected based on their cell surface Annexin-V expression. Tube formation assay: SECs were treated with/without MTX (1μM), icariin or genistein (100nM-10μM) and effects on angiogenesis were examined based on formation of tubes by SECs on Matrigel.
Results: Histological image analyses of H&E-stained tibial sections showed significant blood vessel damage in the bone marrow of rats on days 6 and 9 and significant but partial recovery on days 11 and 14 following the first MTX dose. Histology analyses suggested that genistein potentially attenuates MTX-induced blood vessel damage in the bone marrow. Examining any cytotoxic effect of MTX on endothelial cells, MTT assays showed that the viability of SECs was not affected after 24 hours of treatment with MTX (10nM-10μM). However, following 48 hour treatment, viability of SECs was reduced in a concentration-dependant manner. Flow cytometry analysis revealed that SECs underwent apoptosis following 48 hours (but not 24 hours) treatment with MTX (1μM). MTT assays also showed that neither genistein nor icariin treatment affected viability of SECs viability. Tube formation assays showed a reduced tube formation potential of SECs treated with MTX (1μM). Interestingly, icariin or genistein (10μM)-treated SECs showed enhanced tube formation and icariin or genistein treatment can prevent MTX-induced decrease in tube formation.
Discussion and Conclusion: Our *in vivo* and *in vitro* studies suggest that MTX causes blood vessel damage in the bone marrow, potentially by inducing apoptosis in endothelial cells and also interfering in the process of angiogenesis. Our *in vitro* tube formation assays showed that icariin and genistein might not only promote angiogenesis but possess some protective effect against MTX damage. Consistently, our *in vivo* studies also showed some positive effects of genistein treatment in reducing MTX-induced blood vessel damage in the bone marrow of rats.
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INCREASED EZH2 COMBINED WITH DECREASED OSTEOBLASTOGENESIS IN LOCAL IRRADIATION INDUCED RAT BONE LOSS

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Radiation therapy is a common treatment for cancer patients. The adverse effects are the insufficiency fractures and bone loss. Epigenetic regulation plays an important role in the BMSCs differentiation. We reported here, the changes of local bone after a single-dose $^{137}C_S$ irradiation exposure in rats. The bone mineral density (BMD) of the femur and the trabecular bone volume in the tibia were significantly decreased at 12 weeks after irradiation. The micro-CT results showed that the tBMD, Tb.h, and Tb.N were also significantly reduced after 12 weeks of irradiation exposure. The ALP-positive OB.S/BS was decreased by 42.3% after 2 weeks irradiation, and decreased by 50.8% at the 12 weeks. In contrast to the decreased expression of Runx2 and BMP2, we found EZH2 expression was significantly increased after 2 weeks of single-dose $^{137}C_S$ irradiation in BMSCs. In conclusion, our results demonstrated that the single-dose $^{137}C_S$ irradiation induces the loss of BMD and bone micro-architecture deterioration in rat skeleton, as well as the increased expression of EZH2 and decrease of osteoblastogenesis after irradiation. The underlying mechanisms may be required to further investigate the relationship.
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MYRICITRIN INHIBITS OSTEOCLASTOGENESIS

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Myricitrin is a botanical flavonol glycoside, extracted from leaves of *Myrica cerifera* and other plants. Abundant evidence supports myricitrin has anti-oxidative, anti-inflammatory, and neuroprotective effects. Osteoclastic bone resorption is

vital to maintain the balance of bone homeostasis. Our early article elucidated myricitrin could protect from osteoporotic bone mass reduction via reducing reactive oxygen species (ROS) in osteoblastic bone formation; however, the influence of osteoclastogenesis by myricitrin is still unclear. Here, we exhibited that myricitrin could decrease the TRAP positive cell number significantly in a dose-dependent manner during osteoclast maturation, but it had no effect to pre-osteoclast proliferation. Consistently, osteoclast maturation markers, and the bone resorptive pits number and area were decreased. Taken together, myricitrin could inhibit osteoclastogenesis.
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ASSOCIATION OF TELOMERE LENGTH AND MITOCHONDRIAL DNA COPY NUMBER IN MUSCULOSKELETAL TUMOURS

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Background: Telomere length plays a vital role in genomic stability and shortened telomeres may cause genomic instability and carcinogenesis. Mitochondria exert a potential role in energy metabolism, free radical production, apoptosis, and may be involved in cancer progression. Both telomere length and alteration of mitochondrial DNA (mtDNA) copy number have been proposed as biomarkers for several cancers. Nevertheless, few studies have examined the association of telomere length and mtDNA copy number in musculoskeletal tumours. This study aimed to examine telomere length and mtDNA copy number in peripheral blood leukocytes, neoplastic tissues, and non-neoplastic adjacent tissues of patients with musculoskeletal tumours. The second objective of this study was to investigate the relationship of telomere length and mtDNA copy number in musculoskeletal tumours.
Subjects and Methods: Peripheral blood leukocytes (n=41), neoplastic tissues (n=46), and non-neoplastic adjacent tissues (n=32) were obtained from patients with musculoskeletal tumours. Relative telomere length and relative mtDNA copy number were evaluated by quantitative real-time polymerase chain reaction.
Results: Relative telomere length in neoplastic tissues was significantly shorter than that in non- neoplastic adjacent tissues ($p = 0.001$). Shorter relative telomere length in neoplastic tissues compared to non-neoplastic tissues was observed in female and male ($p = 0.018$ and $p = 0.019$, respectively). In addition, the relative telomere length in malignant tissues was significantly decreased as compared with that in adjacent non-neoplastic tissues ($p = 0.016$). While the relative telomere length in malignant tissues seemed to be lower than that in benign tissues, there was no significant difference. The relative telomere length in peripheral blood leukocytes was not correlated with that in neoplastic tissues. In contrast, relative mtDNA copy number in neoplastic tissues was not different when compared to that in non-neoplastic adjacent tissues. The relative mtDNA copy number in peripheral blood leukocytes was not associated with that in neoplastic tissues. Interestingly, relative mtDNA copy number in neoplastic tissues was significantly higher than that in peripheral blood leukocytes ($p < 0.001$). Further analysis showed that there was a negative association between relative telomere length and mtDNA copy number in patients with musculoskeletal tumours ($r = -0.306$; $p = 0.104$).
Discussion and Conclusion: Relative telomere length in neoplastic tissues was significantly shorter than that in non-neoplastic adjacent tissues, suggesting that neoplastic tissues (especially malignant tissues) may undergo several cell divisions which could lead to progressively shorter telomeres. This finding demonstrated that relative mtDNA copy number in tissues was significantly higher than that in peripheral blood leukocytes. The explanation of this finding could be due to specificity of mtDNA copy number in different types of tissues. The relative telomere length is inversely associated with the relative mtDNA copy number, indicating that telomere length attrition and mtDNA alteration are necessary events in musculoskeletal tumour progression.
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DOWN-REGULATION OF μ-OPIOID RECEPTOR MEDIATED EPIGENETICALLY BY NEURON-RESTRICTIVE SILENCER FACTOR IS INVOLVED IN THE REDUCED MORPHINE ANALGESIA IN A BONE CANCER PAIN ANIMAL MODEL

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Background: Primary and metastatic cancers that affect bone are frequently associated with severe and intractable pain. Bone cancer pain has been reported with unique mechanisms and is resistant to morphine treatment. Previous studies have indicated that change of μ-opioid receptor (MOR) expression may be involved in the pathogenesis of bone cancer pain. In addition, neuron-restrictive silencer factor (NRSF) has been recently reported to modulate transcription of the MOR gene. The present study elucidates the regulatory mechanisms of MOR and its potential to effect bone cancer pain.
Methods: Using a sarcoma inoculated murine model, pain behaviours that represent continuous or breakthrough pain were evaluated. Immunofluorescent staining was used to check the expression of NRSF in the dorsal root ganglion (DRG). Reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analysis were used to quantify expression of NRSF at the transcriptional and translational levels, respectively. Additionally, chromatin immunoprecipitation assays were used to detect NRSF binding to the promoter of MOR. Furthermore, NRSF was genetically knocked out by antisense oligodeoxynucleotide (AS-ODN), and the expression of MOR and the effect of morphine were subsequently analysed.
Results: Our results indicated that in a sarcoma murine model, expression of NRSF is upregulated in the DRG neurons and the expression of NRSF mRNA is significantly negatively correlated with expression of MOR mRNA. Additionally, chromatin immunoprecipitation analysis revealed that NRSF binding to the neuron-restrictive silencer element within the promoter area of the MOR gene is significantly promoted with a hypo-acetylation state of histone H3. Furthermore, genetically knocking down of NRSF with AS-ODN rescued the expression of MOR, with potentiation of system morphine analgesia.
Discussion and Conclusion: The present results suggest that in sarcoma induced bone cancer pain, NRSF induced downregulation of MOR is involved in the reduction of morphine analgesia. Epigenetically, up-regulation of MOR could substantially improve the effect of system delivery of morphine. The results indicate that NRSF plays an important role in the modulation of MOR transcription and may represent a novel analgesic target for bone cancer pain. What should be noted is that the expression of opioid receptors are not only regulated at the transcriptional level, but are also controlled by extensive post-transcriptional processing. Further studies are needed at both the preclinical and clinical levels to develop pharmacological therapy and to effectively block/relieve bone cancer pain with the goal of increasing the functional status and quality of life of humans with bone cancer pain.
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miR-138-5p TARGETS MACF1 TO INHIBIT BONE FORMATION

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Introduction: MicroRNAs (miRNAs) play important roles in the regulation of target gene expression to coordinate a broad spectrum of biological processes. There is increasing evidence that multiple miRNAs serve as important regulators of osteoblast differentiation and bone formation. Significantly, recent studies have discovered that miR-214, miR-103a, and miR-103, were sensitive to mechanical stimulation to regulate osteoblast differentiation or proliferation. However, functional roles of miRNAs in mechano-transduction in bone formation and further mechanisms *in vivo* and *in vitro* have not been well-characterised and, therefore, particularly remain to be elucidated. This study aimed to identify specific miRNAs and their regulatory roles in the process of bone loss induced by mechanical unloading condition.
Subjects and Methods: Bone specimens from 70 osteoporotic individuals with bedridden states were collected. Twenty-one-month mice and HLU mice were used as the osteoporosis model. The RPM machine as an unloading model was used to culture cells.
Results: We assessed the expression of miRNAs involved in bone formation in bone specimens from 70 osteoporotic individuals with bedridden states in clinical settings. The expression of miR-138-5p altered with bedridden time and was negatively correlated with the expression of the bone formation marker genes *ALP* in bedridden women and men. Moreover, consistent results were found in bone tissue and ALP positive cells in hind limb unloading (HLU) and 20-month aging mice. In addition, we found that high miR-138-5p expression increased gradually and ALP activity decreased in osteoblasts after RPM treatment for 12, 24, 48 hours. Target prediction analysis tools and luciferase activity were used to confirm microtubule actin crosslinking factor 1 (MACF1) as a direct target of miR-138-5p, and miR-138-5p inhibited MACF1 expression and osteoblast differentiation *in vitro*. We treated mouse preosteoblast MC3T3-E1 cells with antagomir-138-5p and cultured cells under RPM condition. The results indicated that miR-138-5p functions as a mechanical unloading sensitive miRNA and plays a negative role in RPM-induced osteoblast differentiation reduction. Predominantly, we found an inhibitory role of miR-138-5p in regulating the bone formation in HLU mice and *in vivo* pre-treatment with antagomir-138-5p partly recovered the bone loss caused by hind limb unloading.
Discussion and Conclusion: Taken together, these results suggest that *in vivo* inhibition of miR-138-5p by antimiR-138-5p could represent a potential therapeutic strategy for ameliorating bone loss.
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