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Matthieu Bourgery

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## University of Turku

Faculty of Medicine
Institute of Biomedicine
Cell Biology and Anatomy
Turku Doctoral Programme of Molecular Medicine

## Supervised by

Adjunct professor, Anna-Marja Säämänen
Institute of Biomedicine
University of Turku
Turku, Finland

Adjunct professor, Tiina Laitala
Institute of Biomedicine
University of Turku
Turku, Finland

## Reviewed by

Professor, Mikko Lammi
Department of Integrative
Medical Biology (IMB)
Umeå University
Umeå, Sweden

Associate professor, Eija Laakkonen
Gerontology Research Centre and
Faculty of Sport and Health Sciences
University of Jyväskylä
Jyväskylä, Finland

## Opponent

Professor, Hanna Taipaleenmäki
Institute of Musculoskeletal Medicine (IMM)
Ludwig Maximilian University of Munich
Faculty of Medicine
Munich, Germany



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*Dedicated to my family*

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## ABSTRACT

Long bone fracture is a common injury which could result from a fall, shock, sports injury or a disease such as osteoporosis. Fracture healing involves multiple partly overlapping steps including inflammation, endochondral ossification, angiogenesis and remodelling. The high burden of bone fractures on public health as well as economy highlights the importance of understanding the healing process and identifying biomarkers for better management of bone health. This thesis study focused on the expression of small non-coding RNAs (sncRNAs) in bone fracture and fracture healing. SncRNAs are important regulators of gene expression, and they also serve as biomarkers for many diseases. MicroRNAs (miRNAs) have well-characterized functions in the regulation of protein-coding mRNAs. Transfer RNA-derived small RNAs (tsRNAs) are a less well-studied class of sncRNAs, but they are also involved in the regulation of gene expression at various levels. In bone and fracture healing callus tissue the role of tsRNAs have not been studied before.

The main aim of the study was to determine the effects of bone fracture on the genome-wide expression profiles of miRNAs and tsRNAs in the callus tissue which forms at the fracture site, as well as in circulation during the healing process in mice. For these purposes, basal (callus tissue) and circulating (serum exosome fraction) RNAs were extracted at different time points after tibial shaft fracture, and tsRNAs and miRNAs were analysed by high-throughput sequencing. Also, mRNA and retrotransposon (LTRs) expression profiles were investigated in fracture callus by high-throughput sequencing.

The study revealed differential expression of 54 miRNAs, 7 tsRNA, and 15 LTRs in callus tissue in comparison to intact bone while in circulation 8 miRNAs and 3 tsRNAs were differentially expressed after fracture. Literature searches were performed to identify the target genes for those 54 differentially expressed miRNAs. A significant negative correlation was observed between the expressions of 164 miRNA-target mRNA pairs in the callus, suggesting a potential role of these miRNAs as fine-tuners of fracture healing by regulation of the expression of their target mRNAs. These results indicate a role for tsRNAs and miRNAs as regulators of fracture healing in vivo and possibly have potential as systemic biomarkers of the fracture healing-related processes in circulation for cell-cell communication.

KEYWORDS: Long bone, fracture, microRNA, tsRNA, callus, serum

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Lääketieteellinen tiedekunta
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## TIIVISTELMÄ

Pitkän luun murtuma on yleinen vamma, joka voi johtua kaatumisesta, shokista, urheiluvammoista tai sairaudesta, kuten osteoporoosista. Murtumien paranemisessa erotetaan useita osin päällekkäisiä vaiheita, joihin kuuluvat tulehdus, endokondraalinen luutuminen, angiogeneesi ja uudelleenmuotoilu. Murtuman paranemisprosessin parempi tunteminen ja sen edistäminen ovat keskeisiä tavoitteita sekä kansanterveydellisesti että kansantalouden kannalta katsottuna. Tässä väitöskirjatyössä tutkittiin pienten ei-koodaavien RNA:iden (sncRNA:iden) ilmentymistä luunmurtumissa ja murtumien paranemisessa. SncRNA:t ovat tärkeitä geenien ekspression säätelijöitä, ja niitä hyödynnetään myös monien sairauksien biomarkkereina. MikroRNA:iden (miRNA:den) toiminta ja merkitys geenien ekspression ja proteiinien ilmentymisessä tunnetaan jo melko hyvin. Siirtäjä-RNA:sta (tRNA:sta) peräisin olevat pienet RNA-fragmentit (tsRNA:t) ovat uusi sncRNA-luokka, joiden tiedetään osallistuvan geenien ekspression säätelyyn sen eri tasoilla, mutta niiden merkitys on vähemmin tunnettu ja erityisesti luukudoksessa ja murtuman paranemisessa niitä ei ole aiemmin tutkittu lainkaan.

Tutkimuksessa selvitettiin luunmurtuman vaikutuksia miRNA:iden ja tsRNA:iden genominlaajuisiin ilmentymisprofiileihin murtuman ympärille muodostuvassa korjauskudoksen sekä verenkierrossa paranemisprosessin aikana hiirillä. Kalluskudoksessa ja seerumin eksosomifraktion RNA:t uutettiin eri ajankohtina kokeellisen sääriluun murtuman jälkeen, ja tsRNA:t ja miRNA:t analysoitiin suurtehosekvensoinnilla. Myös mRNA:n ja retrotransposonien (LTR:t) ilmentymisprofiileja tutkittiin kalluskudoksessa.

Korjauskudoksessa havaittiin 54 miRNA:n, 7 tsRNA:n ja 15 LTR:n poikkeava ilmentyminen ehjään sääriluuhun verrattuna. Verenkierrossa kahdeksan miRNA:ta ja kolme tsRNA:ta ilmentyivät kontrollieläimiin nähden poikkeavasti murtuman jälkeen. Kirjallisuushakuun perustuen tunnistettiin kohdegeenit e.m. 54:lle poikkeavasti ilmentyvälle miRNA:lle. Näistä yhteensä 164 miRNA-kohdegeeni/mRNA -parin ilmentymistasot korreloivat negatiivisesti keskenään, osoittaen niiden mahdollisen roolin murtuman paranemisen hienosäätäjinä. Tulosten perusteella tsRNA:t ja miRNA:t osallistuvat murtuman paranemisen säätelyyn in vivo. Verenkierrossa niillä voi olla merkitystä solujen välisessä systeemisessä viestinnässä sekä murtuman paranemiseen liittyvinä biomarkkereina.

AVAINSANAT: Pitkä luu, murtuma, mikroRNA, tsRNA, kallus, seerumi

# Table of Contents

# Abbreviations

| | |
|---|---|
| Acan | Aggrecan |
| Adam9 | A disintegrin and metalloproteinase domain 9 |
| ADSC | Adipose-derived stem cell |
| Ago | Argonaute |
| ALKBH3 | AlkB Homolog 3, Alpha-Ketoglutarate Dependent Dioxygenase |
| Alp | Alkaline phosphatase |
| Alpl | Alkaline phosphatase gene |
| ANG | Angiogenin |
| ANOVA | Analysis of variance |
| ATF4 | Activating transcription factor 4 |
| Bglap | Bone gamma-carboxyglutamate protein |
| BMP | Bone morphogenetic protein |
| bp | Base pair |
| Cbfa1 | Core binding factor α1 |
| circRNA | Circular RNA |
| Col | Collagen |
| Comp | Cartilage oligomeric matrix protein |
| CSF | Colony Stimulating Factor |
| CSF1R | Colony Stimulating Factor 1 Receptor |
| Ctnnb1 | β-catenin |
| CXCR-4 | Chemokine receptor 4 signaling axis |
| D | Day |
| DE | Differentially expressed |
| DEA | Differential expression analysis |
| DGCR8 | DiGeorge syndrome critical region gene 8 |
| Dmp1 | Dentin matrix acidic phosphoprotein 1 |
| DNA | Deoxyribonucleic acid |
| Dnmt2 | DNA methyl transferase 2 |
| E11/Gp38 | Podoplanin gene |
| ECM | Extracellular matrix |
| EIF4 | Eukaryotic Translation Initiation Factor 4 |

| | |
|---|---|
| ERV | Endogenous retrovirus |
| FGF | Fibroblast growth factor |
| Fmod | Fibromodulin |
| FZD3 | Frizzled class receptor 3 |
| Fzd4 | Frizzled class receptor 4 |
| G6PC | Glucose-6-phosphatase catalytic subunit |
| GABBR2 | Gamma-Aminobutyric Acid Type B Receptor Subunit 2 |
| GTP | Guanosine triphosphate |
| Hapln1 | Hyaluronan and proteoglycan link protein 1 |
| HDAC | Histone deacetylase |
| Hif1α | Hypoxia inducible factor 1 subunit alpha |
| Hmgb1 | High mobility group box 1 |
| Hoxa1 | Homeobox A1 |
| HSC | Hematopoietic stem cell |
| Ibsp | Integrin binding sialoprotein |
| Ihh | Indian hedgehog protein |
| IL | Interleukin |
| iPSC | induced pluripotent stem cell |
| Kdm6b | Lysine demethylase 6B |
| Klf4 | Kruppel like factor 4 |
| LATS2 | Large tumor suppressor kinase 2 |
| LINE | Long interspersed nuclear element |
| lncRNA | Long non-coding RNA |
| log2FC | Log2 fold change |
| LTR | Long terminal repeat |
| m5C | 5-methyl cytosine |
| m7G: | 7-methyl guanosine |
| Map3k8 | Mitogen-activated protein kinase kinase kinase 8 |
| M-CSF | Macrophage colony-stimulating factor |
| MERVL | Mouse endogenous retrovirus type-L |
| miRISC | MicroRNA-induced silencing complex |
| miR/miRNA | Micro ribonucleic acid |
| miRNA* | Passenger miRNA |
| Mitf | Microphthalmia-associated transcription factor |
| Mmp | Matrix metalloproteinase |
| mRNA | messenger ribonucleic acid |
| ms2i6A | 2-methylthio-*N*6-isopentenyl adenosine |
| MSC | Mesenchymal stem cell |
| MTPAP | Mitochondrial poly(A) polymerase |
| ncRNA | non-coding RNA |

| | |
|---|---|
| Nfatc1 | Nuclear factor of activated T cells 1 |
| NF-kB | Nuclear Factor Of Kappa Light Polypeptide Gene Enhancer In B-Cells |
| NGS | Next generation sequencing |
| Nsun 1-6 | NOP2/Sun RNA Methyltransferase 1-6 |
| OA | Osteoarthritis |
| Oct4 | Octamer-binding protein 4 |
| OPG | Osteoprotegerin |
| ORF | Open Reading Frame |
| Osr1 | Odd-skipped related transcription factor 1 |
| PAPD | Poly(A) RNA polymerase D |
| PDGF | Platelet derived growth factor |
| piRNA | piwi-interacting RNA |
| PPARγ | Peroxisome Proliferator Activated Receptor Gamma |
| pre-miRNA | precursor miRNA |
| pri-miRNA | primary miRNA |
| PTEN | Phosphatase and tensin homolog |
| PTHrP | Pth-related protein receptor |
| qPCR | Quantitative polymerase chain reaction |
| RAN-GTP | RAs-related Nuclear protein |
| RANKL | Tumor necrosis factor (ligand) superfamily, member 11 |
| RISC | RNA-induced silencing complex |
| RNA | Ribonucleic acid |
| RNH1 | Rnase inhibitor 1 |
| RPA1 | Replication Protein A1 |
| rRNA | ribosomal RNA |
| rsRNA | ribosomal RNA small RNA |
| RT-PCR | Real time polymerase chain reaction |
| Runx2 | Runt-related transcription factor 2 |
| SDF-1 | Stromal cell-derived factor-1 |
| SINE | Short interspersed nuclear element |
| siRNA | Small interfering RNA |
| Smad | SMAD family member |
| sncRNA | Small non-coding RNA |
| Snorc | Secondary ossification centre associated regulator of chondrocyte maturation |
| snoRNA | Small nucleolar RNA |
| snRNA | Small nuclear RNA |
| SOX | Sex-Determining Region Y-type high motility group Box |
| TAR | Trans-Activation Responsive |

| | |
|---|---|
| TF | Transcription factor |
| TGF-β | Transforming growth factor β |
| tiRNA | Stress-induced tRNA fragment |
| TLR4 | Toll Like Receptor 4 |
| TNF | Tumor necrosis factor |
| TNFSF11 | TNF Superfamily Member 11 |
| TRAcP | Tartrate-resistant acid phosphatase |
| Traf | TNF receptor associated factor |
| TRBP2 | TAR RNA-binding protein 2 |
| tRF | tRNA fragment |
| tRNA | Transfer RNA |
| Trnt1 | tRNA nucleotidyl transferase 1 |
| tsRNA | tRNA-derived small RNA |
| TUT1 | Terminal uridylyl transferase 1 |
| UTR | Untranslated region |
| VEGF | Vascular endothelial growth factor |
| YBX1 | Y-Box Binding Protein 1 |
| ZCCHC | Zinc finger CCHC-type containing |

# List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I **Bourgery, M.**, Ekholm, E., Fagerlund, K., Hiltunen, A., Puolakkainen, T., Pursiheimo, J.-P., Heino, T., Määttä, J., Heinonen, J., Yatkin, E., Laitala, T., & Säämänen, A.-M. (2021). Multiple targets identified with genome wide profiling of small RNA and mRNA expression are linked to fracture healing in mice. *Bone Reports*, *15*, 101115. https://doi.org/10.1016/j.bonr.2021.101115.

II **Bourgery, M.**, Ekholm, E., Hiltunen, A., Heino, T. J., Pursiheimo, J. P., Bendre, A., Yatkin, E., Laitala, T., Määttä, J., & Säämänen, A. M. (2022). Signature of circulating small non-coding RNAs during early fracture healing in mice. *Bone Reports, 17,* 101627. https://doi.org/10.1016/j.bonr.2022.101627.

# 1 Introduction

Long bone fracture is a recurrent trauma which may occur due to an accident, sports-related injury, or various bone diseases. Osteoporosis is a major factor of bone fractures, especially in elderly people. The prevalence of osteoporosis in Europe was reported to be around 16.7% in elderly people between 50-85 years of age (Salari et al., 2021). Although fractures take place in all long bones, tibial bone is the most prone to fracture.

Fracture healing of long bones involves a succession of six intricate metabolic phases which are closely connected and partly overlapping with each other. The main steps are inflammation, mesenchymal stem cell (MSC) recruitment and migration to the fracture site, endochondral ossification, intramembranous ossification, vascularization, and finally bone remodelling. The healing process requires the intervention of numerous molecules and pathways tightly regulated by multiple molecules. The main steps cited above are roughly present in mice at day 0 - day 5 (D0-D5) (hematoma and inflammation), D3-D5 (MSCs recruitment and migration to the fracture site, D7-D21 (endochondral ossification), D3-D21 (intramembranous ossification and angiogenesis), and D21-D35 (bone remodelling) post-fracture (Einhorn & Gerstenfeld, 2015). The analysis of regulatory molecules affecting the tibial fracture healing process in mice is crucial to understand the complex molecular mechanism behind fracture healing steps.

It is critical to understand and identify the circulating indicators of fracture healing status as well as be the factors that systematically affect the healing process to further potentially identify undetected or non-union fractures. Circulating molecules are found in three main types of extracellular vesicles, which are exosomes, microvesicles, and apoptotic bodies. The ribonucleic acid (RNA) content of exosomes is mainly composed of non-coding RNAs (ncRNAs). Non-coding RNAs were also found freely in circulation associated with lipid particles and RNA-binding proteins (Etheridge et al., 2013). Circulating non-coding RNA biomarkers are already under intensive investigation for a wide range of diseases, viral and bacterial infections and various types of cancers. These molecules may also have applications in skeletal biology and function as biomarkers to indicate, e.g., a micro-fracture or even a long bone non-union fracture which is the result of a fracture

healing failure or a delayed union. Earlier studies have clearly indicated the vital role of microRNAs (miRNAs) in the post-transcriptional regulation of messenger RNA (mRNA) expression. Recent studies have also pointed out the non-canonical role of tRNA-derived small RNAs (tsRNAs), which are newcomers among the factors regulating mRNA transcription and protein translation (Avcilar-Kucukgoze & Kashina, 2020; S. Li et al., 2018; Oberbauer & Schaefer, 2018; J. Park et al., 2020).

Understanding the mechanisms and molecules involved in fracture healing will facilitate the development of regenerative medicines. Differential expression of miRNA and tsRNAs in callus and serum tissues as shown by the studies in this thesis project suggests their putative role during fracture healing with possible applications as novel tools in the development of various therapeutic approaches.

# 2 Review of the Literature

Bone tissue is part of the skeleton providing mechanical support to the body and protecting internal vital organs. Bone is a highly vascularized and dynamic tissue undergoing permanent changes through modelling and remodelling. The cellular composition of the bone is mainly osteoblasts (bone-forming cells), osteocytes (mature osteoblastic cells), and osteoclasts (bone-resorbing cells). Also, chondrocytes (cartilage cells), are closely associated with bone tissue via endochondral ossification during bone development and fracture healing (Blumer, 2021). The long bones are mainly divided into four regions: the diaphysis (1) which is the middle region, the epiphysis (2) at the distal parts of the bone in between the metaphysis (3) and the growth plate (4), located at the border between epiphysis and metaphysis, which is a thin layer of cartilage crucial for bone development. In humans, the growth plate slowly disappears (closes) in adulthood but in mice, it never totally closes. Macroscopically, two main parts can be conserved in long bones. Cortical bone contains osteons with a central Haversian canal system for blood supply. The trabecular bone is found in the inner part of the bone and at the bone ends, which are devoided of osteons (Figure 1) (Blumer, 2021). Osteocytes are embedded in the mineralized matrix. The medullary cavity contains the bone marrow stroma with MSCs, progenitor cells, immune cells, osteoblasts, osteoclasts, adipocytes, red blood cells, and haematopoietic stem cells (HSCs). The periosteum, covering the outer surface of the cortical bone, contains osteoblastic cells, MSCs, nerves and blood vessels. The endosteum, covering the inner surface of the cortical bone, contains osteoblasts, osteoclasts, and MSCs. Osteoblasts and chondrocytes both originate from MSCs whereas osteoclasts originate from HSCs (Blumer, 2021; Colnot, 2009; Murao et al., 2013).

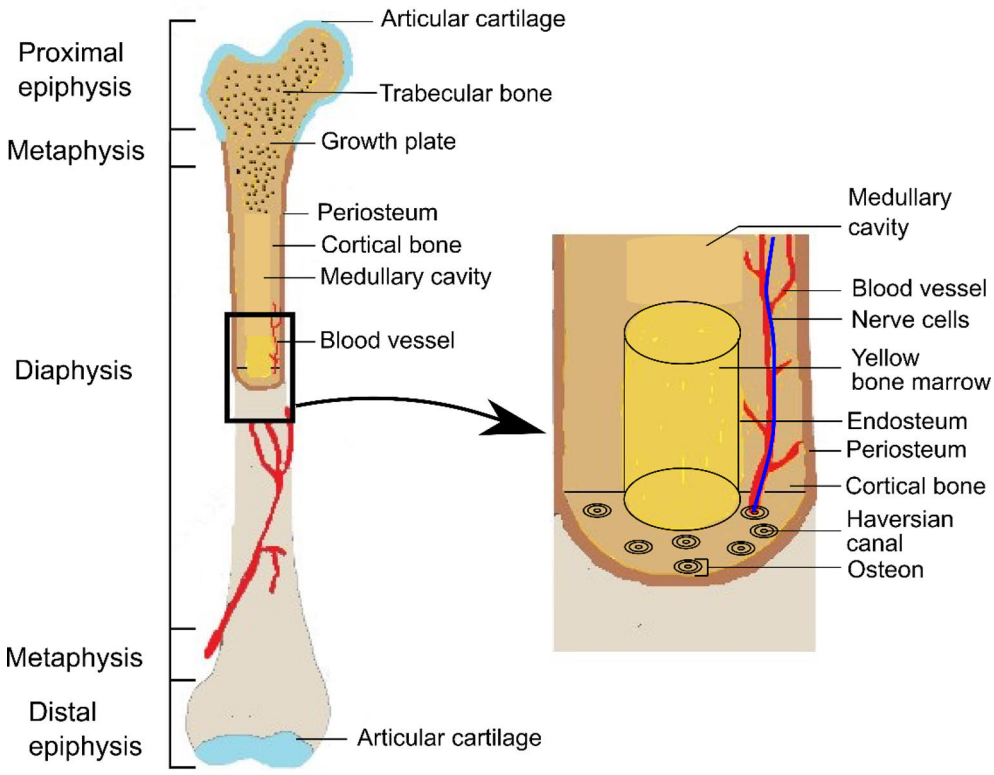


**Figure 1. Schematic diagram of long bone structure.**

Hyaline cartilage, called articular cartilage, covers the bone epiphyses at the ends of the long bone and provides gliding surfaces with minimal friction to the joints. Hyaline cartilage is also crucial during limb development or after fracture injury where it functions as a platform for the future bone via a process of endochondral ossification. Cartilage tissue is unique as it lacks blood vessels and nerves. Essential nutrient supply occurs by diffusion while most metabolism is anaerobic. Three main types of cartilage are defined: fibrocartilage, elastic cartilage, and hyaline cartilage. Fibrocartilage provides better support than hyaline cartilage and is mostly found between intervertebral disks and in menisci (Benjamin & Evans, 1990). Elastic cartilage found e.g. in ears has a morphology comparable to hyaline cartilage including the presence of elastic fibres (Cox & Peacock, 1977). Finally, the hyaline cartilage has a crucial role in the mechanical support, movement, and growth of the skeleton (Archer & Francis-West, 2003). Furthermore, hyaline cartilage accounts for only 2% of the MSCs population. It is a highly specialized connective tissue, mainly composed of water (65-80%), collagens (mainly type II collagen), and proteoglycans such as aggrecan, decorin, biglycan, and fibromodulin (Sophia Fox et al., 2009).

Limb development in mice is initiated at embryonic day E9.5, starting with the condensation of MSCs in limb buds (Figure 2). Bone morphogenetic proteins (BMPs) are important growth factors in the regulation of MSCs condensation (Harkness et al., 2009; K. Hata et al., 2017; Heinonen et al., 2011). Several bones such as long bones, ribs, vertebrae, a part of the clavicle, and some craniofacial bones in mammals develop during embryogenesis via endochondral ossification from the cartilage anlage surrounded by the perichondrium. The skull, some craniofacial bones, and some parts of the clavicle develop via intramembranous ossification. During limb development, condensed MSCs differentiate into chondrocytes which proliferate and eventually undergo terminal differentiation to hypertrophic chondrocytes starting from the central diaphyseal part of the bone. Subsequently, hypertrophic chondrocytes either undergo apoptosis or transdifferentiate into osteoblasts. Osteoclasts resorb the mineralized cartilage which allows the invasion of blood vessels and the formation of bone marrow. In the meantime, perichondrial ossification takes place at the perichondrium. This process is similar to intramembranous ossification with direct differentiation of MSCs into osteoblasts. Collar forms around the hyaline cartilage anlage by perichondrial ossification, followed by endochondral ossification inside the cartilage. The cartilage is slowly resorbed and the bone marrow cavity appears. Finally, osteoblasts invade from the perichondrium to the bone marrow and deposit hydroxyapatite crystals on type I collagen fibres to form a trabecular bone matrix (Blumer, 2021; Cervantes-Diaz et al., 2017). Fracture healing phases of long bones are similar and comparable to limb development except for the hematoma and inflammation phases which are absent during limb development.

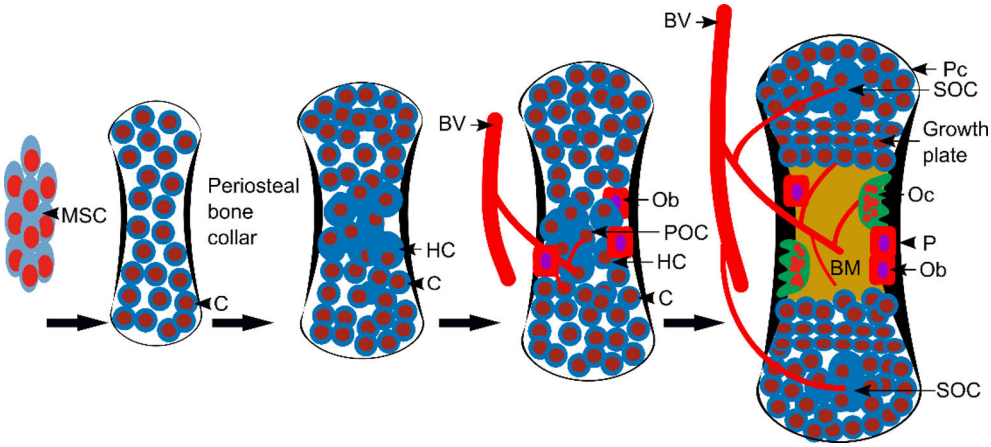


**Figure 2. Schematic diagram of endochondral ossification during limb development.** MSC: mesenchymal stem cell, C: chondrocyte, HC: hypertrophic chondrocyte, BV: blood vessel, Ob: osteoblast, POC: primary ossification centre, SOC: secondary ossification centre, BM: Bone marrow, Oc: osteoclast, P: periosteum, Pc: perichondrium.

## 2.1 Healing of long bone fracture

Two different mechanisms contribute to fracture healing which are called primary and secondary bone healing. Primary bone healing occurs after a partial crack in the bone when the remaining bone is still holding the main structure together. Primary ossification is a straightforward mechanism which does not require the formation of hematoma and/or soft callus tissue. This type of ossification happens only in specific circumstances if the fracture is stabilized and if the fracture gap is reduced by surgery.

During the primary bone healing process, the fractured bone will be remodelled and filled with osteoblasts differentiated from osteoprogenitor cells with the assistance of vascular endothelial cells and perivascular mesenchymal cells, originating from blood vessels of the Haversian system, expressing BMPs and therefore stimulating osteogenesis (Bahney et al., 2019; Dimitriou et al., 2005). Secondary bone healing occurs when a gap is created in the bone with the instability of the fracture site.

Secondary fracture healing involves the formation of a callus and requires the intervention of several healing phases including hematoma (D0-D3), inflammation (D0-D5), migration and proliferation of MSCs (D3-D5), endochondral (D7-D21) and intramembranous ossification (D3-D21), angiogenesis (D3-D21), and bone remodelling (D21-D35) (Figure 3). These steps are interconnected and partly overlap with each other and are similar to the limb development steps observed during embryogenesis (Figure 2) (Dimitriou et al., 2005; Gerstenfeld et al., 2003; Marsell & Einhorn, 2011). The gap generated by the fracture will create an unstable and weak structure for the bone. Callus formation is a response of the body toward this unstable environment. The callus evolves in time via cell differentiation and matrix formation giving a gradually more stable callus to ensure the stability of the fracture. Due to the fragility of the callus, especially at the early stages of callus development, it is really important to keep the fracture site motionless or at least to a minimum to avoid a non-union of the fracture (Keramaris et al., 2008; Komatsu et al., 2021; S. H. Park et al., 1998).

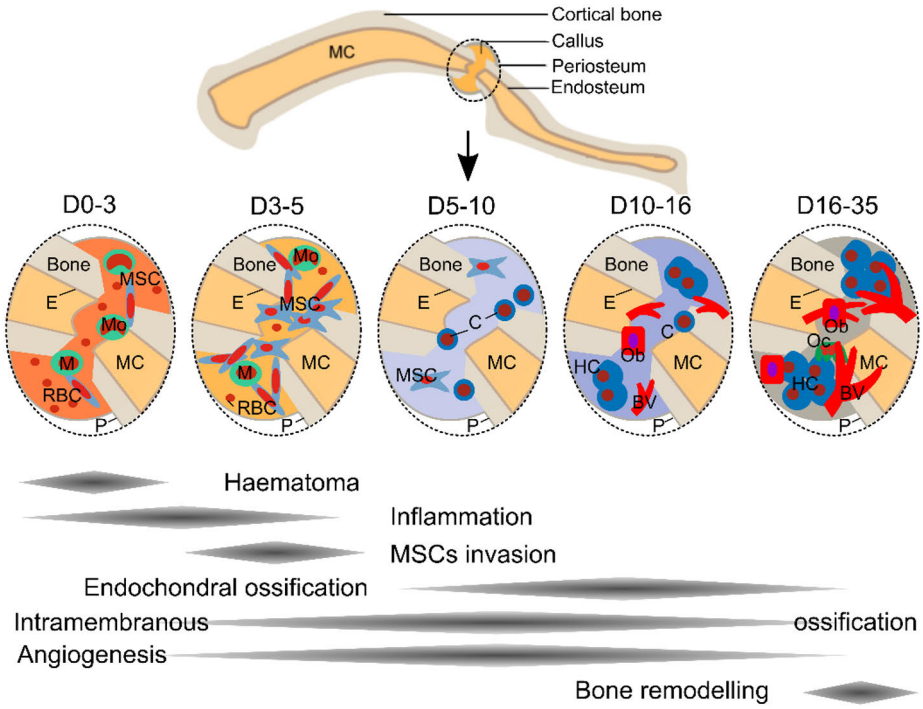


**Figure 3. Stages of long-bone fracture healing in mice.** D: Day, MC: medullary cavity, P: periosteum, E: endosteum, MSC: mesenchymal stem cell, RBC: red blood cell, M: macrophage, Mo: monocyte, C: chondrocyte, HC: hypertrophic chondrocyte, Ob: osteoblast, Oc: osteoclast, BV: blood vessel. The grey beams depict the temporal expression patterns for haematoma, inflammation, MSCs invasion, endochondral and intramembranous ossifications, angiogenesis, and bone remodelling phases in relation to time frames (D0-3, D3-5, D5-10, D10-16, and D16-35) and their associated cellular composition.

### 2.1.1 Inflammatory phase

Immediately after fracture, a hematoma forms at the fracture site due to blood vessel disruption, resulting in a fibrin-rich granulation tissue for initial stability to the fracture. Hematoma represents the first phase of inflammation (Kolar et al., 2010; Schell et al., 2017). Inflammation takes place strictly after fracture involving the upregulated expression of different molecules such as interleukin-1β and 6 (IL-1β, IL-6), and tumour necrosis factor α (TNF-α) through MSCs, macrophages, and other immune cells infiltrating the hematoma (Dimitriou et al., 2005; Einhorn et al., 1995; Kon et al., 2001). These inflammatory molecules are necessary to initiate of fracture repair and angiogenesis (Einhorn & Gerstenfeld, 2015), and anti-inflammatory drugs have been demonstrated to have a negative impact on fracture healing with a delay in ossification and vascularization (Bissinger et al., 2016; Holstein et al., 2008; Satoh et al., 2011).

Macrophages originating from bone lining tissues (osteomacs) and from inflammatory tissue have been shown to have an important role during endochondral ossification. Depletion of macrophages delays intramembranous ossification as well as cartilage mineralization during endochondral ossification (Alexander et al., 2011; Schlundt et al., 2018). Also, T-cells and B-cells have a major role in fracture healing, and depletion of these cell types can lead to either a delay or acceleration in the fracture healing process depending on the cell subsets (Ono & Takayanagi, 2017). IL-1β promotes osteogenesis and inhibits chondrogenesis as well as proliferation and differentiation of MSCs *in-vitro* although its impact on fracture healing *in-vivo* is limited (Lange et al., 2010). Furthermore, IL-1β facilitates the formation of fibrin clots formation important for fracture healing initiation (X. Wang, Friis, et al., 2016; X. Wang, Luo, et al., 2016). IL-6 modulates the activity of osteoblasts and osteoclasts by having both positive and negative effects on osteoblast and osteoclast differentiation (Blanchard et al., 2009). TNF-α is an important cytokine in the recruitment and chondrocyte differentiation of MSCs, apoptosis of hypertrophic chondrocytes as well as in cartilage resorption. A loss of TNF-α results in a delay in these steps (Gerstenfeld et al., 2003). Degranulating platelets present in the hematoma release transforming growth factor β1 (TGFβ1) and platelet-derived growth factor (PDGF) necessary to initiate the fracture healing process (Bolander, 1992; Dimitriou et al., 2005). Furthermore, TGF-β released from degranulating platelets, bone, and extracellular matrix (ECM) enhances osteo-chondrogenic differentiation by stimulating MSCs (Patil et al., 2011; Tsiridis et al., 2007). Altogether, these signalling molecules are very important to ensure complete fracture repair. Most of them remain expressed throughout all stages of fracture healing (Dimitriou et al., 2005).

### 2.1.2 Recruitment and invasion of MSCs

Stem cells are divided into four types depending on their differentiation potency. *Unipotent* stem cells can differentiate into only one cell type, *oligopotent* cells can differentiate into a restricted niche of cells, *multipotent* stem cells differentiate into a broader range of cells than oligopotent, *pluripotent* stem cells also called embryonic stem cells will give rise to all cell types of the embryo proper whereas totipotent stem cells give rise to all type of cells including embryonic cells. In addition to these cell types found in animals, there is another artficial cell type called induced pluripotent stem cells (iPSCs), which are somatic cells reprogrammed to become pluripotent. In 2006, Takahashi and Yamanaka reprogrammed fibroblasts to become iPSCs, by overexpressing Octamer-binding protein 4 (Oct4), The Sex-Determining Region Y-type high motility group Box 2 (Sox2), Kruppel like factor 4 (Klf4), and c-Myc transcription factors (K. Takahashi & Yamanaka, 2006). MSCs

are multipotent cells which can differentiate into osteoblasts, chondrocytes, myocytes, stromal cells, fibroblasts, and adipocytes (Shariatzadeh et al., 2019). They are important in the initiation of fracture healing and are recruited to the fracture site to initiate the formation of the soft callus via differentiation into chondrocytes. MSCs are also crucial to form the hard callus, by differentiating into osteoblasts in the process of endochondral and intramembranous ossifications. MSCs are adherent cells and express specific surface antigens such as CD73, CD90, and CD105 (Bragdon & Bahney, 2018). MSCs originate from the periosteum (membrane present on the outer layer of bone), endosteum (membrane lining the medullary cavity of long bones), and bone marrow. Osteoblasts derived from MSCs originated from all three locations whereas chondrocytes are derived from MSCs mainly originating from the periosteum (Colnot, 2009; Murao et al., 2013). MSCs recruitment and migration are proven to be crucial steps for bone regeneration, angiogenesis, and bone remodelling. The recruitment of MSCs has been shown to be mediated by stromal cell-derived factor-1 (SDF-1) and its receptor chemokine receptor 4 signalling axis (CXCR-4) (Kitaori et al., 2009; J. Ma et al., 2005). These studies indicated increased SDF-1 expression at the periosteum during the inflammatory phase of fracture healing. The complex SDF-1/CXCR-4 induces MSCs recruitment and migration, and thereby, enhances the endochondral ossification. SDF-1 also enhances neo-angiogenesis during endochondral ossification (Kawakami et al., 2015). Migration of MSCs has been found to be induced by TGF-β1 (Y. Tang et al., 2009) having an important role in osteogenesis and osteoclastogenesis (Janssens et al., 2005).

## 2.1.3 Endochondral ossification

### 2.1.3.1 Chondrogenesis

Endochondral ossification necessitates the migration of the MSCs from the periosteum to gather, proliferate, and condensate between both fracture ends. BMPs, expressed by osteoprogenitors, MSCs, osteoblasts, and chondrocytes, are important in chondrocyte and osteoblast differentiation, and angiogenesis (Deckers et al., 2002; Dimitriou et al., 2005; Shu et al., 2011). BMP2 in particular is an important growth factor in the initiation of the fracture healing (Dimitriou et al., 2005; Shu et al., 2011; Tsuji et al., 2006). Undifferentiated MSCs will subsequently differentiate into chondrocytes and secrete type II collagen and proteoglycans to create the cartilage matrix (Phillips, 2005). Chondrocyte differentiation is triggered by the expression of different transcription factors (TFs). Sox9, Sox6, and Sox5 are crucial TFs in the differentiation process of chondrocytes; together, they form the so-called Sox trio that is important in the regulation and maintenance of chondrocyte phenotype

(Akiyama et al., 2002; de Crombrugghe et al., 2001; Han & Lefebvre, 2008; Wegner, 1999). Sox9 is a major TF and has a key role in the chondrogenic differentiation of MSCs. Sox9-deficient embryonic stem cells fail to form cartilage (Bi et al., 1999). Sox9 regulates the expression of a wide number of key molecules associated with chondrocyte phenotype, including aggrecan (*Acan*), hyaluronan, proteoglycan link protein 1 (*Hapln1*), cartilage oligomeric matrix protein (*Comp*), type II collagen (*Col2a1*), type IX collagen (*Col9a1*), type XI collagen (*Col11a2*), and secondary ossification centre associated regulator of chondrocyte maturation (*Snorc*) among many other molecules (Bridgewater et al., 1998, 2003; Genzer & Bridgewater, 2007; Han & Lefebvre, 2008; Heinonen et al., 2011; Jaiswal et al., 2020; Kou & Ikegawa, 2004; Lefebvre et al., 2007; C. Liu et al., 2007). Proliferative chondrocytes synthesize type II collagen and aggrecan which together form the basic structural components of the cartilage primordia and future cartilage (K. Hata et al., 2017).

During endochondral ossification, chondrocytes cease proliferating and become pre-hypertrophic. Pre-hypertrophic chondrocytes express Pth-related protein receptor (PTHrP) as well as Indian hedgehog protein (Ihh). Pre-hypertrophic chondrocytes ultimately differentiate into hypertrophic chondrocytes and start expressing the matrix metalloproteinase 13 (Mmp13) and type X collagen (Sandberg et al., 1989). The formation of a perichondrium starts in the outer layer of aggregated MSCs, and at the centre of the callus, chondrocytes become hypertrophic (Kronenberg, 2003). Formation of a cartilage matrix gives greater stability to the callus which at this stage is also called a soft callus (Figure 4).

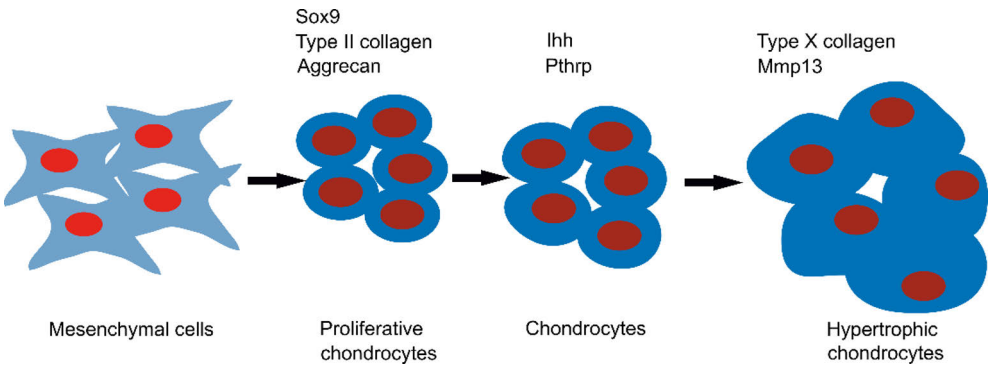


**Figure 4. Stages of chondrocyte differentiation.**

### 2.1.3.2 Osteogenesis

Following the chondrocyte hypertrophy, the soft callus undergoes mineralization to form a hard callus made of woven bone along with vascularization (Barnes et al., 1999; Dimitriou et al., 2005). Osteoblasts originate from different sources during

endochondral ossification. Earlier the prevailing understanding has been that the hypertrophic chondrocytes undergo apoptosis and osteoblast invade the callus from the perichondrium, from the pericytes or via differentiation of osteochondral progenitors from the vasculature (Colnot et al., 2004, 2005). However, several studies have provided evidence on the ability of hypertrophic chondrocytes to directly transdifferentiate into osteoblastic cells rather than undergoing apoptosis (Bahney et al., 2014; D. P. Hu et al., 2017; L. Yang et al., 2014; Zhou et al., 2014).

MSCs differentiation into osteoblasts requires the expression of several specific genes. The crucial TFs regulating osteoblastogenesis are runt-related transcription factor 2 (Runx2), also known as core binding factor α1 (Cbfa1) (Komori et al., 1997), and Sp7/Osterix (Nakashima et al., 2002). A lack of either of those factors results in osteoblastogenesis failure and consequently no bone formation. Runx2 activates type I collagen (*Col1a1*), alkaline phosphatase (*Alpl*), bone sialoprotein (integrin binding sialoprotein, *Ibsp*), osteocalcin (bone gamma-carboxyglutamate protein, *Bglap*), and osterix (*Sp7*, *Osx*) genes (Florencio-Silva et al., 2015; Nishio et al., 2006). Osterix inactivation in preosteoblastic cells prevents osteoblastic differentiation (Akiyama et al., 2005). Runx2 is considered the main TF to determine the commitment of MSCs to osteoblastic lineage (Stein et al., 2004). Osteoprogenitors secreting Runx2 and type I collagen proliferate and become pre-osteoblasts characterized by the secretion of alkaline phosphatase. After morphological changes, osteoblasts become mature osteoblasts and secrete osteocalcin, osteopontin, bone sialoprotein, and MMPs forming the ECM (Figure 5) (Florencio-Silva et al., 2015; Jensen et al., 2010).

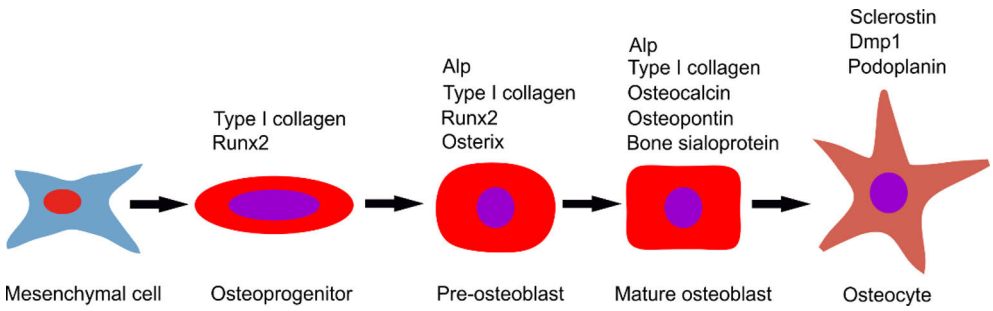


**Figure 5. Stages of osteoblast differentiation**

Mature osteoblasts are active cells with secretory vesicles, large Golgi apparatus, and a large rough endoplasmic reticulum. Osteoblasts secrete to the fracture site unmineralized connective tissue called osteoid which is rich in type I collagen and contains minor amounts of osteocalcin, osteopontin, bone sialoprotein, decorin, and biglycan. New woven bone forms once hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] deposits

mineralize the fibrillar type I collagen network (F. Long, 2011). The transition between mature osteoblastic cells towards the osteocytic cells, called osteocytogenesis, is accompanied by morphological and anatomical changes such as rough endoplasmic reticulum and Golgi apparatus decreases. Sclerostin, dentin matrix acidic phosphoprotein 1 (Dmp1), and podoplanin (E11/gp38 gene) are highly expressed in osteocytes (Florencio-Silva et al., 2015). Podoplanin protein is actively involved in osteocytogenesis, important in cytoskeletal development, and more specifically in the development of dendrites. The lacunocanalicular system connects osteocytes together and facilitates cell-cell communications as well as the transport of nutrients, oxygen, and signalling molecules (Florencio-Silva et al., 2015) (Figure 5). Osteocytes are mature osteoblasts embedded into their secreted mineralized matrix. They are mostly star-shaped (Florencio-Silva et al., 2015). Osteocytes are the most abundant cell type found in bone tissue representing 90 to 95% of all bone cells. Osteocytes have a long lifespan (up to 25 years) compared to mature osteoblasts (about 150 days) (Tresguerres et al., 2020). Osteoblast can also become a bone lining cell if present at the surface of the bone or can even undergo apoptosis (F. Long, 2011). Bone lining cells are quiescent flat-shaped bone cells lining the bone tissue surface. These cells are present in the bone where no formation or resorption occurs, some have cytoplasmic processes for cell-cell communications reaching gap junctions and caniculi. Their function is not well understood but can be involved in osteoclast differentiation by producing osteoprotegerin and Rankl similarly to osteocytes. They also have a role to prevent interactions between osteoclasts and bone matrix (Florencio-Silva et al., 2015).

### 2.1.4 Intramembranous ossification

Intramembranous ossification takes place side-by-side with endochondral ossification, mainly at the periosteum, progressing towards the fracture gap, to increase the stability of the callus (Marsell & Einhorn, 2011). This type of ossification requires the osteoblasts to directly differentiate from MSCs. The osteoblasts start forming new woven bone also called hard callus surrounding the soft callus tissue providing a greater mechanical stability to the fracture site. It was previously shown that inflammatory macrophages and osteomacs were crucial during endochondral ossification. Osteomacs are localised in bone lining tissues, therefore at the site of intramembranous ossification and have been found to enhance intramembranous ossification. Osteomacs produce anabolic factors to enhance osteoblast recruitment, maturation and function (Alexander et al., 2011).

### 2.1.5 Angiogenesis

Blood supply is crucial for successful bone regeneration during endochondral ossification. During the early healing phase, blood is primarily provided by the hematoma which supplies the callus with the necessary cell populations (MSCs, immune cells, macrophages, white and red blood cells), nutrients, and oxygen. After soft matrix formation, the action of MMPs triggers vessel invasion into the callus (Dimitriou et al., 2005; Keramaris et al., 2008). Blood vessel invasion inside the cartilage matrix requires reorganization of the soft callus with the intervention of osteoclast-like cells. MMP9 and MMP13 facilitate blood vessels to transverse the soft callus and allow hypertrophic expansion (Thompson et al., 2015). Vascular endothelial growth factor (VEGF) is an angiogenic stimulator produced by hypertrophic chondrocytes which can also induce migration and differentiation of osteoblasts and osteoclasts (Brouwers et al., 2006). Fibroblast growth factor-2 (FGF-2) and TGF-β1 are important cytokines which were shown to stimulate the expression of VEGF (Saadeh et al., 1999, 2000). MMP9 and MMP13 or VEGF deficiency lead to a delay in endochondral ossification (Gauci et al., 2019). Angiopoietin 1 and 2 are expressed throughout the fracture healing process and contribute to the formation of large vessels as well as collateral branches (Dimitriou et al., 2005).

### 2.1.6 Bone remodelling

After the formation of a hard callus by osteoblasts, osteoclasts are constantly resorbing the callus. In human, a complete remodelling of the fractured area may take several years. However, bone remodelling per se is a continuous process to ensure the maintenance of bone function (W. Wang & Yeung, 2017). The bone remodelling process is a balance between hard callus resorption mainly driven by osteoclasts and lamellar bone formation driven by osteoblasts (Marsell & Einhorn, 2011).

The proliferation and differentiation of osteoclasts are driven by Macrophage Colony-Stimulating Factor (M-CSF) also known as Colony-Stimulating Factor 1 (Csf1). M-CSF is secreted by numerous cells such as osteoblasts, osteocytes, and bone lining cells but also by monocytes, fibroblasts, endothelial cells, and tumour cells (Chockalingam & Ghosh, 2014). After being excreted, M-CSF binds to its receptor (CSF1R). M-CSF is highly expressed in osteoclast precursors and activates their differentiation towards multinucleated osteoclasts. Microphthalmia-associated transcription factor (Mitf) and PU.1 are expressed throughout all stages of osteoclasts differentiation. Mitf, and PU.1 act together to activate tartrate-resistant acid phosphatase gene expression (Tracp, *Acp5*) (Soltanoff et al., 2009). Nuclear Factor Of Kappa Light Polypeptide Gene Enhancer In B-Cells (NF-kB) induces

Tracp expression in osteoclast precursors (Q. Zhao et al., 2007). TNF (ligand) superfamily, member 11 (TNFSF11, Rankl) is mainly produced by osteocytes to form mature osteoclasts and activates its adaptor called TNF receptor-associated factor 6 (Traf6) and nuclear factor of activated T cells 1 (Nfatc1) important for terminal osteoclast differentiation (Soltanoff et al., 2009). Cathepsin K may have a role in osteoclast apoptosis, together with Tracp and calcitonin receptors which are considered markers of mature osteoclasts (Soltanoff et al., 2009) (Figure 6).

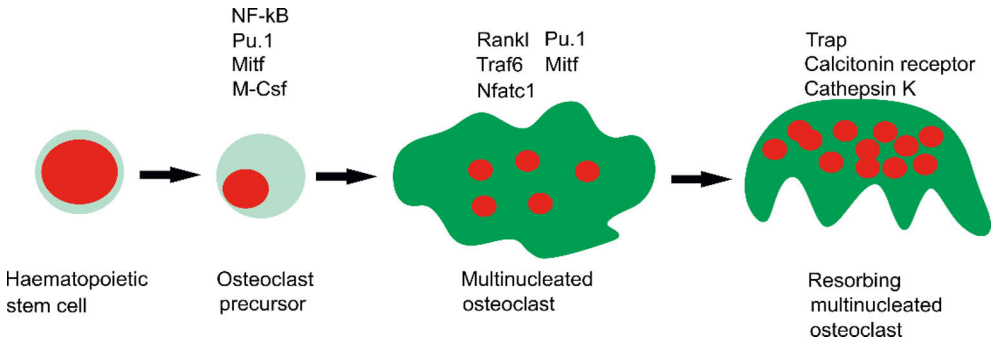


**Figure 6. Stages of osteoclast differentiation.**

Osteocytes act as mechanosensors by detecting pressure on the skeleton and transmit signals to osteoblasts and osteoclasts by releasing cell mediators to control their differentiation and activation (Goldring, 2015). After mechanical or biochemical signalling osteocytes produce Rankl to activate osteoclastogenesis. Furthermore, mechanical loading and unloading affect the Sclerostin expression in osteocytes by regulating the expression of Rankl and osteoprotegerin (OPG) among others. Rankl activates osteoclastogenesis, while OPG represses osteoclastogenesis by competitively binding to Rankl, therefore, repressing Rank/Rankl interaction (Palumbo & Ferretti, 2021).

## 2.2 Non-coding RNAs

The majority of non-coding RNAs were for long classified as 'junk' RNAs or degraded mRNA molecules. They include a large variety of RNA classes such as ribosomal RNAs (rRNAs), transfer RNAs (tRNAs) long non-coding RNAs (lncRNAs), small interfering RNAs (siRNAs), circular RNAs (circRNAs), small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), piwi-interacting RNAs (piRNAs), miRNAs, and more recently discovered tsRNAs and ribosomal-derived small RNAs (rsRNAs). This review focuses on miRNAs and tsRNAs as they were the research topic of this thesis study (Slack & Chinnaiyan, 2019).

### 2.2.1 MicroRNAs

MiRNAs are abundant and widely conserved through evolution. They are small single-stranded, about 22 nucleotides long, non-coding RNA molecules. Mostly, they target the 3' untranslated region (UTR) of the mRNAs to induce translational repression or degradation depending on the extent of complementarity with their targets (Bartel, 2004). The translational repression of the mRNA is less effective if the miRNA binds to the coding region of the mRNA; possibly due to competition with the ribosomal complex (Gu et al., 2009). After their discovery, a miRNA database was established (miRBase) (Kozomara & Griffiths-Jones, 2014). Database version 22.1 from January 2019 contains 38589 entries for hairpin precursor miRNAs and 48885 mature miRNAs in 271 species (http://www.mirbase.org/). The first miRNA was identified in 1993 (R. C. Lee et al., 1993) from *Caenorhabditis elegans* during larval development. Since then, miRNA expression has been associated with practically all biological processes, including organ and tissue development and diseases e.g., osteoarthritis (OA), cancer, and in chondro-, and osteosarcomas (Engin, 2017; Husain & Jeffries, 2017; Maurizi et al., 2018; Oliveto et al., 2017; Oliviero et al., 2019; Palmini et al., 2017; Scimeca & Verron, 2017; Tahamtan et al., 2018; J. Wang et al., 2018; Weiner, 2018; C. X. Yu & Sun, 2018).

#### 2.2.1.1 Biogenesis and function of microRNAs in mammals

MicroRNAs originate from intergenic, intronic, polycistronic, mirtrons or even from exonic regions Figure 7 (Ha & Kim, 2014). In animals, miRNA genes are present in all except male chromosomes. A pri-miRNA is transcribed by RNA polymerase II or III (Borchert et al., 2006; Y. Lee et al., 2004). Primary miRNAs (pri-miRNAs) are capped and polyadenylated, presenting a hairpin-like structure. Drosha (RNase III-type enzyme) in association with DiGeorge syndrome critical region 8 (DGCR8) co-factor are together called the microprocessor. This microprocessor is responsible for the trimming of the pri-miRNA and consequently for the formation of about 70 nucleotides long hairpin precursor-miRNA (pre-miRNA). Pri-miRNAs originate from mirtrons (by-products of intron splicing) by-pass the Drosha-DGCR8 step but instead, the pre-miRNA is generated through mRNA splicing.

Then, the pre-miRNA is exported to the cytoplasm through the Exportin 5 transporter and RAs-related Nuclear protein (RAN-GTP) to be processed by Dicer (RNase III-type enzyme) in association with its co-factor Trans-Activation-Responsive (TAR) RNA-binding protein 2 (TRBP2). Together they trim the pre-miRNA terminal loop into a duplex miRNA (miRNA-miRNA*) of about 20 base pair (bp). The miRNA duplex will be bound by an Argonaute (AGO) protein which is part of a multiprotein complex called RNA-induced silencing complex (RISC).

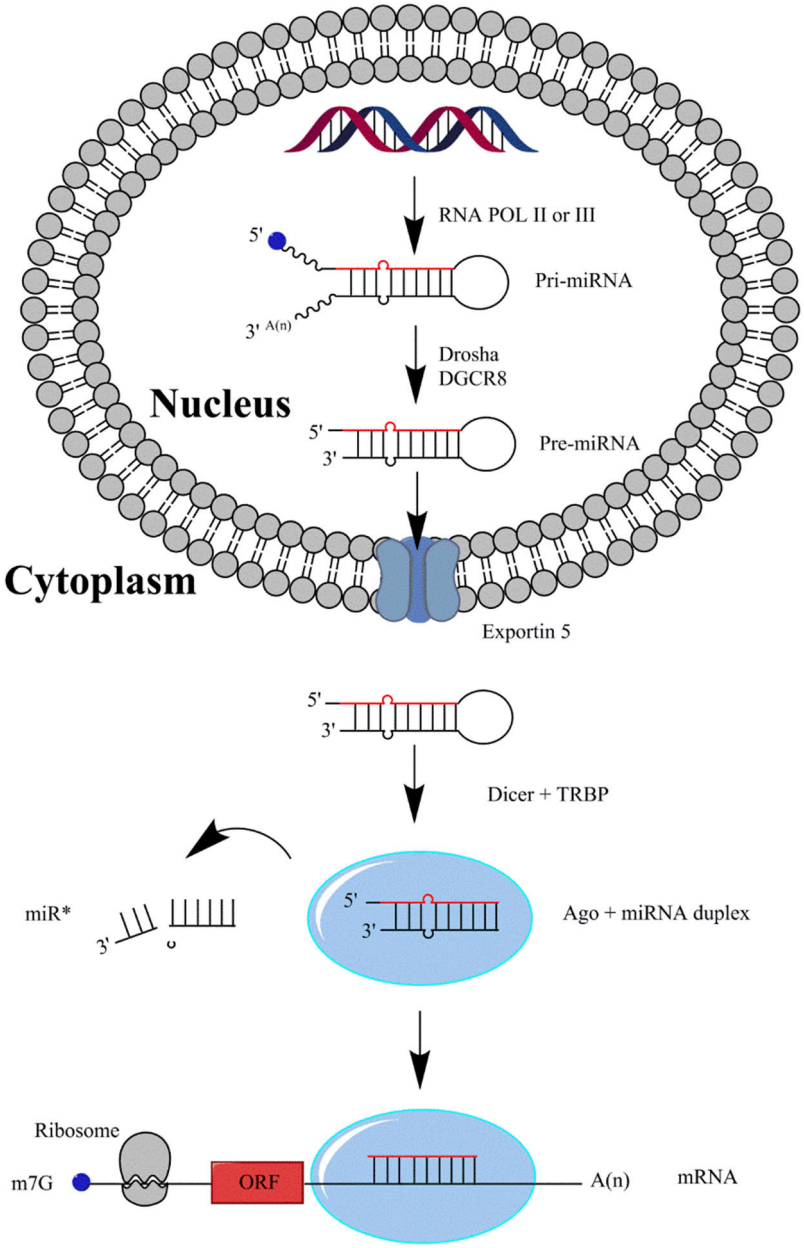


**Figure 7. Canonical miRNA biogenesis pathway.** The miRNA biogenesis requires multiple steps starting from the nucleus where the miRNA precursors are processed by Drosha and DGCR8. Then, miRNA precursors are exported to the cytoplasm through the Exportin 5 and processed by Dicer, TRBP, and by the RISC complex. Following those steps, the mature miRNA will recognize and bind to its target mRNA in association with the RISC complex. m7G: 5′-end capping with 7-methyl guanosine, A(n): poly-A mRNA tail, ORF: Open Reading Frame, pri-miRNA: Primary-miRNA, pre-miRNA: precursor-miRNA, DGCR8: DiGeorge syndrome critical region 8, TRBP: RNA-binding protein, Ago: Argonaute protein, miR*: Passenger miRNA, RISC: RNA-induced silencing complex.

Next, the guide strand of the miRNA duplex will be incorporated into the microRNA-induced silencing complex (miRISC) complex whereas the passenger strand (miR*) is released. The passenger strand is the complementary strand from the pre-miRNA molecule. The miR* strand is either degraded or incorporated into another miRISC complex (Beermann et al., 2016; Ha & Kim, 2014; Krol et al., 2010). The strand selection follows the thermodynamic asymmetry rule. The 5'ends of miRNAs tend to possess a Uracyl (low thermodynamic stability) whereas 3'ends of miRNAs tend to possess a Cytosine (high thermodynamic stability). As a consequence, the miRISC complex will favoured the less thermodynamically stable strand over the high thermodynamically stable (Hutvagner, 2005; Medley et al., 2021). Following the maturation process, occurring in the nucleus and the cytoplasm, the mature miRNA will finally be able to silence the targeted mRNAs. Target recognition is triggered by the seed sequence of a mature miRNA (nucleotides 2 to 8 from the 5' end) within 3′ UTR of the targeted mRNA, dictated by Watson-Crick pairing (Lewis et al., 2005).

MiRNAs are also present in the nucleus (Khudayberdiev et al., 2013; H. Liang et al., 2013; Liao et al., 2010; C. W. Park et al., 2010). Nuclear miRNAs induce post-transcriptional gene silencing, transcriptional gene silencing, gene activation, and modulation of alternate splicing (Roberts, 2014). Recently miRNAs were shown to regulate the maturation of other miRNAs; miR-709 negatively regulates miR-15a and miR-16-1 in mice in the nucleus by targeting the pri-miRNA (R. Tang et al., 2012).

#### 2.2.1.2 Nomenclature

Regarding the miRNA nomenclature, it is important to avoid any confusion between species and types of miRNAs. MicroRNAs of animal origin are annotated with a ``-´´ (e.g., miR-1 for *Homo sapiens* and miR161 for *Arabidopsis thaliana*). Precursor animal miRNAs are annotated in lowercase and italics (e.g., *mir-1*) whereas when the letter ``r´´ is capitalized it means that the miRNA is matured (e.g., miR-1). For plants, when all letters are capitalized and in italics, it refers to the gene(s) necessary for the miRNA biosynthesis (e.g., *MIR161*). When the same mature miRNA sequence is shared between different species then a three-letter prefix is attached to the miRNA name (e.g., mmu-miR-16 and hsa-miR-16) where ``mmu´´ stands for *Mus musculus* and ``hsa´´ stands for *Homo sapiens*. When two pre- or pri- miRNAs produce a similar mature miRNA sequence, the mature miRNA is annotated with a number (e.g., mmu-miR-194-1 from chromosome 1 and mmu-miR-194-2 from chromosome 19). Two evolutionary-related miRNAs with similar sequences are annotated with a small letter (e.g., mmu-miR-181a and mmu-miR-181b). When two mature miRNAs originate from opposite arms of the common pre-miRNA, the

mature miRNA will be annotated with a ``-5p´´ or a ``-3p´´ suffixes; the two mature miRNAs have different sequences and therefore different targets (Ambros et al., 2003). Most likely one mature miRNA will be more abundant than the other, an ``*´´ will be added to the least abundant mature miRNA to differentiate them (e.g., mmu-miR-127-5p* and mmu-miR-127-3p) also known as passenger strand. Nonetheless, it also happens that both mature miRNAs are equally represented in the cell as a co-maturation (Beermann et al., 2016; Fromm et al., 2015; Ha & Kim, 2014; Krol et al., 2010).

#### 2.2.1.3 Regulation of microRNA expression

MicroRNA expression is regulated by multiple mechanisms. The first obvious type of regulation is linked to random mutations occurring in genes coding for Drosha, DGCR8, Exportin 5, Dicer or TRBP. Such mutations, disturbing the microRNA processing, were previously linked to generate cancers (A. Hata & Kashima, 2016). Another mechanism for miRNA regulation is their biological half-life partially depending on the percentage of adenosine and uracyl nucleotides, in the miRNA sequence. Several enzymes such as mitochondrial poly(A) polymerase (MTPAP), poly(A) RNA polymerase D4 (PAPD4), poly(A) RNA polymerase D5 (PAPD5), zinc finger CCHC-type containing 6 (ZCCHC6), zinc finger CCHC-type containing 11 (ZCCHC11), and terminal uridylyl transferase 1 (TUT1) are known to destabilize the mature miRNA, by adding nucleotides to the 3' end. Some molecules, however, protect mature miRNA from degradation such as AGO itself which increases miRNA half-life when overexpressed. MicroRNA editing could affect the half-life of the molecule as well as target recognition. Adenosine deaminases were found to edit mature as well as pri-miRNAs by transforming the adenosine to inosine (A-to-I), and therefore potentially can affect the miRNA seed sequence crucial for target recognition (Gebert & MacRae, 2019; Gulyaeva & Kushlinskiy, 2016).

Depending on their genomic location, miRNAs are classified as intergenic or intragenic. Furthermore, intragenic miRNAs are found in intronic, exonic or junction locations or even in the antisense strand of a gene. Intragenic miRNA expression is sometimes co-expressed with their host gene, where the miRNA is encoded. Particularly, this co-expression happens when the miRNA does not possess a promoter on its own. MicroRNAs originating from mirtrons belong to this category. Co-expressions have been described in several microarray analyses between miRNA and their host genes (Baskerville & Bartel, 2005; Luedde, 2010; Ronchetti et al., 2008). This co-expression is the result of crosstalk between the RNA splicing complex and the miRNA microprocessor complex (Agranat-Tamir et al., 2014; B. Liu et al., 2018). It was reported that co-expression is less likely to happen for evolutionary less-conserved miRNAs (He et al., 2012).

On the other hand, competition occurs in some cases between the microprocessor and the spliceosome. This is found when the miRNA originates from the junction site. In cases of competition, the spliceosome complex does not recognize the exon, and instead, the microprocessor complex binds to the RNA transcript and generates a pre-miRNA skipping the exon in mRNA production. In some cases, the exon is processed by the spliceosome complex before the miRNA microprocessor recognizes the miRNA promoter. Therefore the pre-miRNA is not generated, and instead, the spliced variant containing the alternative exon is produced (Melamed et al., 2013; Q. Sun et al., 2020).

Intra- and intergenic miRNA expression is regulated by the expression of TFs binding to the regulatory elements of the pre-miRNA (when present) (Arora et al., 2013). Alternative processing from Drosha and Dicer could affect miRNA length and/or sequence and ultimately generate an isomir, which is a variant of a miRNA. Isomirs may ultimately recognize a different target than the original miRNA (Gebert & MacRae, 2019). Long non-coding RNAs have been reported to interact with miRNAs by binding on the mature miRNA molecule to ultimately prevent the miRNA-mRNA binding (López-Urrutia et al., 2019; Tornesello et al., 2020). Similarly, circRNAs have been reported to sequester miRNAs in cervical cancer tissues (Tornesello et al., 2020).

Also, multiple other factors such as methylation of miRNA promoters, hormones, stress factors, anoxia or xenobiotics affect miRNA expression (Gulyaeva & Kushlinskiy, 2016).

#### 2.2.1.4 Evolution

MicroRNAs are widely expressed in plants and throughout the metazoan group comprising all multicellular animals (Cui et al., 2017; Wheeler et al., 2009). Some miRNA families are highly conserved amongst animal taxa. For example, the mir-200 family (miR-200a, miR-200b, miR-200c, miR-141, and miR-429) is highly conserved within the Animalia kingdom. The mir-200 family is divided into two gene clusters; the tricistronic miR-200a/200b/429 transcribed from a common promoter located on mouse chromosome 4 and human chromosome 1p36 and the bicistronic miR-200c/141 also transcribed from a common promoter located on mouse chromosome 6 and human chromosome 12p13 (Trumbach & Prakash, 2015). Those two clusters differ by one nucleotide located at position 4 on the miRNA seed sequence. The same miRNA family specifically from the bicistronic cluster is found in *Drosophila melanogaster* under the name of miR-8. Due to this nucleotide difference, the two groups have different targets but also have some common targets. Any mutation in the seed sequence will result in a different miRNA name with different target recognition. This miR-200 miRNA family was

found to regulate neurogenesis throughout all taxa (Trumbach & Prakash, 2015). It was shown that the evolution of miRNAs is very slow across the metazoan group and therefore they are highly conserved. For the last 800 million years, the acquisition of new miRNAs remained constant, mostly erupting by gene duplication, seed shifts, and 5' edits from pre-existing miRNAs with a minimal miRNA loss (Wheeler et al., 2009).

#### 2.2.1.5 Functions in fracture healing

The role of miRNAs is under constant investigation. A convenient way to evaluate their role, in general, is to perform a conditional knockdown of Dicer under a specific promoter. Dicer is an essential enzyme for miRNA biogenesis and therefore for cell differentiation and survival. The role of Dicer has been evaluated at different post-embryonic stages and in adult mice (Bendre et al., 2018; Bernstein et al., 2001, 2003; Finnegan & Pasquinelli, 2013; Gaur et al., 2010; Mizoguchi et al., 2010). Dicer deletion in embryos triggers skeletal malformation. These studies indicate that miRNAs are important regulators of bone homeostasis and skeletal tissue maintenance. In 2006, mir-140 was found to be enriched in cartilage and involved in the formation and maintenance of cartilage by targeting histone deacetylase 4 (*Hdac4*) in mouse embryos (Tuddenham et al., 2006). Cartilage is an important tissue for skeletal development and fracture healing as it forms an anlage for the endochondral ossification of future bone tissue.

MicroRNAs are expected to regulate all steps of fracture healing including angiogenesis, chondrogenesis, and bone formation and resorption. Angiogenesis was found to be inhibited by miR-222 which targets c-Kit and therefore represses endothelial cells proliferation and migration and capillary density (Groven et al., 2021; Komatsu et al., 2021). Angiogenesis was found to be repressed by miR-92a, which decreases blood vessel density. Injection of anti-miR-92a combined with anti-miR-335-5p enhanced fracture healing in rats (Komatsu et al., 2021).

Bone tissue is under permanent remodelling via a balance between formation (osteoblast-mediated) and resorption (osteoclast-mediated). Numerous miRNAs have been reported to play a major role in the maintenance of this balance. For instance, miR-455-3p, miR-455-5p, miR-133, miR-433, miR-217, miR-375, miR-505, and miR-497 were found to directly target and inhibit *Runx2* expression and therefore inhibit osteogenesis (Groven et al., 2021; Nugent, 2017; Xiao et al., 2018; Z. Zhang et al., 2015). In C2C12 myoblast cells, miR-214-3p inhibits osteoblast differentiation by targeting *Sp7/Osx* expression (K. Shi et al., 2013). In addition, miR-214 inhibits osteogenesis by targeting the activating transcription factor 4 (*Atf4*) and enhances osteoclastogenesis by targeting phosphatase and tensin homolog (*Pten*) (X. Wang et al., 2013; C. Zhao et al., 2015). MiR-144-3p targets SMAD

family member 4 (*Smad4*) and therefore inhibits osteogenic differentiation and proliferation in C3H10T1/2 cells (Huang et al., 2016).

### 2.2.2 Bioinformatics tools to study miRNA target prediction

Several bioinformatics tools have been designed to identify putative miRNA-mRNA interactions based on the miRNA seed sequence and mRNA 3' UTR. TargetScan, miRanda, and DIANA microT are amongst the most popular tools to identify new miRNA-mRNA duplex interactions. TargetScan has been identified as the most reliable tool as it takes into account if the interaction is conserved, its database includes mRNA isoforms, and its database is up to date (Riffo-Campos et al., 2016). It is still advised to use a combination of several tools to predict miRNA-mRNA interactions as different tools have different algorithms.

Although miRNA-mRNA interactions can be predicted by several tools, a large number of false positives are predicted, possibly due to the complex mechanisms existing between miRNA and target recognition but also most likely due to all possible post-transcriptional miRNA modifications.

### 2.2.3 Transfer RNA-derived small RNAs

Transfer RNAs were first described in *Escherichia coli* in response to bacteriophage infection (Levitz et al., 1990). Transfer RNAs are part of the most abundant group of non-coding RNAs with a size ranging from 73 to 90 nucleotides harbouring a "clover leaf"-shaped secondary structure. In complement to cytoplasmic tRNA, mitochondrial tRNAs are small in size and enriched in adenine and uracil nucleobases affecting their stability. In brief, their canonical role is crucial in the translational machinery by delivering amino acids to ribosomes to translate the genetic information into an mRNA template. The structure of a tRNA molecule contains a T-loop, a variable loop, an anticodon loop, and a D-loop (see Figure 8) (Kirchner & Ignatova, 2015; S. Li et al., 2018; Schimmel, 2018). Non-canonical roles of tRNAs were suggested in response to a cellular stress event including post-transcriptional mRNA regulations (Torres et al., 2019; Tosar et al., 2021). These non-canonical functions are supported by the differential abundance observed between tsRNAs and parental tRNA molecules (Torres et al., 2019).

For decades tsRNAs were considered as degradation products from tRNA molecules while recent studies have shown that they also are important in the regulation of tissue metabolism (Ivanov et al., 2011; H. K. Kim, 2019; Kumar et al., 2016; S. Li et al., 2018; Mleczko et al., 2018; Oberbauer & Schaefer, 2018; Pandey et al., 2021; J. Park et al., 2020; Sharma et al., 2016).

A total of 401 highly confident mature tRNAs and 22 mitochondrial tRNAs are documented in GtRNAdb for the mouse genome (http://gtrnadb.ucsc.edu/) (Thornlow et al., 2020).

#### 2.2.3.1 Biogenesis

The nuclear pre-tRNA molecule is transcribed by RNA polymerase III and undergoes numerous post-transcriptional modifications such as 5' end trimming by RNase_P and 3' end trimming by RNase_Z as well as CCA addition at the 3' terminus by tRNA nucleotidyl transferase 1 (Trnt1) (Cao et al., 2020; Hopper & Nostramo, 2019). Most modifications are required for tRNA stabilization which potentially also affects the biogenesis of tsRNAs. The pre-tRNA molecule is then, folded in a "clover-leaf" shape. According to the tRNA/pre-tRNA molecule cleavage site, tsRNAs are classified into several categories (Figure 8).

RNA Z cleaves pre-tRNA molecule into tRNA-fragment type-1 (1-tRFs), also called 3'U tRFs, 1-tRF molecules do contain a poly-U tail (S. Li et al., 2018). 1-tRF fragments are produced in the nucleus and exported to the cytoplasm by an unknown mechanism (Kumar et al., 2016).

Cleavage of the D-loop and T-loop of the tRNA molecule results in 5-tRFs and 3-tRFs, respectively. According to their length, they are classified with small letters (a, b or c) (Figure 8). Their processing enzymes are still under debate as Dicer was first thought to be the main enzyme processing the 3-tRF and 5-tRF fragments. However, studies have shown that Dicer depletion does not affect the expression of 3-tRFs or 5-tRFs (Cao et al., 2020). Later, a ribonuclease A family member angiogenin (ANG) was found to be the processing enzyme which cleaves mature tRNA to generate 3-tRF and 5-tRF fragments. (Cao et al., 2020; S. Li et al., 2018; Z. Li et al., 2012; J. Park et al., 2020). ANG has multiple functions besides regulating nucleic acid metabolism such as being involved in vascularization, immune responses, and tumorigenesis (Sheng & Xu, 2016).

The internal tRNA fragment (itRF) contains one-half of D-loop with its arm and one part of the anticodon arm with half of the anticodon loop. 2-tRFs, also originating from the internal structure of the tRNA molecule, contain anticodon stems and loop, but their endonuclease is unknown. (Cao et al., 2020).

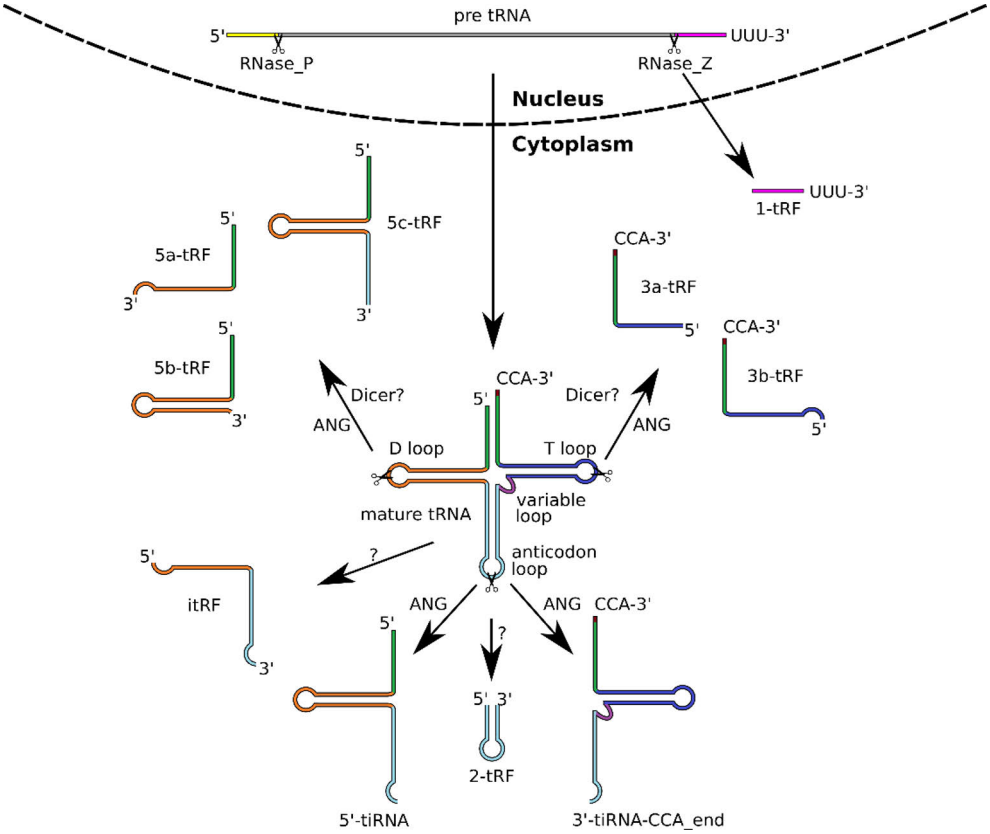


**Figure 8. Biogenesis of tsRNAs** from pre-tRNA molecule in the nucleus and mature tRNA molecule in the cytoplasm. Rnase Z cleaves pre-tRNA to generate 1-tRF fragments. Endonucleases (Angiogenein (ANG) and possibly Dicer) cleave mature tRNA to generate 3'tRFs, and 5'tRFs. The naming of 3' and 5'tRFs depends on their cleavage sites: D-loop (5a-tRF), T-loop (3b-tRF), T-stem (3a-tRF), full D-loop including both arms of the D-stem (5b-tRF) or one arm of the anticodon stem (5c-tRF). 2-tRFs contain the anticodon loop and are generated from the mature tRNA by an unknown endonuclease. The itRF fragments originate from the internal part of mature tRNA containing one arm of the T-loop and one arm from the anticodon loop. Angiogenin (ANG) is a ribonuclease involved in the generation of 3' and 5' tiRNAs. Modified from Qin et al., 2020 (C. Qin et al., 2020).

Under cellular stress such as damage, infection or toxin exposure, ANG cleaves the tRNA molecule at the anticodon loop resulting in two tRNA halves which are also known as tRNA-derived stress-induced fragments (tiRNAs) (Saikia & Hatzoglou, 2015). TiRNAs have a size ranging from 28 to 36 nt and originate from the 5' end or 3' end of the tRNA molecule with a CCA end if originating from the 3' end. Under homeostatic conditions, ANG remains inactive due to its high affinity with RNase inhibitor 1 (RNH1). Under stress conditions, however, ANG dissociates from RNH1 and becomes active resulting in tRNA cleavage. It is

speculated that ANG might not be the only endonuclease-producing tiRNA fragments as tiRNA$^{Tyr}$ fragments were not produced after tissue starvation or ANG overexpression (Fu et al., 2009; Krishna et al., 2019; Saikia & Hatzoglou, 2015; Su et al., 2019). It is still unknown in which conditions ANG is acting but it could be cell-specific or depend on the nature of stress conditions. Altogether, it is plausible that tRF but also tiRNA fragments have different biogenesis pathways depending on their cellular locations.

Transfer RNAs are mostly affected by post-transcriptional modifications which give better stability to the tRNA but can also impact ribosomal interactions and facilitate the "clover leaf"-shaped secondary structure. One specific modification is the 5-methyl cytosine (m5C) which is triggered by DNA methyltransferase 2 (Dnmt2) and NOP2/Sun RNA Methyltransferase 1-6 Nsun1-6. TiRNA biogenesis rate was enhanced in Nsun2-deficient mice thereby suggesting that modification by 5' methylation of cytosine protects tRNA from cleavage by ribonucleases (Krishna et al., 2021). In agreement with that observation, the demethylation of tRNA molecules by AlkB Homolog 3, Alpha-Ketoglutarate Dependent Dioxygenase (ALKBH3) was shown to increase tsRNA production (Z. Chen et al., 2019). Other modifications such as pseudouridylation, or queuosine at the anticodon by substituting guanine were shown to impact the production and/or function of tsRNAs. Pseudouridylation was shown to be required for translational control in stem cells by targeting specific 5' tRFs (Guzzi et al., 2018). Queuosine modification at the tRNA molecule was shown to protect the tRNA$^{His}$ and tRNA$^{Asn}$ from ANG cleavage and therefore reduce their fragment expressions in human cells (X. Wang et al., 2018).

#### 2.2.3.2 Functions

With the use of high-throughput sequencing technology and bioinformatics, tsRNAs have been recently identified in a wide variety of cells and organisms and have a great variety of functions in cellular metabolism. They were discovered to be regulators of mRNA stability in a miRNA-fashioned way, epigenetic regulators by targeting retrotransposons, controlling RNA reverse transcription, inhibiting translational inhibition and elongation, and preventing apoptosis by binding on cytochrome C (Figure 9) (S. Li et al., 2018).

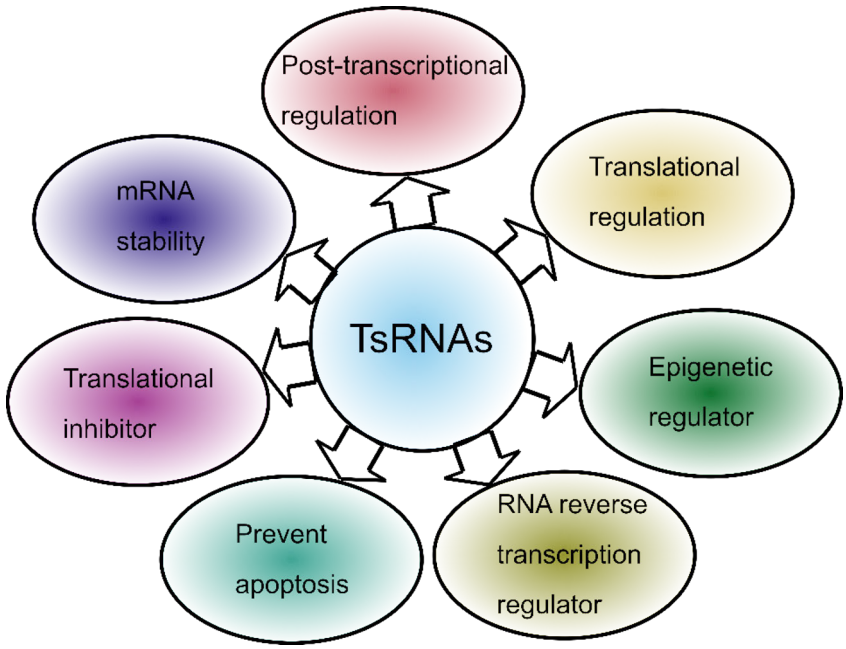


**Figure 9. Identified functions of tsRNAs.**

Under cellular stress, especially under alkaline stress conditions, Val-tRF has been found to inhibit mRNA translation by competing with ribosome binding (Gebetsberger et al., 2017). 5' tsRNA have been reported to inhibit the translation of targeted mRNAs into protein (Sobala & Hutvagner, 2013). 3-tRFs and 5-tRFs were reported to act similarly to miRNAs by promoting the degradation of their target mRNAs. For example, tRNAGly-GCC was found to have an affinity with all four human AGO proteins and destabilize mRNA by binding on 3'UTR of the Replication Protein A1 (*RPA1*) gene similarly to miRNAs (Maute et al., 2013). Similarly to miRNAs, tRFs can regulate gene expression by incorporating into AGO protein. Larger tsRNAs (26-34 nucleotides) were found to behave like piRNAs, by interacting with Piwi proteins and therefore have a potential role in translation regulation in human breast cancer cells (Keam et al., 2014). Also, direct post-transcriptional regulation of mRNA has been observed between tsRNAs and mRNAs based on sequence complementarity (Krishna et al., 2019).

TiRNAs have slightly different functions, compared to tRFs, partially due to their size (28-36 nt). 5'tiRNAs can bind on Y-Box Binding Protein 1 (YBX1) and enhance the formation of stress granules. They were also found to bind on Eukaryotic Translation Initiation Factor 4A,G (eIF4G/eIF4A) to inhibit cap-dependent mRNA translation. In addition, 5' tiRNAs were reported to inhibit mRNA expression via different mechanisms than tRFs without the need for

sequence complementarity (Pandey et al., 2021; Sobala & Hutvagner, 2013). Also, tiRNAs preferentially load into Ago3, due to their larger size and therefore behave like piRNAs, they were also found to load into Ago1 and Ago2 like miRNAs (Chai et al., 2021; Krishna et al., 2021; Tao et al., 2021). Taken together, under stress, tiRNAs with or without the cooperation of YBX1 inhibit translation as a possible protecting mechanism against stress conditions (Emara et al., 2010; Ivanov et al., 2011, 2014; S. Li et al., 2018). TiRNAs have been found to competitively bind on the RNA-binding protein YBX1 and therefore destabilize oncogenic targets of YBX1 (Krishna et al., 2021).

Furthermore, tsRNAs have been found to inhibit the expression of retrotransposons. Retrotransposons are genetic elements which are found in several genomic locations resulting from a mechanism of reverse transcription. In contrast, transposons result from the action of transposases by moving a transposable element from one genomic location to another (J. Park et al., 2020). Retrotransposons are classified into two categories: long terminal repeats (LTRs) and non-LTR including short interspersed nuclear elements (SINEs) and long interspersed nuclear elements (LINEs) (Mita & Boeke, 2016). Retrotransposons are responsible for genomic reorganization and potentially creating genetic diseases. If retrotransposons become active and inserted inside a coding region of the genome they can alter the target gene expression (Mita & Boeke, 2016). Retrotransposons can create genetic disruptions after being inserted in an exon, intron, promoter, enhancer, or even UTRs, and they are also able to create new exons (exonisation) and block transcription once methylated (Figure 10). In humans, retrotransposon DNA sequences compose even about 35 % of the genome (Gorbunova et al., 2021). In mammals, LTRs derive from the endogenous retroviruses (ERV) superfamily and represent about 8% of the human genome. The same ERVs are further classified into sub-families with the most dominant being ERVL, ERVK, and ERV1 (Stocking & Kozak, 2008; Teissandier et al., 2019).

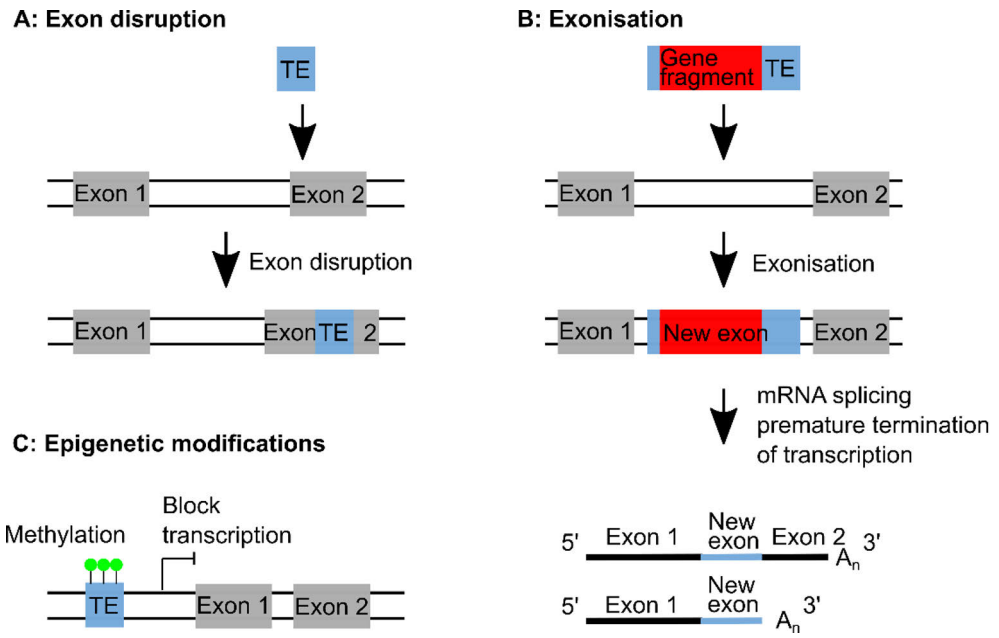


**Figure 10. Principal functions of retrotransposons. A: Exon disruption** resulting from a TE (transposable element) inserted inside an exon. The same could be true for introns, gene enhancer, gene promoter and UTRs; **B: Exonisation** which is the result of a TE incorporated, or not, inside a gene fragment, to form a new exon resulting in some possible alternative mRNA splicing; **C: Epigenetic modifications** which result in blockage of gene expression. Modified from Pradhan and Ramakrishna, 2022 and Savage et al., 2019 (Pradhan & Ramakrishna, 2022; Savage et al., 2019).

Eighteen and 22 nucleotides long 3' CCA-capped tsRNAs have been reported to inhibit the LTR translation by competitively binding to the primer binding site (Schorn et al., 2017). Indirectly, 5' Gly-GCC tiRNA has been found to suppress gene transcription related to mouse endogenous retrovirus type-L (MERVL) LTR-retrotransposon (Sharma et al., 2016). 3' tsRNAs were found to competitively bind on the primer binding site of LTRs, therefore inhibiting the reverse transcription of LTRs by blocking the binding of the mature tRNA (J. Park et al., 2020).

# 3 Aims

Recently, sncRNAs have been recognized as a novel group of factors that regulate body metabolism by interfering with gene expression in multiple and even non-canonical ways. Their potential as tools in novel therapeutic approaches and biomarkers is under eager investigation and data is also emerging on their role in fracture healing. The development of genome-wide sequencing technologies have opened up novel and efficient possibilities to study these molecules in a broader spectrum in multiple tissues and pathological conditions. In this thesis project, genome-wide sequencing approach was utilized to study the expression profiles of small non-coding RNAs (sncRNAs) during fracture healing focusing mainly on tsRNAs and miRNAs. Also, the relationship between DE miRNAs and mRNAs was investigated. Furthermore, retrotransposons were studied as they have been shown to interact with tsRNAs and their connection with fracture healing process has not been studied before.

The specific aims were to

1. Carry out genome-wide profiling of mRNAs and sncRNAs in normal cartilage and bone, as well as in callus tissue during early fracture healing in mice.
2. Study the effect of bone fracture on the expression profiles of sncRNAs, including tsRNAs and miRNAs, during fracture healing from callus tissue in comparison to healthy bone and articular cartilage.
3. Identify miRNA – mRNA target pairs potentially involved in the regulation of fracture healing.
4. Identify sncRNAs from circulation after fracture to serve as biomarkers of fracture healing and potential systemic regulators.

To address these aims, experimental closed tibial fractures were introduced to C57BL/6 male mice. Callus tissue and serum were collected during fracture healing and total RNA was isolated to carry out next-generation sequencing for genome-wide expression analyses of mRNA and sncRNAs.

# 4 Materials and Methods

## 4.1 Research material

### 4.1.1 Animals (**I**, **II**)

C57Bl/6N male mice were used in this study. At the age of two months (70 - 74 days), a standard closed fracture on mouse tibia was performed under anaesthesia using an impact device and after drilling the cortical bone at the proximal head of the tibia, a stainless-steel rod was inserted for fracture stability (Hiltunen et al., 1993; Puolakkainen et al., 2017). When collecting the samples, mice were euthanized using $CO_2$ overdose and blood samples were taken by cardiac puncture. Further, post-natal 10-day old male mice (P10) were used in this study. Mice received a soya-free diet and water *ad libitum.*

### 4.1.2 Ethics (**I**, **II**)

The study plan and use of animal material were approved by the National Animal Experiment Board ELLA (project license ESAVI/6129/04.10.03/2011) and animal care was in accordance with their guidelines following 3R's (Replacement, Reduction, and Refinement) principles. Animals were maintained in the Central Animal Laboratory of the University of Turku.

### 4.1.3 Samples (**I**, **II**)

Samples were collected from two months old control mice (D0, serum, tibial diaphyseal bone and hip articular cartilage), as well as from mice with a fracture at D1 (serum samples), and at D5, D7, D10 and D14 (serum and callus samples) Figure 11 and Table 1. In addition, epiphyseal cartilage was collected from 10-days old male mice for the quantitative polymerase chain reaction (qPCR) normalization analysis of miRNAs (**I**, Supplemental File 1).

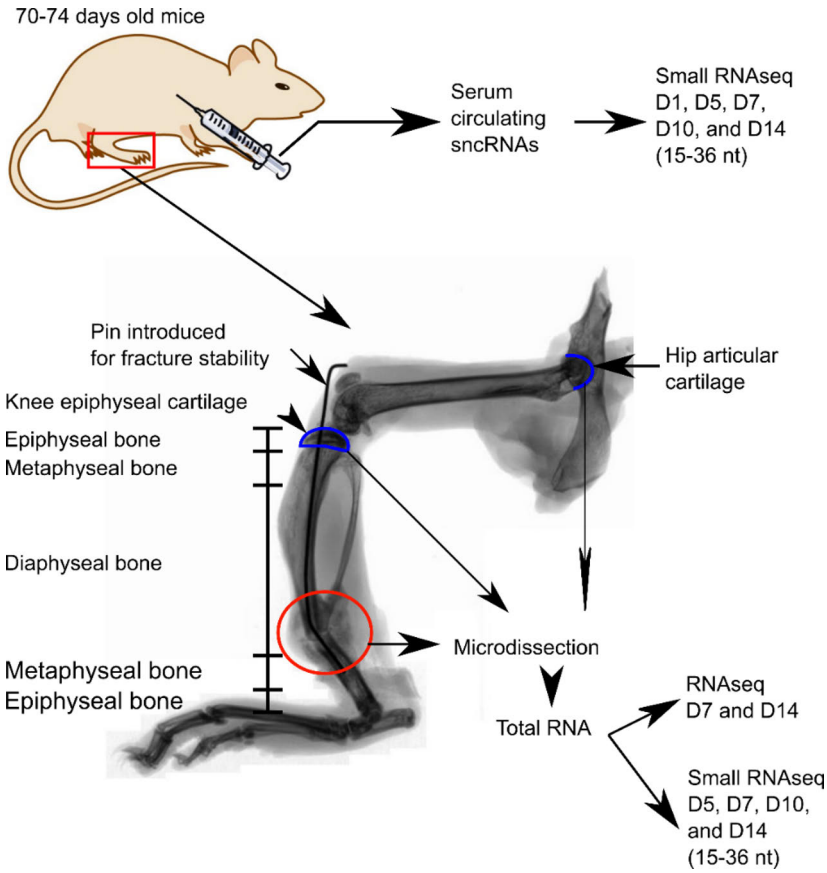


**Figure 11. Schematic representation of the workflow procedure for the sample collection.** X-ray image of an operated hind limb at D14 post-fracture from 2month old male mouse. Intact hip articular and knee epiphyseal cartilage, epiphyseal, metaphyseal and diaphyseal bones were collected from intact mice (control) and screened by next-generation sequencing or qPCR. Serum samples from intact mice (D0, control) were also collected. nt: nucleotide. D: Day.

**Table 1. Samples collected for genomics analyses.**

| Samples | Time points | Analyses | Study |
|---|---|---|---|
| Tibial diaphyseal bone | D0 (n=4) | small RNAseq (n=4) | I |
| Tibial diaphyseal bone | D0 (n=4) | RNAseq (n=4) | I |
| Callus | D5 (n=3), D7 (n=5), D10 (n=4), and D14 (n=2) | small RNAseq | I |
| Hip articular cartilage | D0 (n=3) | small RNAseq | I |
| Callus | D7 (n=4) and D14 (n=4) | RNAseq | I |
| Serum | D1 (n=4), D5 (n=4), D7 (n=4), D10 (n=4), and D14 (n=4) | small RNAseq | II |
| Serum from intact mice | D0 (n=3) | small RNAseq | II |

## 4.2 Research methods

### 4.2.1 Basic evaluation of the fracture model (**I**)

Hind limbs were dissected by removing skin and excess fat and muscle tissue. X-ray imaging was performed to evaluate the position, state, and stability of the fracture after skin removal using Faxitron X-ray MX-20. Thereafter limbs were fixed overnight in 4% paraformaldehyde and decalcified in 10% Na2-EDTA, 0.1 M phosphate buffer, pH 7.0. Samples were embedded in paraffin and cut into 5 mm sections and stained with haematoxylin and eosin or Safranin-O. Slides were imaged for histological analysis using Pannoramic 250 Slide Scanner (3DHISTECH, Budapest, Hungary).

### 4.2.2 Preparation of samples for RNA analyses (**I**, **II**)

Basal (callus, bone, and cartilage) and blood samples were collected for RNA analyses to study RNA and smallRNA expression after fracture in mice (Figure 11, Table 1).

#### 4.2.2.1 Callus, bone, and cartilage samples (**I**)

All samples were dissected free of surrounding muscle tissue, and removed under a stereomicroscope. After dissection, samples were snap-frozen into liquid nitrogen and stored at –80°C for later examination. For RNA isolation, tissues were pulverized at liquid nitrogen and homogenized in TRIsure (Bioline) usingULTRA-TURRAX T 25 (Janke&Kunkel IKA Labortechnik). Subsequently, total RNA was isolated by the addition of phase separation reagents (chloroform and isopropyl alcohol). Total RNA concentration was measured using Nanodrop (Thermo Fisher Scientific).

#### 4.2.2.2 Serum samples (**II**)

Blood samples were collected via cardiac puncture. After blood extraction, samples were allowed to clot for 60 minutes and centrifuged for 10 minutes at 3,000 x g. The supernatant was further centrifuged for 2 minutes at 3,000 x g to remove all cells and serum was snap-frozen into liquid nitrogen in 500 µl aliquots. Following the manufacturer's instructions, the exosomes were isolated from serum samples using ExoQuick (System Biosciences, ref# EXOQ5A-1), and RNA fraction was isolated using miRNeasy Mini Kit (Qiagen, cat# 217004). RNA concentration was measured using Nanodrop (Thermo Fisher Scientific).

### 4.2.3 Quantitative real-time PCR analysis (**I**)

Quantitative PCR analyses were performed using Bio-Rad instruments, models CFX384™ and CFX96™.

Relative messenger RNA expression was quantified using SensiFAST ™ cDNA Synthesis kit (Bioline) for cDNA synthesis from 500 ng of total RNA followed by a qPCR step performed using DyNAmo Flash SYBR Green qPCR kit (ThermoFisher Scientific) with 10 ng of cDNA as a template. ΔCT was calculated using the geometric mean from *Tubb_5* and *Actb* gene expression.

Relative miRNA expression was quantified using miScript II RT Kit (Qiagen, cat# 218161) for reverse transcription step from 500 ng of total RNA followed by a qPCR step using miScript SYBR Green PCR Kit (Qiagen, cat#: 218073) with 10 ng of cDNA as a template. ΔCT was calculated using the geometric mean from a set of stable miRNAs including let-7g-5p, miR-98-5p, and let-7d-3p. MiRNAs stability was analysed in I, Supplemental file 2, Figure 6.

### 4.2.4 Library preparation and sequencing (**I**, **II**)

Small RNA libraries were prepared using TruSeq Small RNA Sample Preparation Kit (Illumina, USA) according to the manufacturer's protocol and sequenced by MiSeq V3 flow cell using Illumina MiSeq reagent kit V3 (**I**, **II**).

RNAseq was done using Illumina TruSeq® Stranded mRNA Sample Preparation Kit and Guide (part #15031047) for HS protocol using 300 ng of total RNA as a starting material and sequenced by the Illumina HiSeq3000 instrument.

Sequencing was carried out at Turku Bioscience Centre, Turku, Finland (**I**).

### 4.2.5 Bioinformatics (**I**, **II**)

The pipeline used for bioinformatics analyses is summarized in Figure 12. All samples were subjected to quality control using FastQC version 0.11.5 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Raw sequence reads were trimmed using FASTX-Toolkit version 0.0.13 for Linux (32Bit).

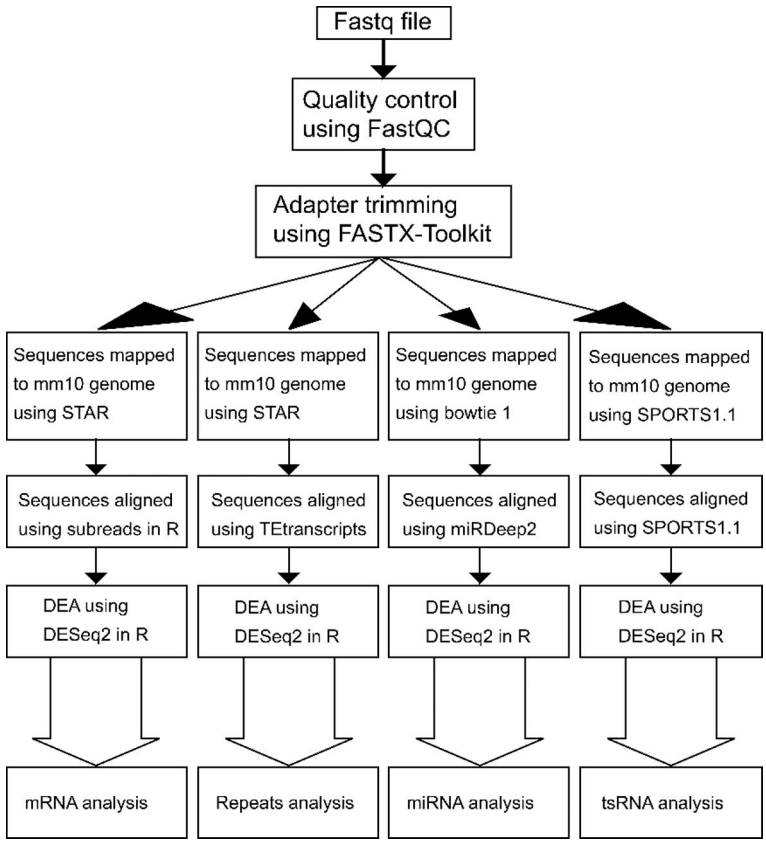


**Figure 12. Simplified diagram of the bioinformatics pipeline for small non-coding next generation sequencing (NGS) data analyses. DEA: Differential expression analysis.** FASTX-Toolkit (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), DESeq2 (Love et al., 2014), STAR (Dobin et al., 2013), miRDeep2 (Friedlander et al., 2012), SPORTS (J. Shi et al., 2018), TEtranscripts (Jin & Hammell, 2018).

#### 4.2.5.1 Mapping and alignment of long RNA sequences (**I**, **II**)

Trimmed sequences were mapped to the mm10 genome using STAR version (v. 2.4.2a) (Dobin et al., 2013b) and aligned using the subreads package (v. 1.5.0) in R (v. 3.0.1). In addition, repeatable elements (including retrotransposons) were mapped using STAR (v. 2.7.3) (Dobin et al., 2013b) with recommended parameters –outFilterMultimapNmax 100 and –outAnchorMultimapNmax 100 and aligned using TEtranscripts (v. 2.2.1) (Jin & Hammell, 2018). Location information of transposable was downloaded from UCSC and provided by RepeatMasker (Smit, AFA, Hubley, R & Green, P. *RepeatMasker Open-4.0*. 2013-2015 <http://www.repeatmasker.org>).

#### 4.2.5.2 Mapping and alignment of small non-coding RNA sequences (**I**, **II**)

Size distribution profiles of the small RNAs falling within the fraction size range of 15 to 36 nucleotides selected for library preparation were analysed in the callus and control bone samples (Figure 13). Most of the reads were annotated as miRNAs and tsRNAs. A small proportion was rsRNAs and no reads belonging to YRNA-derived fragments category were detected. YRNA-derived fragments range between 22-36 nucleotides mostly present in human tissues (Dhahbi, Spindler, Atamna, Boffelli, et al., 2013; Guglas et al., 2020).

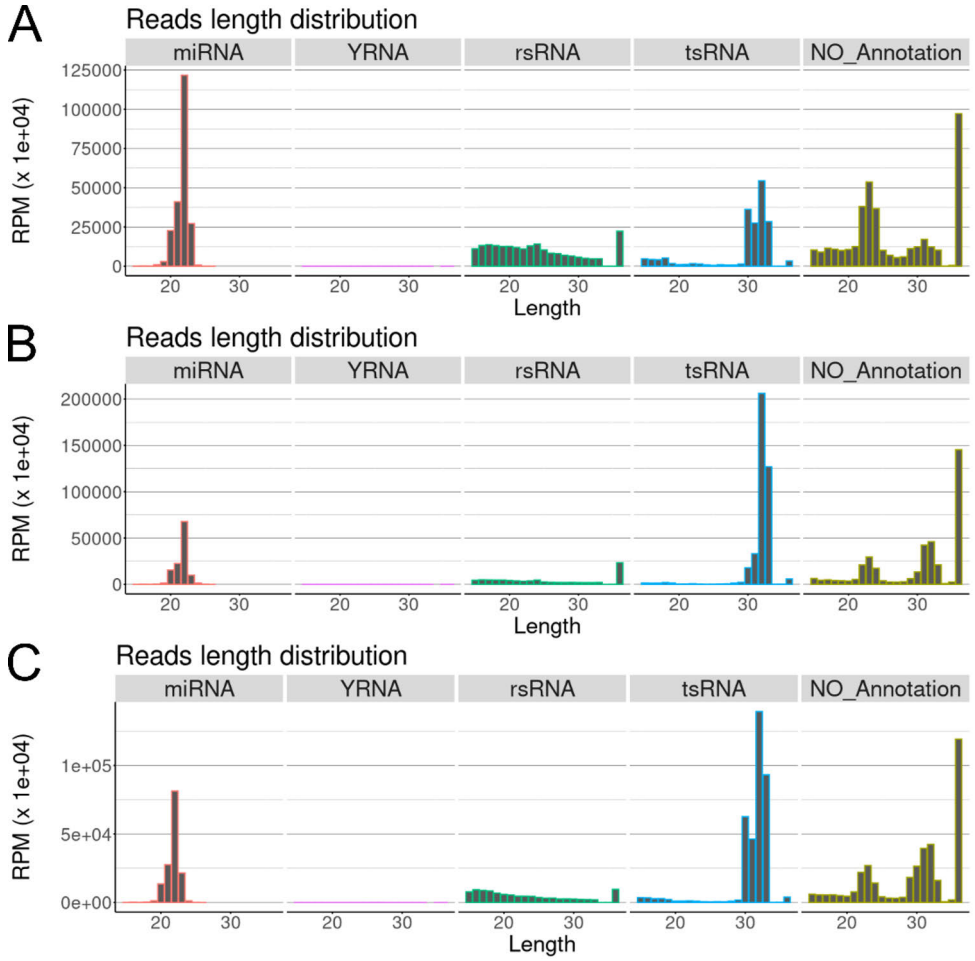


**Figure 13. Distribution of the small RNA reads by size.** Average Reads per million (RPM) **(A):** in control tibial diaphyseal bone (D0, 2 months old mouse) (n = 4); **(B)** in callus tissue at D7 post-fracture (n = 5) and **(C):** in callus tissue at D14 post-fracture (n = 2).

For miRNA analysis, trimmed sequences were mapped to the reference genome (mm10) using Bowtie (v. 1.2.2) and aligned using miRDeep2 (v. 2.0.1.2) (Friedlander et al., 2012). For tsRNA analysis, trimmed sequences were mapped and aligned using SPORTS1.1 (v. 1.1.1) (J. Shi et al., 2018). TiRNAs were analysed from serum and callus samples due to their observed prevalence in size distribution profiles of the small RNAseq reads from callus samples.

#### 4.2.5.3 Differential expression analyses (**I**, **II**)

Differential expression (DE) analyses were performed using DESeq2 (v. 1.20.0) (Love et al., 2014) in R. Raw data were filtered to a minimum raw count of 10 reads for each molecule.

### 4.2.6 Data mining (**I**)

To understand the possible role of DE miRNAs during fracture healing, data mining was performed by searching their verified mRNA targets in published data available via PubMed. All available literature by June 2020 covering the validated targets of 54 DE miRNAs was uncovered. Then, Spearman correlation analysis was performed to determine whether the miRNA-mRNA relationship was detected in smallRNAseq and RNAseq data, evidenced by a significant negative or positive correlation between their expression levels.

### 4.2.7 Statistical analyses (**I**, **II**)

In qRT-PCR analysis for gene expression, statistical significance between two groups was analysed by T-test. Group comparisons were determined by analysis of variance (ANOVA) with Tukey post hoc test after assessing the normal distribution using a Shapiro-Wilk test and calculating the equality of variances using Levene's test. When any assumption for T-test or ANOVA test was violated such as data distribution or normality of the data, a non-parametric equivalent test was used instead.

$P_{adjusted}$ values reported by DESeq2 corresponds to P-values adjusted with Benjamini and Hochberg methods (Benjamini & Hochberg, 1995).

A Spearman rank-order correlation test was used to determine the correlation between miRNA-mRNA expressions or between NGS and qPCR results.

P-values below 0.05 were considered significant for callus tissue **I** and 0.1 for serum samples **II**.

# 5 Results

## 5.1 Fracture healing model (**I**, **II**)

To study the effect of fracture on sncRNA, mRNA, and retrotransposon profiles, a closed tibial fracture was generated on C57BL/6 male mice to subsequently carry out genome-wide sequencing (**I**). The fracture was evaluated macroscopically via X-ray imaging and microscopically using both haematoxylin and eosin and Safranin O staining (**I**, Figure 1). Both methods were used to evaluate the progression of the fracture healing and to detect the gradual formation of the soft-callus, which itself is reorganized and replaced with the hard-callus. In addition, several mRNA targets including *Sox5*, *Runx2*, *Col2a1*, *Sp7*, *Acan*, and *Bmp2*, and miRNA targets including miR-148a-3p, 140-3p, 214-3p, 150-5p, and 340-5p were further quantified by qPCR and correlated with NGS data for validation (Figure 11, Figure 14, **I**, Figure 1). Figure 14, shows the expression of cartilage- and bone-associated molecules following the progression of the callus transformation. Macroscopic and microscopic imaging point out the transformation of the callus tissue which becomes clearly defined macroscopically and hardens over time. Microscopically, at D5, the invasion of MSCs is clearly seen, which subsequently differentiate into chondrocytes (observed at D7, **I**, Figure 1). Then, hypertrophic chondrocytes were observed at D10 onwards (**I**, Figure 1). Finally, at D25, a replacement of the chondrocytic cells by bony cells was observed (**I**, D25, Figure 1). Expression of *Sox5*, *Col2a1*, *Acan*, and miR-140-3p indicated progression of the chondrogenic phase of fracture healing with a peak at D5-D14. Expression of *Runx2*, *Sp7*, *Bmp2*, and miR148a-3p was progressively increased with a peak at D14, indicating a major osteogenic phase at D14. The expression of miR-340-5p and miR-150-5p were associated with bone tissue (**I**, Figure 4) with a peak of expression at D0 ad D25 to maintain bone-tissue homeostasis (**I**, Supplemental Figure 7).

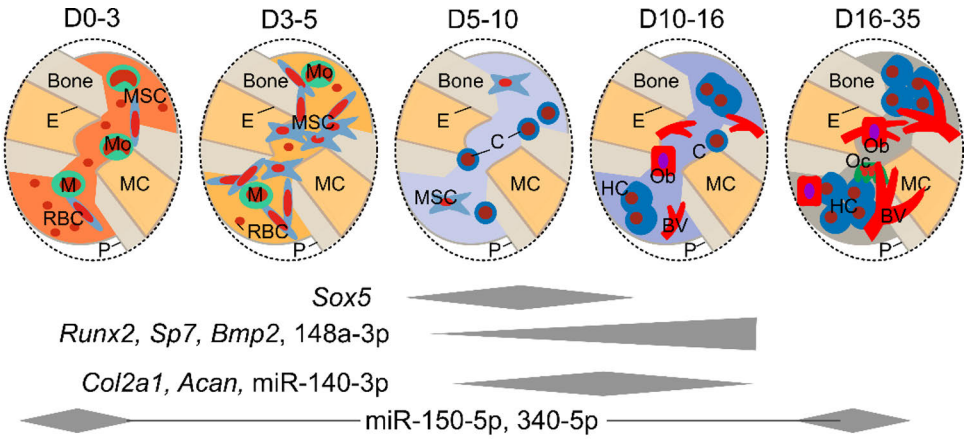


**Figure 14. Validation of the fracture healing model in conjunction with the expression of selected mRNAs and miRNAs.** Cartilage-specific mRNA targets such as *Sox5*, *Col2a1*, and *Acan* and cartilage-specific miRNAs (identified in **I**, Figure 4) as miR-148a-3p and miR-140-3p. Bone-specific mRNA targets such as *Runx2*, *Sp7*, and *Bmp2* and bone-specific miRNAs (identified in **I**, Figure 4) as miR-150-5p and miR-340-5p. Targets were validated via qPCR (**I**, Figure 1, Supplemental Figure 7). D: Day, MC: medullary cavity, P: periosteum, E: endosteum, MSC: mesenchymal stem cell, RBC: red blood cell, M: macrophage, Mo: monocyte, C: chondrocyte, HC: hypertrophic chondrocyte, Ob: osteoblast, Oc: osteoclast, BV: blood vessel. The grey beams depict the temporal peaks of expression for the molecules cited above in relation to the time frames shown above (D0-3, D3-5, D5-10, D10-16, and D16-35) and their associated cellular composition.

## 1.1 RNA expression is affected in the fracture healing model (**I**, **II**)

Small non-coding RNAs were analysed after fracture to study the effect of fracture healing on their expression and the possible effects they may have on the expression of their mRNA targets in the tissue. Samples containing small RNA fractions ranging between 15 and 36 nucleotides were processed for NGS analyses (**I**, **II**). This size range covers miRNAs and tsRNAs, including tiRNAs and tRFs, as shown above in Figure 13.

The majority of tiRNAs originated from the 5' end (> 95%) of the tRNA molecule (**I, II**). The observed low amount of 3' end tiRNA is most likely linked to the used library preparation chemistry which is not adapted to the detection of modifications present at the 3' end of the tiRNA molecule (Honda et al., 2015; J. Shi et al., 2021). Under cellular stress, angiogenin expression increases triggering the synthesis of tiRNAs (Saikia et al., 2012; Yamasaki et al., 2009). In callus tissue samples, as well as in bone, 191 tsRNAs were identified. In serum, 36 tsRNAs were identified.

For target prediction purposes of miRNAs, also RNAseq was performed in control diaphyseal bone and callus tissues at D7 and D14 (**I**). This data covers the

genome-wide expression of mRNAs and repeatable elements, including retrotransposons which were analysed afterwards (unpublished). In callus tissue samples, as well as in bone, and hip cartilage samples, 806 miRNAs were identified. In serum, 290 tsRNAs were identified. In addition, 18700 mRNAs were found in RNAseq data in callus tissue and bone samples.

Interestingly, tiRNAs have been shown to directly target mRNAs and therefore to play a role as post-transcriptional regulators. However, the role of tiRNAs is not limited to target mRNAs but also retrotransposons and particularly LTRs (Sharma et al., 2016). For this reason, differential expression of retrotransposons was also performed in callus NGS RNAseq data to find out if retrotransposons have a role during fracture healing. A total of 1148 repeatable elements including retrotransposons (LTRs, SINEs, LINEs) but also simple repeats, DNA and RNA fragments, and satellites were found at D7 and D14 post-fracture in callus tissue and bone samples.

### 5.1.1 The expression of tiRNAs is affected by the fracture

The expression levels of tsRNAs, including tiRNAs and itRFs, were assessed in callus tissue and serum after fracture. Altogether, eleven tsRNAs were highly expressed, in callus tissue at a level over 1000 reads (baseMean). The high threshold was arbitrarily selected to only focus on highly expressed tsRNAs as they are expected to have a bigger impact on fracture healing and therefore considered as promising biomarker candidates. Amongst these, expression levels of five tiRNAs including Gly-CCC-5', Asp-GTC-5', His-GTG-5', Lys-CTT-5', and Cys-GCA-5' , as well as one itRF His-GTG were increased post-fracture by log2 fold change (log2FC) >2 (Table 2, and **I**, Figure 3). In serum, the expression levels of two tiRNAs Lys-CTT-5' and Lys-TTT-5' were reduced and one tiRNA His-GTG-5' was increased after fracture, with no correlation to their expression levels in callus tissue (Table 2 and **I**, Figure 3; **II**, Figure 2). Val tRNA isoacceptor derived tiRNAs Val-CAC-5' and Val-AAC-5' were also DE in callus tissue but their log2FC was little a lower than the selected threshold value of 2, being 1.92 and 1.86, respectively. In serum, they composed the major type of 5'-tiRNAs but were not affected by the fracture (**I**, Figure 1).

**Table 2. Differentially expressed tiRNAs** in serum or callus after fracture.

| tsRNA | Callus | Serum | Function | Target | References |
|---|---|---|---|---|---|
| Lys-CTT-5' | Up: D10, D14 | Down: D1, D10, D14 | Increases glucose metabolism | glucose-6-phosphatase catalytic subunit (G6PC) | (P. Zhu et al., 2021) |
| His-GTG-5' | Up: D5, D7, D10, D14 | Up: D1, D5, D7, D10, D14 | Promotes colorectal cancer | LATS2, GABBR2, TLR4, and GABARAP | (Chai et al., 2021; Tao et al., 2021) |
| Cys-GCA-5' | Up: D7, D10, D14 | NDE | Represses cell proliferation, migration, and transformation | STAT4 | (Zong et al., 2021) |
| Lys-TTT-5' | NDE | Down: D1, D5, D7, D10, D14 | Regulates cell proliferation | Unknown | (Krishna et al., 2019) |
| Asp-GTC-5’ | Up: D5 | NDE | Unknown | Unknown | NA |
| Gly-CCC-5’ | Up: D5, D7, D10, D14 | NDE | Unknown | Unknown | NA |

*NDE, not differentially expressed.*
*NA, No available publication found in literature.*

### 5.1.2 Retrotransposons expression is destabilized during fracture healing

In this study, retrotransposon expression was evaluated for the first time during fracture healing in addition to mRNA from callus tissue samples. Retrotransposons have a role in epigenetics, transcriptional regulation, cell differentiation, and reprogramming (Mita & Boeke, 2016). The expressions of retrotransposons and more specifically LTRs were analysed in RNAseq data of the callus tissue focusing on LTR subfamily, as they have been described to be targeted by tiRNAs (J. Park et al., 2020; Schorn et al., 2017; Sharma et al., 2016). A total of 576 LTRs with baseMean over 10 were observed and 15 of them were DE and met the criteria of the absolute value of log2FC > 2 and $P_{adjusted}$ value < 0.05 (Figure 15).

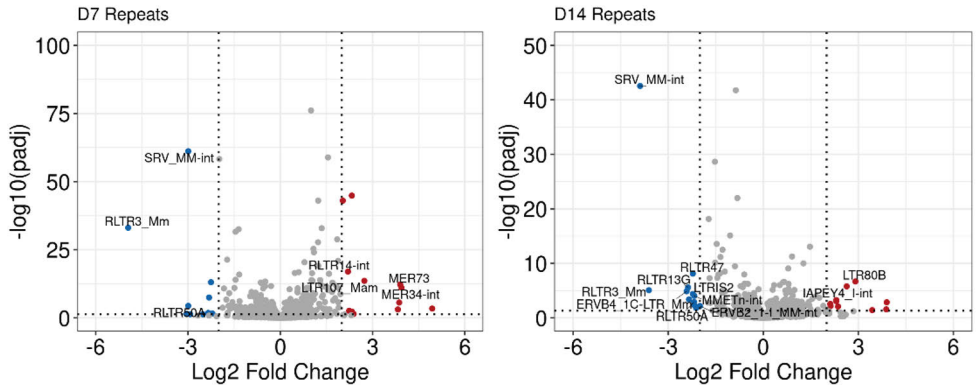


**Figure 15. Differential expression of retrotransposons after fracture retrieved from RNAseq data visualized by volcano plots.** Red dots show upregulated retrotransposons (log2FC > 2 and $P_{adjusted}$ value < 0.05) whereas blue dots show downregulated retrotransposons (log2FC < 2 and $P_{adjusted}$ value < 0.05) compared to intact bone tissue (D0). D0 (n = 4), D7 (n = 4), and D14 (n = 4). Only DE repeats belonging to LTR family with a baseMean > 10 are annotated in the figure.

The majority of the DE retrotransposons derive from LTRs which mostly belong to the ERVK sub-family (Figure 15, Table 3). Expression levels of six LTRs were increased by 2.2 to 3.94 log2FC while the levels of nine LTRs were decreased by 2.0 to 4.94 Log2FC. ERVK is a genetic parasite called ERV. This retrovirus family is partly responsible for the human genome evolution. ERVK is particularly active in cases of inflammatory diseases and viral infections (Manghera & Douville, 2013).

**Table 3. Differentially expressed LTRs observed in callus RNAseq data at D7 and D14 post-fracture,** extracted from Figure 15.

| Repeat_id | Sub-family | baseMean | log2FC | 0.00E+00 | DE Day |
|---|---|---|---|---|---|
| SRV_MM-int | ERVK | 673.30 | -2.99 | 7.28E-62 | D7 |
| RLTR3_Mm | ERVK | 163.99 | -4.94 | 8.48E-34 | D7 |
| RLTR14-int | ERV1 | 2437.51 | 2.20 | 1.07E-17 | D7 |
| LTR107_Mam | LTR | 32.09 | 2.73 | 2.60E-14 | D7 |
| MER73 | ERVL | 142.92 | 3.90 | 6.19E-13 | D7 |
| MER34-int | ERV1 | 13.61 | 3.94 | 8.54E-12 | D7 |
| RLTR50A | ERVK | 16.96 | -2.99 | 3.96E-05 | D7 |
| SRV_MM-int | ERVK | 1182.40 | -3.89 | 2.85E-43 | D14 |
| RLTR47 | ERVK | 269.74 | -2.22 | 7.71E-09 | D14 |
| LTR80B | ERVL | 131.55 | 2.91 | 2.09E-07 | D14 |
| IAPEY4_I-int | ERVK | 58289.38 | 2.63 | 1.66E-06 | D14 |
| RLTR13G | ERVK | 16067.07 | -2.38 | 2.61E-06 | D14 |
| RLTR3_Mm | ERVK | 320.50 | -3.61 | 8.48E-06 | D14 |
| ERVB4_1C-LTR_Mm | ERVK | 6247.14 | -2.42 | 1.35E-05 | D14 |
| LTRIS2 | ERV1 | 40581.97 | -2.22 | 4.17E-05 | D14 |
| MMETn-int | ERVK | 68505.00 | -2.18 | 9.10E-05 | D14 |
| RLTR50A | ERVK | 33.26 | -2.18 | 1.31E-03 | D14 |
| ERVB2_1-I_MM-int | ERVK | 171.78 | -2.00 | 6.57E-03 | D14 |

### 5.1.3 Comparison of the miRNA populations in bone, cartilage and callus tissues and their association with mRNA targets

MicroRNA populations were first identified in control hip cartilage and diaphyseal bone by simply comparing one tissue against the other (**I**, Figure 4). This analysis was done to identify cartilage and bone homeostasis-associated miRNAs in the fracture healing data where D5-D7 represent mainly ongoing chondrogenesis and D10-D14 osteogenesis. By comparing healthy bone and cartilage miRNA profiles at the age of two months, expression levels of several miRNAs were high in one tissue while low in the other, suggesting that these miRNAs are characteristic of their homeostasis while the remaining miRNAs associated with the maintenance of general skeletal tissue balance. So, logically during fracture healing, bone homeostasis-associated miRNAs are reduced during the chondrogenic phase compared to control bone while cartilage homeostasis-associated miRNAs are upregulated. With a similar logic, levels of bone homeostasis-associated miRNAs would be increased during the osteogenic phase or bone remodelling.

In cartilage, altogether 29 miRNAs were expressed at a high level while being low in bone, suggesting their association with the maintenance of chondrogenic phenotype. Further, in bone tissue 25 miRNAs were highly expressed compared to cartilage and are suggested to be associated with the maintenance of bone phenotype (**I**, Figure 6).

Altogether, 54 DE miRNAs out of 806 were identified in the fracture callus tissues with the criteria of baseMean > 100, the absolute value of log2FC > 2, and adjusted p-value < 0.05 (**I**, Figure 4). Out of them, 36 miRNAs, including 16 cartilage-associated miRNAs (miR-410-3p, 411-5p, 541-5p, 434-3p, 434-5p, 136-3p, 127-3p, 455-3p, 455-5p, 140-3p, 140-5p, 6240, 182-5p, 181-1-3p, 125b-2-3p, and 148a-3p) were DE with increased levels in callus after fracture (I, Figure 6).

Further, 18 miRNAs, including 12 bone homeostasis-associated miRNAs (miR-144-3p, 451a, 142a-5p, 340-5p, 181c-5p, 142a-3p, 150-5p, 144-5p, 486a-5p, 10a-5p, 223-3p, and 652-3p) were DE with decreased levels in callus tissue after fracture. MicroRNAs were divided into four clusters (D5, D7, D10, D14) based on the date of their highest absolute log2FC indicating differential expression.

In serum, a total of 290 miRNAs were identified, and out of them, eight miRNAs were DE with the criteria of baseMean > 100, the absolute value of log2FC > 1.5, and adjusted p-value < 0.1 (Table 4; **II**, Figure 3, Supplemental file 1). Levels of five miRNAs, including miR-328-3p, miR-133a-3p, miR-375-3p, miR-423-5p, and miR-150-5p were significantly increased after fracture and levels of two miRNAs miR-451a and miR-143-3p were decreased.

To better understand the possible role of these miRNAs during the fracture healing process, data mining was carried out to search for the verified targets of all

DE miRNAs in callus and serum data. A total of 750 potential miRNA=mRNA target pairs were first identified for the 54 DE miRNAs in callus tissue by June 2021. Thereafter, a correlation analysis was carried out between expression levels of a given miRNA and its target mRNA in callus and bone tissues (**I**, Table 1 and 2 and Supplemental file 5). A total of 164 miRNA=mRNA pairs with significant negative correlation were identified.

Recent literature search updates on mRNA targets of DE miRNAs in callus and in circulation indicated their involvement in all the steps which are also essential in the fracture healing process (Table 4). These functions include regulation of MSC differentiation into chondrocytes, and further terminal differentiation into hypertrophic chondrocytes, osteogenesis, angiogenesis, and finally osteoclastogenesis and bone remodelling.

Out of the remaining miRNA=mRNA target pairs (**I**, supplemental file 5), expression levels of 171 miRNAs correlated positively with their verified target mRNA, and 415 target mRNAs did not correlate significantly with their miRNAs, suggesting that expression of these mRNAs may either be regulated by other factors or be tissue specific. They may also be so local or restricted to a certain cell type that the possible correlations were not observed due to the mixed cell population in callus tissue.

**Table 4. Differentially expressed miRNAs and their target mRNAs in serum and callus tissue after fracture.** Current knowledge on targets, pathways and functions in bone, cartilage and fracture healing of recently and earlier identified targets with negatively correlated expression levels (updated in November 2022 and **I**, Table 1 and 2, Supplemental file 5).

**A. DE miRNAs in serum compared to their expression in callus tissue**

| miRNA/tissue | Target mRNA/ Pathway | Function | References |
|---|---|---|---|
| **143-3p**<br>Callus, NDE () | *SOX5*, *IGFBP5*, *BMPR2*<br>MAPK signaling pathways | (-) chondrogenesis<br>(-) osteogenesis<br>(-) osteoclastogenesis | (D. Gao et al., 2022; Jiang et al., 2021; J. Tian et al., 2018; C. Yang, Xu, et al., 2022) |
| Serum, Down D5 (160) | | | |
| **375-3p**<br>Callus: NDE (1) | *LRP5*, b-catenin<br>YAP1/LEKTI pathway | (-) inflammation<br>(-) osteogenesis | (Cheng et al., 2020; T. Sun et al., 2017) |
| Serum, Up D1-D14 (107) | | | |
| **328-3p**<br>Callus, NDE (54) | *COL1A1*, *PTEN*<br>PTEN/PI3K/AKT pathway | (-) endochondral ossification | (T. Liu et al., 2020; Xie et al., 2020) |
| Serum, Up D1 (114) | | | |

| | | | |
|---|---|---|---|
| **133a-3p**<br>**Other**<br>Callus, Up D5 (7149) | *VEGFA*, *ANKRD44*<br>PI3K/AKT pathway | (-) angiogenesis<br>(-) osteogenic diff. | (Ahmed et al., 2022; M. Li et al., 2021; Y. Tang et al., 2018) |
| Serum, Up D1 (270) | ‡: ***Met*** | | |
| **451a**<br>**bone-associated**<br>Calllus, Down D5-D14 (1056) | *CDKN2D*, *Bmp6, Osr1* | (-) angiogenesis,<br>(-) osteogenic diff. | (Karvande et al., 2018; Lu et al., 2019; H.-Y. Zhu et al., 2021) |
| Serum, Down D1 (598) | ‡: ***Osr1†, Cav1, Trim66, Mif, Tbx1, Oxtr*** | | |
| **423-5p**<br>**Other**<br>Callus, Down D14 (142) | *Sufu, Tnip2* | (+) angiogenesis<br>(-) osteoclastogenesis | (W. Wang et al., 2017; F. Xu et al., 2019) |
| Serum, Up D1-D14 (243) | ‡: ***Tnip2†, Cdkn1a, Igf2bp1*** | | |
| **150-5p**<br>**bone-associated**<br>Callus, Down D7, D10 (177) | *Vezf1*, *VEGFA*, Mmp14 | (-) angiogenesis<br>Modulates ECM | (Z. Chen et al., 2018; Perales et al., 2022; Vimalraj et al., 2021) |
| Serum, Up D1-D14 (164) | ‡: ***Mmp14†, Socs1, Rab9, Slc2a1, Elk1*** | | |

**B. Cartilage homeostasis associated DE miRNAs – increased expression**

| MiRNA | Target mRNA/ Pathway | Function | References |
|---|---|---|---|
| **410-3p** | *Hmgb1*<br>NF-κB signaling pathway | (-) chondrogenesis | (Pan et al., 2020) |
| D5-D14 | ‡: ***Cxcr5, Fmr1, Yy1, Pten*** | | |
| **411-5p** | *Gata4*<br>GATA4/Runx2 pathway | (+) osteoblast diff. | (X. Gao et al., 2020) |
| D5-D14 (847) | ‡: ***Txnip, Vasp, Grp2*** | | |
| **541-5p** | | | |
| D5-D14 (448) | ‡: ***Cdk6*** | | |
| **434-3p** | *Gata4*<br>GATA4/Runx2 pathway | (+) osteoblast diff. | (X. Gao et al., 2020) |
| D5-D14 (510) | ‡: ***Eif5a1*** | | |
| **136-3p** | *PTEN* | (+) osteoblast diff. | (Y. Chen et al., 2020) |
| D5-D14 (132) | ‡: ***Pten†*** | | |

| | | | |
|---|---|---|---|
| **127-3p** | *CDH11*<br>Wnt/β-catenin pathway | (-) Wnt/β-catenin pathway | (J. Dong et al., 2021) |
| D5-D14 (3002) | ‡: ***Kif3b*** | | |
| **455-3p** | *Twist1*, *Runx2* | (-) angiogenesis<br>(-) osteogenesis | (Z. Zhang et al., 2015; L. Zhao et al., 2022) |
| D5-D14 (142) | ‡: ***Fam83f*** | | |
| **455-5p** | *Runx2* | (-) osteogenesis | (Xiao et al., 2018) |
| D5-D14 (133) | ‡: ***Lgals9, Jak1, Myd88, Dnmt1*** | | |
| **140-3p** | *Kmt5b*, *Smad2, Mcf2l* | (+) osteogenesis<br>(-) preosteoblasts viab.<br>(+) chondrogenesis | (X. Liu et al., 2020; J.-H. Mao et al., 2020; H. Zheng et al., 2021) |
| D7-D14 (9311) | ‡: ***Cxcl12, Trpm2, Cd38, Tnfa, Jak1, Sirt1, Atp1b, Myb, Mcf2l, Atp8a1, Bcl2*** | | |
| **140-5p** | *Igf1r, Hmgb1*<br>Mtor pathway | (-) osteogenesis | (Y. Tang et al., 2022; Y. Wang et al., 2020) |
| D7-D14 (350) | ‡: ***Tnf, Nfe2l2, Map3k11, Stat1, Bloc1s2, Creb1, Birc5, Glul, Hdac4, Pin1, Adam10, Igf1r†, Hmgb1†, Hmgn5, Tlr4*** | | |
| **182-5p** | *Smad4, PTHLH* | (-) chondrogenesis | (Bai et al., 2019) |
| D7-D14 (454) | ‡: ***Rab27a, Flot1, Foxo3, Cdkn1b, Bcl2l12, Sesn2, Cfl1, Creb1, Pten*** | | |
| **181a-1-3p** | | fracture in diabetic rats | (Takahara et al., 2018) |
| D7-D14 (290) | | | |
| **148a-3p** | Kdm6b | (-) osteogenesis | (L. Tian et al., 2017; Yuan et al., 2019) |
| D14 (19385) | ‡: ***Dnmt1, Ikbkb, Mcl1, Kdm6b†, Snhg4*** | | |

| **C. Other DE miRNAs, increased expression** | | | |
|---|---|---|---|
| **MiRNA** | **Target mRNA/ Pathway** | **Function** | **References** |
| **381-3p** | FGF7<br>MEK/ERK signaling pathway | (-) osteogenesis | (L. Qiu et al., 2022) |
| D5-D14 (129) | ‡: ***Map3k8†, Ube2c, Cdk6, Cxcr4*** | | |
| **335-3p** | | OsteomiR | (Avendaño-Félix et al., 2019) |
| D5-D14 (318) | | | |

| | | | |
|---|---|---|---|
| **351-5p** | | | |
| D7-D14 (1737) | ‡: ***Mapk13*** | | |
| **214-3p** | *ATF4, Sp7* | | (K. Shi et al., 2013; X. Wang et al., 2013; C. Zhao et al., 2015) |
| D5-D14 (318) | ‡: ***Nlrc5, Hmga1, St6gal1, Atg12, Ezh2, Ezh1, Pim1, Stat6, Cadm1, Pten*** | | |
| **99b-5p** | *FGFR3* | (-) osteoblast prolif. | (Ding et al., 2021) |
| D7, D14 (2368) | | | |
| **133b-3p** | *FBN1* | (-) angiogenesis | (G. Liang et al., 2022) |
| D7 (375) | | | |
| **152-3p** | | osteoporotic fractures | (Zarecki et al., 2020) |
| D7 (276) | ‡: ***Atg12, Fasl, Brd4, Dnmt1*** | | |
| **34c-5p** | | MSC osteogenesis | (B. Liu et al., 2021) |
| D5-D14 (304) | ***‡Gucy1b3, Flot2, Sp1, Etv6, Atg4b, Ccl22*** | | |
| **214-5p** | *ITGA7* | (+) osteoclastogenesis | (L.-L. Liu et al., 2022) |
| D5-D10 (113) | ‡: ***Rock1, Klf5, Cxcr5, E2f2, Ciz1*** | | |
| **335-5p** | | (+) bone formation regeneration | (L. Zhang et al., 2017) |
| D5-D14 (269) | | | |
| **99a-5p** | | (-) osteogenesis (+) osteoclastogenesis | (Moura et al., 2020) |
| D7, D14 (894) | | | |
| **152-5p** | *Atg14* | (-) osteogenesis | (S. Li et al., 2022) |
| D5-D14 (144) | ‡: ***Txnip*** | | |
| **199a-5p** | Tet2 | (+) osteoblast diff. | (Qi et al., 2020) |
| D7, D14 (1874) | ‡: ***Ccr7, Mst1, Ccnb1, Slit1, Nfkb1, Rela, Ccn2, Rock1, March8, Fkbp5, Sirt1*** | | |
| **322-5p** | *Smad7* | (+) chondrogenesis (-) chondrocyte hypertr. | (Zeng et al., 2021) |
| D10-D14 (580) | ‡: ***Fam3b, Nfkb1*** | | |

| D. Bone homeostasis associated DE miRNAs, decreased expression | | | |
|---|---|---|---|
| **MiRNA** | **Target mRNA/ Pathway** | **Function** | **References** |
| **144-3p** | *PTEN, Bmpr1b, Bmp2, Tet2, Fzd4, Smad4* PI3K/AKT pathway | osteoblast diff., (-) chondrogenesis | (Huang et al., 2016; N. Li et al., 2020; Ling et al., 2022; M.-L. Mo et al., 2022; Peng et al., 2022; Z. Sun et al., 2019) |
| D5-D10 (148) | ‡: ***Hif1a, Pbx3, Fn1, App, Fzd4†, Smad4†, Fosb, Ctbp2, Hoxa7, Tie2*** | | |
| **340-5p** | *Runx2, Ctnnb1, Hif1a, Fmod* | (-) osteogenesis down during osteoclastogenesis | (Du et al., 2017; Y. Ma et al., 2016; X. Wang et al., 2021; W. Zhang et al., 2018) |
| D7-D10 (428) | ‡: ***Hif1a†, Fmod†, Yap1, Arg1, Stat3, Nrp1, Ctnnb1†*** | | |
| **181c-5p** | *SMAD7, SFRP1* Wnt3a/β-catenin pathway | (+) chondrogenic diff. (+) osteogenic diff. (+) angiogenesis (-) osteoclastogenesis | (X. Yu et al., 2021; Q. Zhang et al., 2022) |
| D7-D10 (368) | ‡: ***Sfrp1†*** | | |
| **142a-5p** | *NFIA* | (+) osteoblast diff. | (Yuan et al., 2021) |
| D7-D10 (2990) | ‡: ***Cyr61, Socs1, Ghr*** | | |
| **142a-3p** | *Adam9, Ctnnb1, Il6* | (-) chondrogenic diff. (-) osteogenic diff. (-) fracture healing | (T. Hu et al., 2016; Y. Liu et al., 2016) |
| D5-D14 (349) | ‡: ***Adam9†, Ctnnb1†, Il6†, Fam98a, Fzd7, Rab3a, Nr2f6*** | | |
| **144-5p** | *Smad1* | (-) fracture healing | (D. Zhang et al., 2021) |
| D5-D14 (108) | ‡: ***Smad1*** | | |
| **10a-5p** | *Hoxa1* | (-) osteogenesis (+) chondrocytes apoptosis | (Y. Ma et al., 2019; Y. Zhang et al., 2020) |
| D10 (8979) | ‡: ***Ccn2, Hoxa1†*** | | |
| **486-5p** | | | |
| D10-D14 (9990) | ‡: ***Dock1, Cemip, Nrp2*** | | |
| **223-3p** | *FGFR2, FOXO3* | fracture healing (-) osteogenesis | (C. Long et al., 2021; B. Wang et al., 2021) |
| D10-D14 (9990) | ‡: ***Fgfr2†, Nf2, Lif, Fat1, Ctnnd1, Skil, Zeb1, Cdh6, Smad3, Fam5c, Rasa1, Il17rd*** | | |

**E. Other DE miRNAs, decreased expression**

| MiRNA | Pathway Target mRNA/ Pathway | Function | References |
|---|---|---|---|
| **92a-3p** | *WNT5A, HDAC2* | (+) chondrogenesis | (G. Mao et al., 2016, 2018) |
| D14 (2978) | ‡: ***Nhg14*** | | |
| **146a-5p** | | bone mass osteoclastogenesis | (Lin et al., 2019; M. Zheng et al., 2021) |
| D14 (292) | ‡: ***Sirt1*** | | |
| **423-5p** | | (-) osteosarcoma glycolysis | (Wan et al., 2021) |
| D14 (142) | ‡: ***Tnip2, Cdkn1a, Igf2bp1*** | | |

*NDE, not differentially expressed.*
*Numbers in parentheses indicate the baseMean expression in I, II.*
*‡, mRNAs with bold fonts were identified as targets of the miRNA and negative correlation was observed between their expression in callus tissue (**I**, Supplemental file 5).*
*†, mRNA found to be negatively correlated with miRNA in I and associated to bone, cartilage or fracture healing.*
*MicroRNA targets were searched in PubMed ("molecule"[All Fields] AND ("angiogenesis"[All Fields] OR "bone"[All Fields] OR "ossification"[All Fields] OR "cartilage"[All Fields] OR "inflammation"[All Fields])).*

# 6 Discussion

## 6.1 RNA expression in callus tissue and in circulation during fracture healing

The expression of ncRNAs was uncovered after fracture from callus and serum samples. Their differential expression in callus tissue is suggested to reflect the active regulation of the fracture healing process by targeting mRNA expression. In circulation, their expression may reflect the biological processes associated with fracture healing either in callus tissue or originate from other tissues affected by the fracture via systemic effects.

Small non-coding RNAs, including tsRNAs and miRNAs, were first studied in callus tissue samples at D5-D14 post-fracture. In addition, mRNA and retrotransposon profiles were studied at D7 and D14 post-fracture. The expression of mRNAs was mainly uncovered to correlate their expression levels with miRNAs and to identify possible miRNA-mRNA interactions in callus tissue.

In circulation, RNAs are carried freely associated with lipid particles, and RNA binding proteins and packaged in extracellular vesicles, including exosomes (Etheridge et al., 2013). In the present thesis project, sncRNA profiles were studied in circulation at D1-D14 after fracture in the exosome-containing fraction and compared to the levels in intact control mice. Exosomal vesicles contain DNA and RNA as well as proteins and lipids, used for cell-cell communication (W. Qin & Dallas, 2019). The sorting mechanism of exosomal ncRNAs has not been established as yet. Several publications refer to motif recognition, interaction with their secondary/tertiary structures, and/or post-transcriptional modifications of the ncRNA recognised by ribonucleoproteins which form a complex ribonucleoprotein-ncRNA subsequently sorted to an exosomal vesicle (Y. Qiu et al., 2021). Exosomal vesicles are playing a role in systemic regulation by carrying non-coding RNAs which might regulate targets in receptor cells (Thomou et al., 2017).

### 6.1.1 Expression and putative functions of tsRNAs after fracture both in the callus and in circulation

For the first time, tsRNAs were analysed after fracture from callus tissue and serum samples. The expression of tsRNAs was previously reported to have similar roles as miRNAs (Krishna et al., 2021). In callus tissue, eleven mature tsRNAs out of a total of 191 were highly expressed at level > 1000 reads (baseMean). Among those, 5' end tiRNAs Gly-GCC-5', and Glu-CTC-5' were most abundant and stably expressed and with baseMean > 15,000 reads. Being so abundant and stably expressed, these 5' end tiRNAs may have an important role in the regulation of the general tissue homeostasis. Val isoacceptor-derived 5' end tiRNAs Val-CAC-5' and Val-AAC-5' were the most abundant tRNA-derived fragments in callus tissue with a baseMean above 35,000 reads. Val isoacceptor tRNA-derived 5'-end tiRNAs have been shown to bind to the human Frizzled class receptor 3 (*FZD3*) and therefore suppress the Wnt signalling pathway (D. Mo et al., 2019; Sarais et al., 2022). They have also been shown to repress *Sirt1* expression by targeting its 3'UTR, leading to an accumulation of Hif1a, and therefore promoting angiogenesis in mice with diabetic retinopathy (Y. Xu et al., 2022). *Hif1a* has been shown to regulate ECM secretion by regulating cellular oxygen level and allowing collagen hydroxylation and maturation, in ER, in hypoxic environment (Bentovim et al., 2012). This is interesting as compared to bone, Val-CAC-5' and Val-AAC-5' expression levels in callus tissue almost reached the differential expression criteria of the absolute value of log2FC > 2 at D5, being 1.92, and 1.86, respectively. Based on these studies, their roles in callus may be related to the modulation of the systemic immune response during early fracture healing as well as in the regulation of angiogenesis. In circulation, Val isoacceptor-derived tiRNAs were also the most abundant 5' end tiRNAs with the baseMean of 1599 and 1640 reads, respectively, although their expression levels were not affected by the fracture.

Expression levels of five 5' end tiRNAs (Gly-CCC-5', Asp-GTC-5', His-GTG-5', Lys-CTT-5', and Cys-GCA-5') were higher in comparison to the healthy intact bone throughout the observation period of D5-D14. In circulation, Gly-CCC-5', Asp-GTC-5' and Cys-GCA-5' were barely present, suggesting that their role is mainly in the fracture callus tissue and that they are not exported to the serum. Not much is known about these three tiRNAs, but in a recent study, Gly-CCC-5'/Gly-GCC-5' was suggested as a mediator of palmitic acid-induced effects in human trophoblasts, based on that, the inhibition of these tiRNAs enhanced palmitic acid-induced apoptosis, including DNA fragmentation and mitochondrial depolarization (C. Yang, Park, et al., 2022). Cys-GCA-5' was found to directly target STAT4 at gene and protein levels, and therefore represses cell proliferation, migration, and transformation (Zong et al., 2021).

In circulation, a total of 36 tsRNAs were identified which was much lower than that in callus where a total of 191 tsRNAs were identified. The reason for this difference is not known but it may be related to the isolation techniques as in callus tissue, total RNA was first extracted while in serum, the analysis was carried out with the exosome fraction. Further studies are required to find out if the freely circulating pool of tsRNAs associated with RNA binding proteins and lipid fraction contains different and richer populations. 5' tiRNAs were reported to be depleted from exosomal vesicles but found in circulation as components of RNA-protein/lipoprotein complexes data. However, in their study, they hybridized tiRNA-Gly-GCC-5' and tiRNA-Val-CAC-5' and detect their presence by northern blotting but these two examples might be different compared to the other tiRNAs from other isoacceptors and/or decoders (Dhahbi, Spindler, Atamna, Yamakawa, et al., 2013). In accordance with Dhahbi et al., Val-CAC was highly expressed in serum (**II**, Table 1), regardless if they originate from extracellular vesicles or as components of RNA-protein/lipoprotein complexes.

In circulation, Lys and His isoacceptor tRNA-derived fragments were expressed among the top ten 5' end tiRNAs and they were found differentially expressed in both serum and callus tissues during fracture healing. Levels of Lys-CTT-5' and Lys-TTT-5' were reduced in serum although increased in callus tissue, with no correlation in expression levels between tissues (**I**, **II**). Lys-TTT-5' together with Gln-GTG-5' and Val-CAC-5' have been suggested to restrain the expression of stemness-promoting genes *Lif* and *Wnt3*, as well as regulation of cell proliferation via RNA binding protein Igf2Bp1/cMyc (Krishna et al., 2019). Further, Lys-CTT-5' was also found to increase glucose metabolism by directly targeting the glucose-6-phosphatase catalytic subunit (G6PC) in human triple-negative breast cancer patients (P. Zhu et al., 2021). Levels of His-GTG-5' tiRNA and His-GTG itRF levels were increased both in the callus and in circulation throughout fracture healing (**I**, **II**). Similarly to miRNA function, tiRNA-His-GTG-5' has been shown to directly target large tumor suppressor kinase 2 (LATS2) 3'UTR after loading into AGO1 and AGO3 and to destabilize Gamma-Aminobutyric Acid Type B Receptor Subunit 2 (GABBR2) by binding to its 3'UTR (Chai et al., 2021; Tao et al., 2021). His-GTG-5' has been found to target and suppress Toll Like Receptor 4 (TLR4), thereby leading to the activation of the Wnt/β-catenin signalling pathway to promote osteoblast differentiation and fracture healing (Chai et al., 2021; H. Xu et al., 2014; C. Zhao et al., 2020).

Functional data on tsRNAs and tiRNAs is slowly emerging, and it is already obvious that the 5'end tiRNAs have multiple functions in the regulation of tissue metabolism, and as shown by the NGS data, they are also involved in the regulation of fracture healing. These data also suggest that circulating tiRNAs may function as regulators of systemic effects and reflect the metabolic changes in tissues and

therefore have value as biomarkers of various disorders. They are also involved in the regulation of retrotransposon expression, as is discussed below. It has been reported that 5' tiRNAs affect gene expression by direct binding to the mRNA molecule but also to regulate the expression of LTR retrotransposons (Advani & Ivanov, 2019; H. K. Kim, 2019; Krishna et al., 2019; J. Park et al., 2020; Sharma et al., 2016).

### 6.1.2 Bone fracture induced expression of LTR retrotransposons in callus

Retrotransposon expression was studied in the RNAseq data to extend the current knowledge on whether their expression was affected by the fracture. Extension of the RNAseq data analysis into retrotransposon-derived fragments indicated, that especially the expression of LTRs was DE in callus tissue compared to the intact bone during fracture healing. For the first time, the presence and differential expression of LTRs were demonstrated in callus tissue. This is of particular interest as also tsRNAs have been connected to the control of retrotransposons (Martinez, 2018).

Expression levels of six LTRs were increased while levels of ten LTRs were decreased at D7 and D14. The major DE LTR sub-family was ERVK which has been described to be active in cases of inflammatory diseases (Manghera & Douville, 2013). Stress is a documented factor in the activation of retroelements (Mita & Boeke, 2016). Cellular stress generated by fracture may be responsible for the increased levels of tiRNAs as well as retrotransposable elements in the callus tissue. The active inflammatory phase during fracture healing may at least partially explain the activity of ERVK LTRs in callus tissue (Dimitriou et al., 2005). Increased expression of transposable elements has been associated with genome instability (Maxwell et al., 2011) which may be one mechanism for the regulation of gene expression in tissues. The expression of retrotransposons is controlled by several mechanisms including TFs, tiRNAs, RNA interference, piRNAs, and self-regulation (Bourque et al., 2018; Levy et al., 2008; Martinez, 2018; Mita & Boeke, 2016; Schorn et al., 2017; Sharma et al., 2016). Transposable elements occasionally also incorporate in the 3'UTR of genes resulting in elongation of the 3' UTR and thus providing more opportunities for binding of miRNAs to the 3' end and post-transcriptional gene regulation (Levy et al., 2008). The role of tiRNAs in the regulation of the transposons and particularly in the protection of the genome against retrotransposon effects is interesting and deserves further investigation (Martinez, 2018).

### 6.1.3 Role of miRNAs as regulators of mRNAs after fracture

MicroRNA expression was analysed in callus samples in connection with the expression of their mRNA targets. The miRNA-mRNA relationships were investigated by correlation analysis to evaluate their role as regulators of fracture healing.

In callus, because miRNAs with increased levels of expression were cartilage-associated in comparison to intact bone (**I**, Figure 6), they were mainly expected to have a role in the maintenance of chondrogenic phenotype. Twenty-eight increased miRNAs negatively correlated altogether with 105 mRNA targets identified by the literature searches (Table 4; **I**, Table 1). Out of these putative interactions during fracture healing, several interactions are associated with the maintenance of the chondrocyte phenotype. Several miRNAs, such as miR-140, miR-181a, and miR-455-3p have been previously shown to be associated with cartilage tissue biology (Razmara et al., 2019). E.g., miR-140-5p and miR-410-3p have been found to target the high mobility group box 1 (*Hmgb1*) mRNA, which suppresses chondrocyte viability. Further, increased levels of miR-140-5p have been shown to repress *Hmgb1* expression through the PI3K/AKT signalling pathway to enhance chondrocyte viability (Pan et al., 2020; Y. Wang et al., 2020). *HMGB1* is enriched in OA tissue compared to healthy cartilage, which is almost depleted of *HMGB1* (Wagner et al., 2021; Y. Wang et al., 2020). In addition, in the present data miR-381-3p is an example of a regulator of angiogenesis, as it has been found to directly target mitogen-activated protein kinase kinase kinase 8 (*Map3k8*) (J. Li et al., 2020). Map3k8 represses angiogenesis; therefore, increased expression of miR-381-3p in fracture callus is suggested to promote angiogenesis via downregulation of *Map3k8*.

As discussed earlier, downregulated miRNAs in callus tissue were expected to facilitate the expression of mRNAs necessary in fracture healing. Fourteen miRNAs were identified to negatively correlate with a total of 59 mRNAs which were their verified targets, based on the published data (**I**, Table 1, Table 4;). Out of these, e.g., miR-142a-3p has been shown to repress the expressions of A disintegrin and metalloproteinase domain 9 (*Adam9*), β-catenin (*Ctnnb1*) (T. Hu et al., 2016), and *Il-6* (Y. Liu et al., 2016). Adam9 has been found to play a role during chondrogenesis by inducing apoptotic death of chondroprogenitors and inhibiting cell migration (D. Kim et al., 2011), *Il-6* is known to initiate fracture repair by enhancing ECM synthesis, angiogenesis, and recruiting endogenous fibrogenic cells to the fracture site (Dimitriou et al., 2005) and *Ctnnb1* is an important factor in Wnt/β-catenin signalling pathway to regulate both chondrogenesis and osteogenesis (Oichi et al., 2020). MiR-451a mediates osteoblastic differentiation by directly targeting odd-skipped related transcription factor 1 (*Osr1*) (Karvande et al., 2018). MiR-144-3p was found to target *Smad4* (Huang et al., 2016) and frizzled class receptor 4 (*Fzd4*) (Z. Sun et al., 2019); therefore, negatively regulating osteogenesis. MiR-150-5p

targets *Mmp14* and therefore indirectly promotes osteoblast mineralization and represses collagen degradation (C.-L. Dong et al., 2015). MiR-340-5p was found to regulate *Ctnnb1* (Du et al., 2017), hypoxia-inducible factor 1 subunit alpha (*Hif1a*) (Du et al., 2017) and fibromodulin (*Fmod*) (W. Zhang et al., 2018), also playing an important role in osteogenesis. Finally, miR-10a-5p targets homeobox A1 (*Hoxa1*) (Y. Ma et al., 2019), which promotes chondrocytes apoptosis, as a result of miR-10a-5p increased expression. MiR-10a-5p was upregulated at D10 post-fracture when most of the chondrocytes have already reached the hypertrophic stage. All downregulated miRNAs cited in the chapter above were enriched in bone compared to cartilage and associated with the regulation of osteogenesis with differential expression. It is also worth mentioning many of the mRNA targets are also important in chondrogenesis and cartilage homeostasis, including *Mmp14*, *Fmod*, and *Hif1a* (Embree et al., 2010; Schipani, 2006; M. Takahashi et al., 2019).

Here, only a few examples of the DE miRNAs and their target mRNAs were discussed, but the data points out that the miRNAs with a differential expression that negatively correlated with their target mRNAs, were associated with bone fracture healing to fine-tune the fracture healing process. While concluding this data, it is important to keep in mind that callus tissue is composed of multiple cell types and stages of differentiation which may dilute the observed effects. Also, in addition to miRNAs, tsRNAs and retrotransposons, as discussed in this thesis, the regulation of mRNA transcription is a complex process including co-factors, microRNAs, epigenetics, systemic factors, circadian rhythm, and the microenvironments (Chan et al., 2021).

### 6.1.4 Role of miRNAs as biomarkers and systemic regulators

In circulation, the role of miRNAs and sncRNAs, in general, is more elusive than it is in tissues. The result of differential expression of miRNAs in circulation can be seen as biomarkers of fracture healing in addition to being regulators of mRNA targets in recipient tissues. An interesting observation was that e.g., in cases of some miRNAs the expression level in serum was reduced although it was highly expressed in callus tissue, and on the contrary, levels of some miRNAs were increased although decreased in callus tissue. A similar observation was also noticed in the expression of tsRNA in callus and circulation. This highlights the complexity of the regulatory mechanisms that also reach beyond local to systemic effects.

In serum, one miRNA (miR-451a) was decreased at D1 post-fracture, while increasing in the callus tissues. On the contrary, levels of three other miRNAs (miR-375-3p, miR-423-5p, and miR-150-5p) were increased at D1, D5, D7, and D14 post-fracture although in callus tissue they were either decreased or stable, suggesting that

callus is unlikely the source for increased levels in serum. This raised a question on the source of the miRNAs in circulation after fracture and hence the role of systemic regulation contributing to the miRNA levels in systemic regulation.

MiR-375-3p regulates osteogenesis by directly targeting LRP5 and β-catenin (T. Sun et al., 2017). MiR-375-3p was found to reduce inflammation in atopic dermatitis by targeting YAP1 (Cheng et al., 2020). It is therefore interesting that miR-375-3p was not DE in callus but enhanced at D1-D14 in serum. It might have a role as a systemic regulator to reduce inflammation in tissue other than callus but its source in circulation is not known. Further, it has been shown that miR-375-3p is released in circulation by human and mouse pancreatic beta cells via high-density lipoproteins complexes to target mRNAs in recipient cells (Sedgeman et al., 2019). However, its tissue/mRNA targets are still unknown.

MiR-423-5p was found to regulate bone remodelling by targeting *Tnip2* which activates NF-κB signalling crucial for osteoclastogenesis (Fischer & Haffner-Luntzer, 2022; W. Wang et al., 2017). miR-150-5p was shown to regulate matrix remodelling by targeting *Mmp14* (C.-L. Dong et al., 2015). Taken altogether, these three miRNAs have a role in fracture healing in-situ but their role in systemic regulation is still unclear.

As discussed above and presented in Table 4, it is not abnormal that a given miRNA will have a pivotal role in the differentiation of MSCs by facilitating the differentiation of MSCs towards one lineage rather than another (C. Yang et al., 2021). For example, Peroxisome Proliferator Activated Receptor Gamma (PPARγ) is known to promote adipogenesis and inhibit osteogenesis (Kawai & Rosen, 2010). Interestingly, PPARγ expression was found to be increased in adipose-derived stem cells (ADSCs) after overexpression of miR-150-5p to trigger adipogenesis (X. Li et al., 2019). Because miR-150-5p expression was found to be increase in serum after fracture and decreased in callus samples, it is reasonable to define miR-150-5p as a pivotal miRNA with the ability to modulate the ECM in callus tissue and play a role in systemic regulation in circulation.

Expression levels of most of the molecules in serum did not correlate to their expression in the callus. This discrepancy suggests that the levels in circulation are mainly independent of their expression levels observed in trauma tissue, and perhaps controlled by a sorting mechanism (Y. Qiu et al., 2021) or other systemic or indirect factors affecting changes such as the physical activity, pain or nutrition due to the trauma. Also, possible changes in sorting for transport may be a cellular response in the trauma and ncRNAs acting as communicators have a role as biomarkers of a physiological phenomenon.

## 6.2 Limitations of the data

Callus tissue is composed of multiple cells undergoing overlapping stages of differentiation including chondrogenesis, osteogenesis, angiogenesis and finally remodelling. Therefore, the data presented here represent the average expression levels in the callus tissue at the particular sampling time point. An option to overcome this problem would be single-cell sequencing. Single-cell sequencing technology would show the spatial segregation of all cell types present in the callus microenvironment and detect from which specific cell type a given mRNA is expressed. Further, it would be easier to allocate which miRNA-mRNA interactions are available in which cell type. In addition, it would be a nice way to detect RNA expression changes during cell differentiation.

While processing the serum samples, one obvious challenge is to avoid blood cell contamination as blood cells are rich in small RNA. E.g., erythrocytes have been shown to deliver exosomes loaded with small RNA cargo into circulation (Harisa et al., 2017). During serum isolation, hemolytic samples were rejected according to their colour to avoid uncontrolled small RNA contamination. Total RNA for smallRNAseq was isolated from exosome isolate. In this study, the ExoQuick kit was used for exosome extraction because of the simplicity and the large number of samples that were taken for the analysis. Precipitation-based exosome isolation methods have been considered equally acceptable as ultracentrifugation (Rekker et al., 2014), although they are limited by the fact that RNA-binding proteins coprecipitate with the exosomes (Karttunen et al., 2019). Therefore, it is possible that in the present data the cargo in the RNA binding complexes has contributed to the observed levels of sncRNAs in circulation (Dhahbi, Spindler, Atamna, Yamakawa, et al., 2013).

The abundance of 5’ tiRNAs, or rather the shortage of 3’ tiRNAs in our data is linked to the used traditional sequencing method which is optimised for miRNA analysis, and not adapted to tsRNA. For example, miRNAs contain a 5’ phosphate (5’-P) and a 3’ hydroxyl (3’-OH) end but tsRNAs possess a 3’ end bearing modifications such as a 3’ phosphate (3’-P) or 2′,3′-cyclic phosphate (2′3′-cP) which blocks the 3’ adapter ligation and therefore prevents the detection of modified 3’ end molecules (Honda et al., 2015; J. Shi et al., 2021). The use of a special kit, such as PANDORA-seq, for RNA library preparation, would allow more accurate detection of modified tsRNA fragments and overcome this problem (J. Shi et al., 2021). PANDORA-seq is designed to remove internal RNA methylations and terminal modifications, detailed above. However, the technique requires further improvement to overcome other tRNA modifications which can interfere with reverse transcription such as 2-methylthio-*N*6-isopentenyl adenosine (ms2i6A) (Wei et al., 2015).

## 6.3 Future perspectives

NGS data are not limited and restricted to only mRNA and miRNAs. Rather the fragment size selected, the chemistry, and the techniques used in the library preparation are the limiting steps on which RNA molecules can be analysed from the given NGS data. To take advantage of this, the thesis aimed at analysing from the RNAseq data also the retrotransposons in addition to mRNAs, and in smallRNAseq data the tsRNAs falling within the same size range with miRNAs, in terms of library preparation, with the limited detection of 3' end modified tiRNA fragments. In addition to miRNAs and mRNAs, also LTR retrotransposons were analysed for their known interaction with tiRNAs. However, it would be great in the future to push this even further by looking more particularly at long non-coding RNAs, snRNAs, or even DNA methylation during fracture healing which is known to be related to the retrotransposons.

The data presented in this thesis project provides a basis for the future studies to look at the role of specific miRNAs and tiRNAs in fracture healing and bone biology by silencing their expression in mouse models, after generating a genomic knockout by selecting the miRNA/tiRNA locus. These types of experiments would allow further understanding on the role and function of selected ncRNAs important in fracture healing. For example, this thesis uncovered the abundant and differential expression of several tiRNAs such as Lys-CTT and His-GTG and miRNAs such as miR-150-5p, miR-340-5p, miR-455-5p, and 455-3p. A similar, but a broader approach would be to delete the expression of endonucleases responsible for tsRNA and/or miRNA biogenesis from a given cell type and to follow the fracture healing process to investigate how important are these tsRNAs/miRNAs originating from a given cell type are in the regulation of fracture healing.

While considering the biomarker applications, the potential of selected sncRNAs presented in this thesis could be tested in human bone fracture cases to follow the fracture healing and efficacy of possible medicines in treating the trauma. Fracture healing is a complexe mechanism and a full recovery is a lengthy process, the use of circulatory biomarkers could indicate a putative non-union of the fracture healing.

Another potential approach to study fracture healing is to combine multi-omics data sets such as RNAseq (found in this study), DNAseq such as ATAC-seq and ChIP-seq, sncRNAseq (found in this study), DNA methylation analysis, proteomics, and metabolomics. This approach allows generation of a whole network interacting biomolecules including their associations and correlations-based on various statistical analyses.

# 7 Summary and conclusions

Expression of sncRNAs (tsRNAs and miRNAs) was assessed during mouse tibial closed fracture healing, in callus, bone, cartilage, and serum samples. Based on the results presented in this thesis, the following conclusions were made (Figure 16):

1. In control bone and cartilage, 25 bone and 29 cartilage homeostasis-associated miRNAs were identified by comparing their expression against each other tissue.

2. Differential expression of 54 miRNAs and seven tsRNAs was shown after fracture in callus tissue at D5-D14, and eight miRNAs and six tsRNAs in circulation at D1-D14 when comparing their expression to the expression of D0 samples (intact diaphyseal bone samples).

3. In callus tissues, 164 miRNA-mRNA interactions were observed, based on statistical findings using a Spearman correlation test. MiRNAs and 5'end tiRNAs are suggested to target mRNAs during fracture healing to fine-tune their expression in callus tissue samples.

4. In circulation, miRNAs and tsRNAs are suggested to have a putative role as biomarkers of fracture healing and as systemic regulators originating even outside the trauma tissue to facilitate fracture healing.

5. Retrotransposon and more specifically LTRs expression differs between intact bone and callus tissue, and it is also DE during fracture healing.

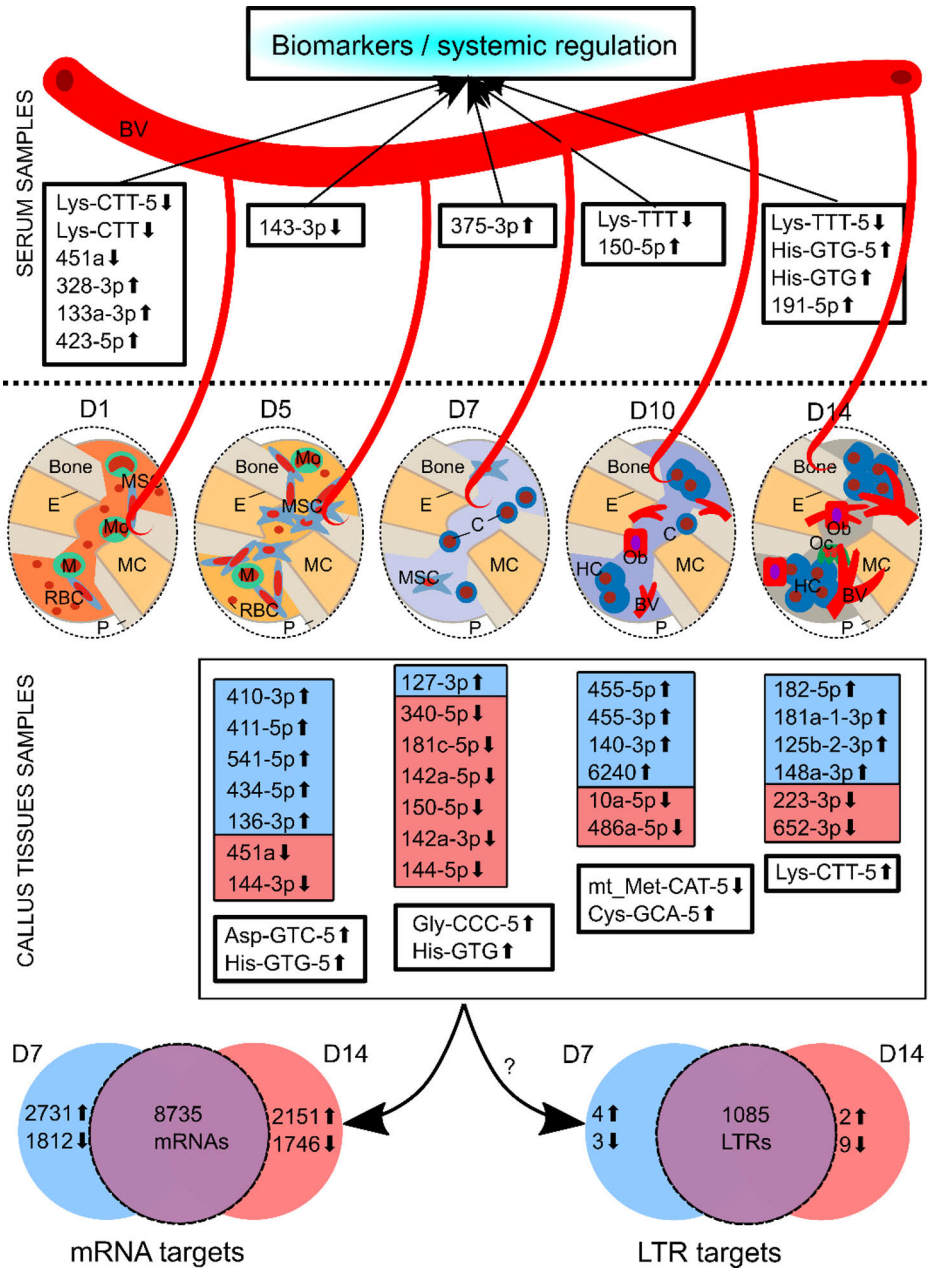


**Figure 16. Summary of the tsRNA and miRNA expressions found in callus and serum samples.** MiRNAs and tsRNAs were clustered based on the date with maximum absolute value of log2FC at D1, D5, D7, D10, and D14 compared to D0. Blue highlighted miRNAs are cartilage-associated while red highlighted miRNAs are bone-associated. ↑: increased expression compared to D0 control, ↓: decreased expression compared to D0 control.D: Day, MC: medullary cavity, P: periosteum, E: endosteum, MSC: mesenchymal stem cell, RBC: red blood cell, M: macrophage, Mo: monocyte, C: chondrocyte, HC: hypertrophic chondrocyte, Ob: osteoblast, Oc: osteoclast, BV: blood vessel, ?: unknown interaction in fracture healing.

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