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REGULATION OF BONE FORMATION AND POST NATAL SKELETAL HOMEOSTASIS – NOVEL ROLE OF FAM3C AND OSTEOBLAST SPECIFIC DICER1

Ameya Bendre

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To my Family

Ameya Bendre: Regulation of bone formation and post-natal skeletal homeostasis – Novel role of Fam3c and osteoblast specific Dicer1. Faculty of Medicine, Institute of Biomedicine, Cell Biology and Anatomy, University of Turku

ABSTRACT

Development of the skeletal system comprises of two major biological processes namely, endochondral ossification and intramembranous ossification. Mesenchymal stromal cells give rise into bone forming osteoblasts, which undergo maturation and mineralization to form bone. Osteoblast differentiation is an intricately regulated process which involves multitude of factors including transcription factors, cytokines, growth factors, microRNAs and functional proteins. A detailed understanding of the molecular mechanisms of osteoblast differentiation and bone formation is essential for the development of novel therapeutic approaches to counter skeletal disorders such as osteoporosis. This thesis combines work on two aspects of bone biology namely, the novel role of Fam3c and osteoblast specific Dicer1 in the regulation of cortical bone formation and post-natal skeletal homeostasis.

Genome wide association studies are studies, which involve large number of individuals and look at genetic variants of individuals to examine their association with different phenotypic traits. In multiple genome wide association studies on independent population cohorts, a novel protein Fam3c (family with sequence similarity 3, member C) was predicted to be associated with bone mineral density at different anatomical locations and fracture risk. However, the role of Fam3c in bone biology was relatively unknown prior to the commencement of this work. First aim of this thesis was to decipher the role of Fam3c in bone biology and to establish its molecular mechanism of action. Fam3c was found to have a multi-faceted role in the regulation of osteogenic differentiation, bone mineral density as well as cortical bone structure. Additionally, in vitro experiments revealed that Fam3c may exert its action via the down-regulation of transcription factor Runx2 in osteoblasts.

MicroRNAs are short (approx. 22 nt) non-coding RNA molecules which negatively regulate gene expression. Dicer1 enzyme is crucial for the processing of pre-miRNAs to mature microRNAs. MicroRNA mediated gene regulation has been established to be a critical part of regulation of a variety of cellular processes including osteoblast differentiation and bone remodelling. However, it was incompletely known whether microRNAs play a role in the maintenance of post-natal bone homeostasis. The second aim of this thesis was to examine whether Dicer1 inactivation and, thus, blocking formation of active miRNAs in bone forming cells affects post-natal bone homeostasis. A novel mouse model was generated (Dicerosx), in which Dicer1 can be inactivated in a time-controlled manner in osterix-expressing bone forming cells. Inactivation of Dicer1 led to dysregulation of post-natal cortical bone formation. Moreover, biomechanical testing revealed increased ductility among the Dicer1 deleted bone samples as compared to the controls.

These results have provided insights into the function of Fam3c in bone biology and role of osteoblast specific Dicer1 in the regulation of post-natal cortical bone homeostasis.

Keywords: osteoblast, Fam3c, osteogenic differentiation, bone formation, post-natal bone homeostasis, microRNAs, Dicer1

Ameya Bendre: Fam3c:n ja osteoblastien Dicer1:n merkitys luun muodostuksen homeostaasin säätelyssä. Turun yliopisto, lääketieteellinen tiedekunta, biolääketieteen laitos, solubiologia ja anatomia.

TIIVISTELMÄ

Luuston kehitystä ohjaa kaksi erilaista luutumismekanismia: välillinen luutuminen ja välitön luutuminen. Luutumisesta vastaavat osteoblasteiksi kutsutut solut, jotka erilaistuvat mesenkymaalisista strooman soluista. Tätä erilaistumisprosessia ohjaavat lukuisat toiminnalliset proteiinit, kasvutekijät, sytokiinit, transkriptiotekijät ja mikro-RNA:t. Luun muodostuksen ja osteoblastien erilaistumisen yksityiskohtainen ymmärtäminen on keskeistä, jotta esim. osteoporoosiin voidaan kehittää uusia hoitomuotoja. Tässä väitöskirjatyössä tutkittiin Fam3c:n ja osteoblastien tuottaman mikro-RNA:n merkitystä kuoriluun muodostuksessa ja tukirangan homeostaasin säätelyssä.

Genominlaajuisissa tutkimuksissa (genome-wide association studies, GWAS) selvitetään laajaa näyteaineistoa hyödyntäen useiden geneettisten lokusten vaikutusta yksilön ilmiasuun. Useiden eri ihmisryhmiin kohdistuneiden GWAS-tutkimusten mukaan Fam3c:n (family of sequence similarity C) geenin alleelien osoitettiin vaikuttavan luun tiheyteen ja luun murtumariskiin. Kuitenkaan Fam3c:n merkitystä luun biologiassa ei ole selvitetty ennen tätä tutkimusta. Tutkimuksen ensimmäisenä tavoitteena oli selvittää, mikä on Fam3c:n merkitys luun biologiassa ja millä molekulaarisilla mekanismeilla se vaikuttaa luuhun. Osoitimme, että Fam3c:llä on monitahoisia vaikutuksia luuta muodostavien solujen erilaistumiseen, luun tiheyteen ja kuoriluun rakenteeseen. Osoitimme myös soluviljelyn avulla, että ilmeisimmin Fam3c estää luutamuodostavissa soluissa keskeisen Runx2 transkriptiotekijän toimintaa.

Mikro -RNA:t (miRNA:t) ovat lyhyitä (n. 22 nt) geenien ilmenemistä negatiivisesti säätteleviä RNA-molekyylejä, jotka eivät koodaa mitään proteiinia. Dicer1-entsyymi muodostaa lyhyet aktiiviset miRNA:t pidemmistä pre-miRNA -molekyyleistä. Aiempien tutkimusten mukaan miRNA:t säätelevät keskeisellä tavalla luun muodostusta, uudelleenmuovausta ja osteoblastien erilaistumista. Kuitenkin miRNA -molekyylien merkitys luun homeostaasin ylläpidossa on huonommin tunnettua. Tämän väitöskirjatutkimuksen toisena tavoitteena olikin selvittää, miten Dicer1-entsyymin inaktivaatio ja siten aktiivisen miRNA:n muodostumisen esto osteoblasteissa vaikuttaa luun homestaasiin. Tätä varten kehitettiin hiirikanta (Dicerosx), jossa Dicer1:n toiminta voidaan haluttaessa estää osterix-transkriptiotekijää ilmentävissä osteoblastin esiastesoluissa. Havaittiin, että Dicer1-entsyymin toiminnan esto näissä soluissa johti kuoriluun syntymän jälkeisen määrän ja muodon säätelyn häiriintymiseen. Myös luun taipuisuuden todettiin lisääntyneen. Nämä tutkimukset toivat lisää tietoa sytokiinin kaltaisen kasvutekijän, Fam3c:n, merkityksestä luun biologiassa ja miRNA -molekyylien merkityksestä kuoriluun homeostaasin säätelyssä.

Avainsanat: osteoblasti, Fam3c, osteogeeninen erilaistuminen, luun muodostuminen, synnytyksen jälkeinen luun homeostaasi, mikro-RNA, Dicer1

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ABBREVIATIONS

ACVR	Activin receptor
AGO	Argonaute
ATP	Adenosine triphosphate
ALK	Activin receptor-like kinase
ALP	Alkaline phosphatase
ATF4	Activating transcription factor 4
APC	Adenomatous polyposis coli
ATCC	American type culture collection
BAPX1	Bagpipe homeobox homolog 1
BMD	Bone mineral density
BMP	Bone morphogenetic protein
BRC	Bone remodelling compartment
BGLAP	Bone gamma-carboxyglutamic acid-containing protein
BMPR	Bone morphogenetic protein receptor
bHLH	Basic helix-loop-helix
BAMBI	BMP and activin membrane bound inhibitor
CTNBB1	Beta catenin
CRIM 1	Cysteine rich transmembrane BMP regulator 1
ClC-7	Chloride channel protein 7
CDK	Cyclin-dependent kinase
CTR	Calcitonin receptor
CHIP	C terminus of HSC70-interacting protein
CPED1	Cadherin-like and PC-esterase domain containing-1
Dhh	Desert hedgehog
DGCR8	DiGeorge syndrome critical region gene 8
DUSP	Dual specificity phosphatase
DKK	Dickkopf
DLL	Delta-like protein
DMP1	Dentin matrix protein 1
DVL	Dishevelled
EGFR	Epidermal growth factor receptor

EMT	Epithelial-to-mesenchymal transition
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FIAT	Factor inhibiting ATF4 mediated transcription
FOXO1	Forkhead box protein O1
FOS	c-Fos proto-oncogene
FosB	FBJ murine osteosarcoma viral oncogene homolog B
FPR	Formyl peptide receptor
FRA-1	Fos-related antigen 1
FRS	Fibroblast growth factor receptor substrate
FZD	Frizzled
GSK	Glycogen synthase kinase
GWAS	Genome wide association studies
GAB	GRB2-associated binding protein
HDAC	Histone deacetylase
HES	Hairy and enhancer of split-1
HEY	Hairy/enhancer-of-split related with YRPW motif protein 1
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HOXA2	Homeobox A2
Ihh	Indian hedgehog
ILEI	Interleukin-like epithelial-mesenchymal transition inducer
IRS	Insulin receptor substrate
iRes	Intracistronic ribosomal entry site
IGF1	Insulin-like growth factor 1
JUN	c-Jun proto-oncogene
JAG	Jagged ligand
LRP	Low density lipoprotein receptor-related protein
LEF	Lymphoid-enhancer-binding factor
LAP	Latency associated peptide
LIF	Leukemia inhibitory factor
M-CSF	Macrophage colony stimulating factor
MLO-Y4	Murine long bone osteocyte Y4
MAPK	Mitogen activated protein kinase
MEPE	Matrix extracellular phosphoglycoprotein

MAF	Avian Musculoaponeurotic Fibrosarcoma (MAF) Proto-oncogene
MEM	Minimum essential medium
MiTF	Microphthalmia-associated transcription factor
MAML1	Mastermind like protein 1
MMP	Matrix metalloproteinase
MSX-2	Msh homeobox 2
NBCn1/SLC4A7	Sodium/bicarbonate co transporter family 4 member 7
NFI-A	Nuclear Factor I/A
NFATc1	Nuclear factor of activated T cell-1
NICD	Notch intracellular domain
NPT	Sodium/phosphate transporter protein
OPG	Osteoprotegerin
PTHrP	Parathyroid hormone related peptide
PHEX	Phosphate-regulating neutral endopeptidase, X-linked
PKA	Protein kinase A
PKB	Protein kinase B
PKcδ	Protein kinase C delta
PKD	Protein kinase D
Pi	Inorganic phosphate group
Pi3K	Phosphoinositide-3-kinase
PLCγ	Phospholipase C gamma
PPi	Inorganic pyrophosphate
PHOSPHO1	Phosphatase orphan 1
PPARϫ	Peroxisome proliferator-activated receptor gamma
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone-related protein
PLAP	Placental alkaline phosphatase
RANK	Receptor activator of nuclear factor κβ
RANKL	Ligand for receptor activator of nuclear factor κβ
RBP-J	Recombining binding protein suppressor of hairless
RISC	RNA induced silencing complex
RSK2	Ribosomal S6 kinase 2
Shh	Sonic hedgehog

SOST	Sclerostin
SFRP	Secreted frizzled-related protein
STAT	Signal transducer and activator of transcription
SMAD	Mothers against decapentaplegic homolog
SMO	Smoothened
SNP	Single nucleotide polymorphism
SOCS1	Suppressor of cytokine signaling 1
SHC	SHC-transforming protein
TRACP	Tartrate-resistant acid phosphatase
TGF-β	Transforming growth factor beta
TNF	Tumor necrosis factor
TAK1	Transforming growth factor β - activated kinase 1
TRPV	Transient receptor potential cation channel
TCF	T cell specific transcription factor
TRAF 1	TNF receptor-associated factor 1
VEGF	Vascular endothelial growth factor
V-ATPase	Vacuolar type proton adenosine triphosphate
VDR	Vitamin D receptor
WIF-1	Wnt inhibitory factor 1
XPO5	Exportin 5

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text as I-III. Additionally, unpublished results have been included in this thesis. The original publications have been reproduced with appropriate permission.

I Määttä JA, **Bendre A**, Laanti M, Buki KG, Rantakari P, Tervola P, Säärimäki J, Poutanen M, Härkonen P & Väänänen K. Fam3c modulates osteogenic cell differentiation and affects bone volume and cortical bone mineral density. **Bonekey Reports** (2016);5:787.doi: 10.1038/ bonekey.2016.14. eCollection 2016.

II **Bendre A**, Buki KG and Määttä JA. Fam3c modulates osteogenic differentiation by down-regulating Runx2. **Differentiation** (2017);93:50-57. doi:10.1016/j.diff.2016.11.005

III **Bendre A,** Moritz N, Väänänen V and Määttä JA. Dicer 1 ablation in osterix positive bone forming cells affects cortical bone homeostasis. **Under Revision.**

1. INTRODUCTION

Human bone is a 'dynamic' tissue regularly undergoing cycles of resorption and formation. The balance between bone resorption and bone formation is one of the most important determinants of bone health during the lifespan of an adult individual. Skeletal disorders such as osteoporosis develop due to the loss of this balance, thereby favouring increased bone resorption rather than formation and leading to chronic complications. A detailed molecular understanding of the process of bone formation and particularly, osteoblast differentiation is therefore essential to channel our efforts into developing novel therapeutic targets to treat such diseases.

Bone formation and homeostasis is regulated by a plethora of factors including hormonal signals, growth factors, cytokines and microRNAs. Growth factors are proteins that are essential for the regulation of cellular proliferation, growth and differentiation. Growth factors, such as bone morphogenetic proteins and fibroblast growth factors play an important role in the regulation of bone formation and homeostasis. Cytokines are small molecular weight proteins (5-20 kDa) which regulate a wide range of cellular processes, such as differentiation, proliferation and tumorigenesis. Cytokines and growth-factors are secreted by cells into the extra-cellular matrix and they regulate the behaviour of the surrounding cells (paracrine regulation) or the secreting cell itself (autocrine regulation). In the bone marrow niche, cytokines such as transforming growth factor beta (TGF-β) and growth factors such as bone morphogenetic proteins (BMPs) and fibroblast growth factors (FGFs) play a crucial role in the regulation of osteogenic differentiation and bone remodelling.

MicroRNAs (miRNAs) are short regulatory RNA molecules widely known for their role in the negative regulation of gene expression in many different species including humans. MicroRNAs bind to their target mRNAs and induce translational repression or mRNA degradation, thereby inhibiting gene expression. According to the latest version of miRBase (version 21), there are over 28000 miRNAs which have been identified in many different species, including humans. A large number of miRNAs are known to be involved in the regulation of bone formation and homeostasis.

Multiple genome-wide association studies have predicted the involvement of novel genes in the regulation of skeletal homeostasis. However, their exact role in bone biology has not been elucidated. Fam3c gene was earlier implicated to have a role in the regulation of skeletal homeostasis by multiple genome-wide studies but its role was not ascertained prior to this study. Similarly, the role of osteoblast specific Dicer1 in the regulation of skeletal homeostasis was not yet characterized.

In this thesis focus is put on understanding and characterizing the role of a poorly characterized cytokine-like growth factor Fam3c (family of sequence similarity 3) and osteoblastic Dicer1 in the regulation of bone homeostasis. This thesis project aimed to characterize the role of Fam3c and osteoblastic Dicer1 in the regulation of bone formation and post-natal skeletal homeostasis respectively. Two independent mouse models (Fam3c knockout) and (Tamoxifen inducible recombination model for time controlled abrogation of Dicer1 gene function) were employed for this purpose. Micro-CT analysis was carried out to study in the detail the differences in trabecular and cortical bone structure. Additionally, biomechanical tests were carried out to determine the differences in mechanical strength post the ablation of Dicer1 gene function.

The following literature review consists of a detailed description of the processes of bone formation, osteoblast differentiation and the regulation of osteoblast differentiation by different factors. It also contains an overview of Fam3c, its role in cancer and its predicted role in bone biology. Finally, the literature review culminates in a concise review of miRNAs, their biogenesis and their involvement in the regulation of osteoblast differentiation and skeletal homeostasis.

2. REVIEW OF LITERATURE

2.1 The process of bone formation

In mammals, bone is primarily formed by two different biological processes during embryonic development. These are called as endochondral ossification and intramembranous ossification respectively. In endochondral ossification, mesenchymal condensations initially form a cartilaginous template, which then further develops into mature bone. On the contrary, during intramembranous ossification, mesenchymal condensations directly give rise to mature bone without the presence of a cartilage intermediate.

2.2 Endochondral ossification

Most of the bones of the mammalian axial and appendicular skeleton (long bones, ribs, vertebrae) with the exception of craniofacial bones and some parts of clavicle are formed by the process of endochondral ossification. During endochondral bone formation, mesenchymal cells adhere to form clusters (also termed as 'condensations') and give rise to chondrocytes (primary cells of cartilage) and perichondrial cells. Chondrocytes proliferate and secrete a matrix rich in type II collagen and the proteoglycan aggrecan leading to the expansion of the cartilage mould. Chondrocytes in the center of the mould undergo hypertrophy (increase in size) and synthesize type X collagen. These hypertrophic chondrocytes direct the perichondrial cells at the border of the cartilage condensation to become osteoblasts. The perichondrial osteoblasts secrete a matrix, forming a bone collar at the outer area of the condensation. Hypertrophic chondrocytes mineralize the surrounding matrix and attract blood vessels by secreting angiogenic factors, such as vascular endothelial growth factor (VEGF). Blood vessels invade the hypertrophic cartilage and surrounding matrix to generate the primary bone marrow cavity. The invading blood vessels supply osteoclast-like resorptive cells (also termed as 'chondroclasts') that degrade the existing matrix to produce the marrow cavity and also allow perichondrial cells to migrate into the newly formed cavity and differentiate into osteoblasts. The osteoblasts then secrete matrix proteins and undergo mineralization, giving rise to the primary ossification center (Kronenberg, 2003; Maes et al., 2010; Olsen et al., 2000).

The cartilage condensation enlarges longitudinally due to continued proliferation of chondrocytes. A portion of these chondrocytes form organized stacks that result in the lengthening of the bone. Secondary ossification centres are established wherein chondrocytes stop proliferating, undergo hypertrophy, facilitate vascular invasion and subsequent osteoblast differentiation. Indian hedgehog (Ihh) and parathyroid

hormone-related protein (PTHrP) are involved in feedback loop that controls the proliferation of chondrocytes and their differentiation into hypertrophic chondrocytes. Ihh also stimulates perichondrial cells to undergo differentiation into osteoblasts of the bone collar. In the long bones of the appendicular skeleton, chondrocytes continue to proliferate between the primary and secondary ossification centres. This region is called as the 'growth plate'(Kronenberg, 2003). The process of endochondral ossification has been depicted in Fig. 1 below.

It has been a widely accepted notion that hypertrophic chondrocytes undergo apoptosis prior to the invasion of blood vessels and osteoblast differentiation. However, a few advanced lineage tracing studies have suggested that hypertrophic chondrocytes can also transdifferentiate into osteoblasts during endochondral bone formation (Ono et al., 2014; Yang et al., 2014; X. Zhou et al., 2014b).

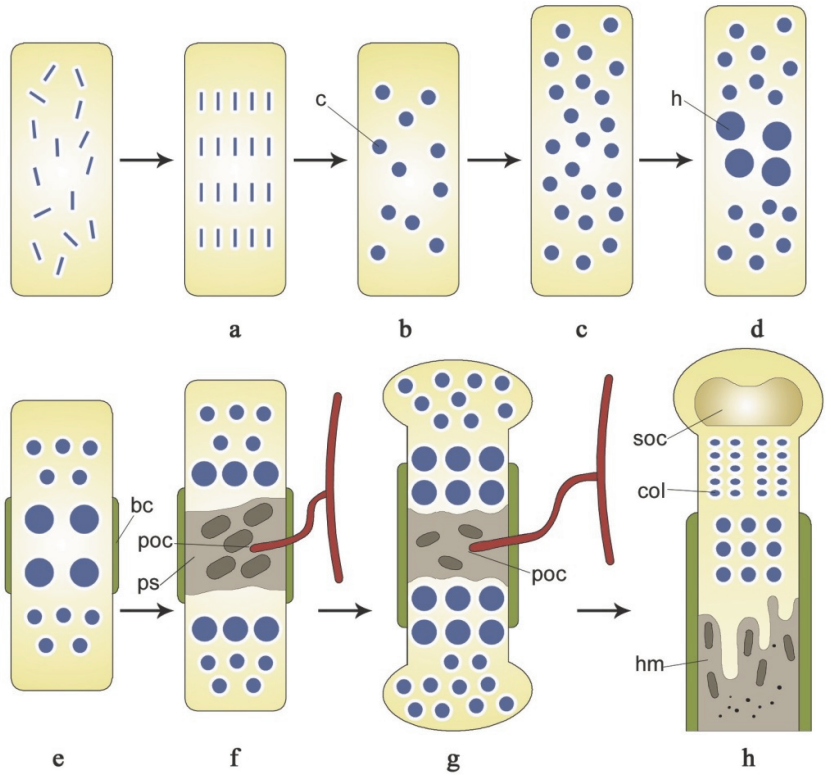

Figure 1. Endochondral bone formation (Modified from Kronenberg HM, Nature, 2003). **a)** Mesenchymal condensation. **b)** Cells transform into chondrocytes [c]. **c)** Chondrocytes proliferate. **d)** Central chondrocytes undergo hypertrophy [h]. **e)** Formation of bone collar by perichondrial cells [bc]. **f)** Formation of mineralized matrix and vascular invasion leads to the formation of primary spongiosa [ps]. Perichondrial cells migrate to the newly formed cavity, differentiate into osteoblasts. The differentiated osteoblasts undergo mineralization and give rise to the primary ossification center [poc]. **g)** Chondrocyte proliferation leading to lengthening of the bone. **h)** Repeated cycles of chondrocyte hypertrophy, vascular invasion and osteoblast differentiation result in the development of a secondary ossification center [soc], generation of a hematopoietic marrow [hm].

2.3 Intramembranous ossification

Flat bones of the skull (frontal, parietal), craniofacial bones (maxilla, nasal bones, lacrymal bones, zygomatic bones) and some parts of the clavicle are formed by intramembranous ossification (Carlson, 2014). Mesenchymal cells in condensations directly transform into osteoblasts without a cartilage intermediate, which then secrete collagenous matrix and subsequently mineralize. The process of intramembranous ossification has been depicted in Fig. 2 below.

The mesenchymal populations that give rise to the bones of the skull and the craniofacial region arise from the neural crest cells or the paraxial mesoderm (Jiang et al., 2002; Noden and Trainor, 2005; Yoshida et al., 2008). Mesenchymal condensations that form frontal and parietal bones commence at the supra-orbital ridge. Mesenchymal cells differentiate into osteoblasts, which proliferate fast and migrate outwards from the condensations (Percival and Richtsmeier, 2013). After rapid expansion of the condensations and initial mineralization, growth of the skull bones is driven towards each other by mesenchymal cells, which proliferate and differentiate (Iseki et al., 1997; Rice et al., 2003). Portions which are initially ossified thicken, and form a trabecular structure. Intramembranous ossification of facial bones is not yet well understood. Angiogenesis is also believed to play an important role during intramembranous ossification. However, this aspect has not been extensively studied.

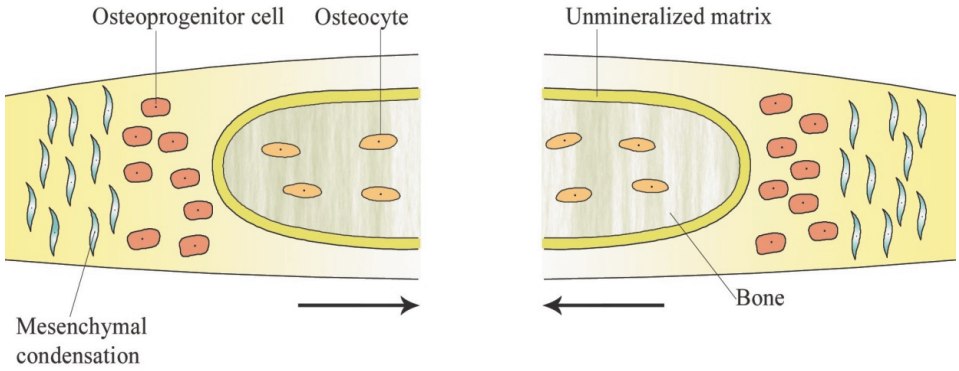

Figure 2. Intramembranous ossification. Mesenchymal condensations differentiate into bone-forming osteoblasts without a cartilage intermediate.

2.4 Cellular composition of bones

2.4.1 Osteoblasts

Osteoblasts are terminally differentiated cells which produce the bone matrix (collagen and non-collagenous proteins). They always function in clusters of

cells on the bone surface and not as independent cells. Individual osteoblasts are connected by gap junctions. Apical surface of osteoblasts is oriented towards the extracellular matrix whereas the basal surface is located away from it. Matrix deposition initially happens towards the bone surface, but subsequently surrounds and engulfs the osteoblasts. Calcification follows matrix deposition, and the buried osteoblasts eventually transform to osteocytes. Some osteoblasts remain on the bone surface and eventually become flat bone lining cells. Ultra-structurally, the osteoblast is characterized by the presence of well-developed rough endoplasmic reticulum, prominent golgi complex with multiple stacks, and a round nucleus at the basal surface. These organelles aid the osteoblast in the production and secretion of collagenous and non-collagenous matrix proteins (Pritchard, 2012).

2.4.2 Osteocytes

Osteocytes originate from osteoblasts that are buried within the bone matrix and which subsequently undergo calcification/mineral deposition. Osteocytes are the most abundant (approx. 95% of the total bone cells) and along with articular chondrocytes, are the most long-lived cells of the adult human skeleton. The transition from osteoblast to osteocyte involves profound morphological changes. There is a decrease in cellular volume along with reductions in endoplasmic reticulum and the golgi apparatus. One of the first morphological changes in this transition is the formation and extension of cellular processes, termed as dendritic processes. Dendritic processes initially extend towards the mineralizing front, and then further towards the bone surface. The osteocyte cell body is confined within a lacuna of 15-20 μm in diameter. The dendritic processes traverse through the calcified bone matrix via narrow canals called as canaliculi, approximately 250-300 nm in diameter. The osteocyte lacunae, along with the canaliculi are called as the ‘lacuno-canalicular system’. The dendritic processes link osteocytes to neighbouring osteocytes and also to the osteoblasts/bone-lining cells on the bone surface Osteocytes were initially thought to be metabolically inert cells. However, recent discoveries have shown that osteocytes are multifunctional cells having key roles in bone remodeling, mineral homeostasis, endocrine regulation of metabolism and as mechano-sensory cells (Dallas et al., 2013).

2.4.3 Bone lining cells

Osteoblasts which do not transform to osteocytes either undergo programmed cell death or become flat bone lining cells. These cells occupy bone surfaces that are neither undergoing bone formation or bone resorption. To date, these cells do not have a defined cellular marker and therefore are difficult to isolate. However, they can be easily distinguished on histological sections under the microscope

due to their 'flat shape'. The functions of bone lining cells are largely unknown. They have been proposed to remove the demineralized matrix on the bone surface prior to bone formation (Everts et al., 2002). Recently, bone lining cells were reported to be a major source of osteoblasts/pre-osteoblasts and, therefore, were suggested to play an important role in bone remodelling in adulthood (Matic et al., 2016).

2.4.4 Osteoclasts

Osteoclasts are terminally differentiated, multi-nucleated cells primarily responsible for resorption of calcified bone. They originate from the hematopoietic myeloid lineage. Multiple peripheral blood mononuclear precursor cells get activated upon stimulation with Macrophage-colony stimulating factor (M-CSF) and Ligand for receptor activator of nuclear factor κβ (RANKL) and fuse to generate a multi-nucleated osteoclast cell (Boyle et al., 2003; Väänänen, 2008). An osteoclast has two prominent morphological features, the ruffled border and the sealing zone. The plasma membrane of the osteoclast which faces the bone matrix, folds inward into numerous deep invaginations to generate the 'ruffled border'. The ruffled border has increased surface area aiding in the efficient secretion of enzymes such as lysozyme and cathepsin K, which are essential for bone resorption. The sealing zone is the area of the osteoclast plasma membrane which attaches to the bone matrix via podosomes. Podosomes are rich in cytoskeletal proteins, focal adhesion molecules, matrix degrading enzymes and molecules involved in vesicular trafficking. This attachment closes off the sub-osteoclastic bone resorption area. Bone resorption occurs by acidification and proteolysis of the bone matrix and the hydroxyapatite crystals in the sealing zone. The demineralized matrix components are then transported across the osteoclast by transcytosis and released into the extracellular area (Salo et al., 1997). Fig. 3 depicts the anatomical structure of long bone and the major types of bone cells.

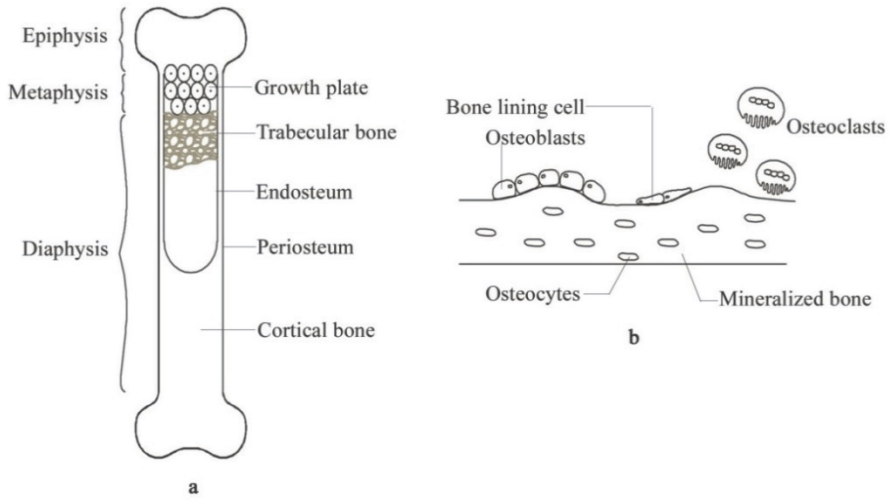

Figure 3. a) Anatomical structure of long bone. Epiphysis is the rounded end of long bone. Metaphysis is a portion between epiphysis and diaphysis. The growth plate is located in this portion. Trabecular (cancellous) bone is located at near the ends of long bones. It is porous and is composed of numerous trabeculae. Cortical bone makes up majority of the bone volume and has a different ultra-structure than the trabecular bone. Periosteum is a membrane which covers the outer surface of the bones whereas endosteum is a membrane which covers the inner surface. **b)** Major type of bone cells. Osteoblasts, osteocytes, osteoclasts and bone-lining cells make up the majority of bone cells.

2.5 Bone strength and mechanical loading

Bone is a dynamic tissue. It gradually responds and adapts to mechanical and hormonal changes in its environment. One of the major functions of the adult skeleton is load-bearing. During physical activities, mechanical forces are exerted on the bones and these loading forces also manifest changes in the bone microstructure (Klein-Nulend et al., 2012). A typical example which illustrates this phenomena is the increase in the bone mass seen in the racquet holding arm of tennis players, as compared to the other arm (Ducher et al., 2004).

Whole bone strength or the ability of bone to withstand fractures, depends upon the bone mass, shape/geometry and the inherent material properties of the bone matrix (elastic modulus, porosity, bone matrix mineralization, trabecular microarchitecture). The mechanical properties of bone also depend upon the orientation of material with respect to the direction of loading force. For instance, cortical bone from femoral diaphysis has more strength when loaded in the longitudinal direction, as opposed to when loaded in the transverse direction (Reilly et al., 1974; Reilly and Burstein, 1975). Bone remodeling or the balance between bone formation and resorption, also influences bone strength (Bouxsein, 2013).

2.6 Osteocytes and mechanotransduction

Julius Wolff in 1892, proposed that bone grows/remodels in response to the forces that are exerted on it (Pearson and Lieberman, 2004; Prendergast and Huiskes, 1995). This later became known as Wolff's law. Osteocytes sense and respond to the stimuli generated by the physical forces which act on bones (Klein-Nulend et al., 2013). Upon mechanical loading, osteocytes sense the stimuli and regulate the production of various signalling molecules, such as bone morphogenetic proteins, Wnt ligands, prostaglandin E2, nitric oxide etc. which can further modulate bone homeostasis (Bonewald, 2011; Robling et al., 2006; Santos et al., 2009; Tan et al., 2007; You et al., 2008). Conversely, in a classic experiment in mice, it was shown that the loss of bone mass in the absence of mechanical loading was prevented if the osteocytes were subjected to targeted deletion (Tatsumi et al., 2007). This observation suggested that osteocytes might influence osteoclast activity in the absence of mechanical loading, which was later verified (Kulkarni et al., 2010; You et al., 2008).

An osteocyte can sense mechanical loading by three major types of stimuli namely, a) physical deformation of the bone matrix, b) fluid-flow shear stress due to load induced flow of canalicular fluid through the lacuna-canalicular network and c) electrical potentials that are generated as a result of canalicular fluid flowing through the lacuno-canalicular network (Dallas et al., 2013). It is therefore difficult to sequester a particular stimulus in vivo since mechanical loading leads to the osteocyte being exposed to all three types of stimuli. Osteocytes are more sensitive than osteoblasts and fibroblasts to mechanical loading when applied in the form of fluid flow shear stress. Which part of the osteocyte, the cell body or the dendritic processes, is most important in the process of mechanosensing has been a question of some contention. Some groups have suggested that the osteocyte dendritic processes are important in mechanosensing, whereas others have propounded that both the osteocyte cell body and the dendritic processes sense mechanical strain (Adachi et al., 2009; Y. Han et al., 2004; Nicolella et al., 2008). Additionally, some groups have proposed that the primary cilium, the hair like projection found on all cells, is the primary strain sensing structure (Malone et al., 2007; Xiao et al., 2006).

There has been ample evidence of osteocyte responses upon mechanical loading. In both primary osteocytes and osteocyte like MLO-Y4 cells, mechanical loading induced fluid flow shear stress resulted in the expression of cyclooxygenase-2, nitric oxide, ATP, prostaglandin E2 and intracellular calcium (Cherian et al., 2005; Forwood, 1996; Gu et al., 2001; Kunnel et al., 2004; Shao et al., 2005; Thompson et al., 2011; Watanuki et al., 2002). Recently, it was found that integrin $\alpha 5\beta 1$ and connexin 43 mediate the opening of hemichannels upon mechanical stimulus in MLO-Y4 cells (Batra et al., 2012). Osteocytes therefore are crucial in the regulation of bone homeostasis in post-natal life.

Table 1. Major non-collagenous proteins in bone and their functions.

Protein	Function
Osteonectin (SPARC)	Calcium binding glycoprotein, mineralization
Osteocalcin	Metabolic regulation via promoting insulin secretion, calcium binding, hydroxyapatite stabilization
Osteopontin (Bone sialoprotein I)	Negatively charged calcium binding protein, anchoring of osteoclasts
Matrix GLA protein	Calcium binding protein, inhibitor of mineralization
Decorin	Collagen binding proteoglycan
Biglycan	Collagen binding proteoglycan, binds to growth factors
Fibronectin	Bone matrix integrity, osteoblast activity

2.7 Bone resorption by osteoclasts

The bone resorption process can be sub-divided into three phases as follows: 1) dissolution of the bone mineral calcium hydroxyapatite, 2) removal of ions released through transcytosis and 3) enzymatic break-down of the organic matrix (Väänänen and Laitala-Leinonen, 2008).

Polarized osteoclasts secrete hydrochloric acid (H+ and Cl- ions) into the resorption lacuna through the ruffled border. Acidic pH is crucial for the solubilisation of the bone mineral. Protons are transported into the resorption lacuna via Vacuolar ATPase (V-ATPase) whereas chloride anions are transported via chloride channel 7 (ClC-7) (Blair et al., 1989; Kornak et al., 2001; Väänänen et al., 1990). Acidification of the resorption compartment facilitates the dissolving of the calcium hydroxyapatite. Mineral dissolution is the rate limiting step in bone resorption, since break-down of the bone matrix can only proceed after the mineral has been dissolved.

Break-down of the mineral results in the release of a large amount of calcium and phosphate ions, which must be transported away from the resorption area. It has been suggested that calcium and phosphate ions are transported through transcytosis from the ruffled border to the apical membrane of the osteoclast (Salo et al., 1997; Yamaki et al., 2005). Other alternative routes for the intracellular transport of calcium ions have also been suggested, one of them being the calcium specific Transient receptor potential cation channel 5 (TRPV5) channel, which is localised to the ruffled border (Li et al., 2007; van der Eerden et al., 2005). Phosphate ions are removed from the resorption lacunae by sodium dependent and sodium independent phosphate transport (Gupta et al., 2001; Ito et al., 2007; Khadeer et al., 2003). Dissolution of hydroxyapatite also releases large quantities of bicarbonate ions, which are removed by sodium-bicarbonate co-transporter NBCn1 (Riihonen et al., 2010).

After break-down of the bone mineral, calcium hydroxyapatite, organic matrix of the bone is exposed to proteolytic and lysosomal enzymes. Cathepsin K plays a major role in the degradation of the organic bone matrix. Cathepsin K is a lysosomal cysteine protease which is expressed primarily in osteoclasts and is secreted into the resorption pit, where it breaks down type I collagen at acidic pH (Bossard et al., 1996; Everts et al., 2006; Väänänen and Laitala-Leinonen, 2008). Osteoclasts also express matrix metalloproteinases (MMPs) such as MMP9, MMP 14 and in some conditions MMP-12, which facilitate the digestion of type I collagen of the bone matrix (Andersen et al., 2004; Hou et al., 2004). Osteoclasts also express tartrate resistant acid phosphatase (TRAcP), and serum levels of its osteoclast-specific isoform (TRAcP 5b) is a marker of bone resorption (Halleen et al., 2006).

2.8 Bone remodelling

Bone remodelling is defined as the process by which bone is broken down and replaced by new bone. It occurs non-synchronously at distinct skeletal sites throughout the adult skeleton. The sites at which bone remodelling takes place are termed as basic multicellular units (BMUs). Old/damaged bone tissue is first resorbed by osteoclasts and then osteoblasts replace it by synthesizing new mineralized bone. Bone remodelling is therefore essential for maintenance of normal bone homeostasis (Sims and Martin, 2014). The work of Harold Frost is credited to have developed the first concepts in this aspect (Hattner et al., 1965).

At the site about to be remodelled, the presence of a canopy-like structure has been suggested (Andersen et al., 2009; Hauge et al., 2001). This canopy is referred to as ‘bone remodelling compartment’(BRC) and contains osteoblasts, bone lining cells and capillaries (Kristensen et al., 2013). The concept of ‘bone remodelling compartment’ suggests that the entire bone remodelling proceeds inside the canopy, with intercellular crosstalk taking place between different cells enclosed in the canopy (Sims and Martin, 2014). There are three phases of bone remodelling, namely, resorption, reversal and formation phases. Osteoclast differentiation and activation occurs in the basic multicellular units in response to appropriate signals, e.g., mechanical strain or bone damage. Osteoclasts then resorb the bone. After bone resorption, osteoclast activity is suppressed. In reversal phase, the degraded remnants of the resorbed bone are removed. It has been suggested that the bone lining cells play a role in this event (Everts et al., 2002). However, osteal macrophages (osteomacs) have also been suggested to remove the degraded matrix (Pettit et al., 2008). Osteoclasts release signalling molecules that facilitate the transition from bone resorption to bone formation (Martin and Sims, 2005; Sims and Martin, 2014). In the formation phase, osteoblasts lay down new bone in the resorbed area which is subsequently mineralized. The remodelling

cycle is concluded when equal amount of new bone is deposited in the resorbed area. Bone remodelling is regulated by both systemic (hormones such as parathyroid hormone (PTH), estrogens, thyroid hormone, vitamin D3, insulin-like growth factor etc) and local factors (osteoprotegerin / ligand for receptor activator of nuclear factor κβ/ receptor activator of nuclear factor κβ system, osteoblast derived Parathyroid hormone-related peptide) (Hadjidakis and Androulakis, 2006; Sims and Martin, 2014). The bone remodelling process has been depicted in Fig. 4 below.

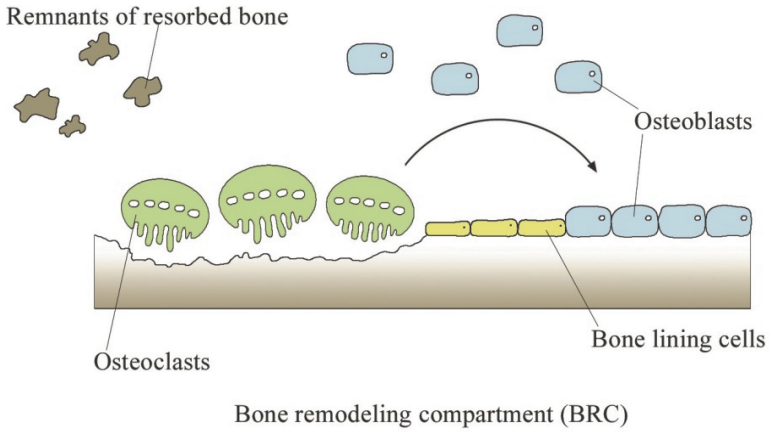

Figure 4. Bone remodeling process. Osteoclasts resorb (break-down) old/damaged bone tissue. In the reversal phase, the remnants of the resorbed bone are removed and osteoclasts secrete signaling molecules that facilitate the recruitment of osteoblasts. In the formation phase, osteoblasts synthesize new bone which is laid down in the resorbed area, completing the remodeling cycle.

2.9 Osteoblast differentiation process

Many systemic and local factors regulate the process of osteoblast differentiation including signalling pathways, transcription factors, growth factors, cytokines, miRNAs and hormones. The regulation of osteoblast differentiation and function is regulated at both local and systemic levels. Locally, factors such as TGFβ, BMPs, Wnts, FGFs regulate osteoblast differentiation. Systemic regulation involves hormones such as insulin, sex-steroids, PTH and calcitonin which play an important role. The following sections will describe the process of osteoblast differentiation in detail along with the major signalling pathways, transcription factors, and systemic regulators of bone homeostasis. The sex steroid hormones 17-B estradiol(E2)/primary estrogen and testosterone/androgen have been known to affect skeletal development and homeostasis during growth, puberty and adulthood and have been extensively reviewed elsewhere (Almeida et al., 2017; Manolagas et al., 2013). MicroRNAs that are involved in the regulation of bone formation and homeostasis have been reviewed in a separate dedicated section.

2.10 Stages of osteoblast differentiation

Osteoblasts differentiate from mesenchymal progenitor cells which are present in the marrow stroma and in the calvariae. Osteoblast differentiation is divided into three distinct stages, namely 1) commitment of mesenchymal progenitors towards osteoblastic lineage 2) maturation and extracellular matrix formation and 3) osteoblast-osteocyte transition followed by mineralization.

2.11 Commitment towards osteoblast lineage

Mesenchymal stem cells are known to differentiate into three distinct cellular lineages: osteoblasts, chondrocytes and adipocytes. Expression of specific transcription factors drives the commitment of the multipotent mesenchymal stromal cells to particular lineages. The transcription factor Sox9 is crucial for chondrogenesis. Furthermore, Sox9 expression marks all osteoblast progenitors (Akiyama et al., 2005; Bi et al., 1999). However, Sox9 is not expressed by mature osteoblasts. The transcription factor Runx2 is considered as the master regulator factor that is vital for osteoblast differentiation. Genetic ablation of Runx2 led to complete lack of osteoblast differentiation and bone formation (Komori et al., 1997; Otto et al., 1997). Mutations in Runx2 cause cleidocranial dysplasia, a congenital skeletal disorder resulting in abnormal growth of bones and teeth. Furthermore, loss of a single allele of Runx2 is sufficient for the disease to manifest (Mundlos et al., 1997). Runx2 expression marks the shift of mesenchymal progenitor cells into the osteogenic lineage.

A few developmental signalling pathways are essential in osteoblast lineage commitment. Wnt/β-catenin mediated signalling plays a role in committing mesenchymal progenitors to the osteoblast lineage (Hill et al., 2005; Rodda and McMahon, 2006). Wnt signalling also prevents adipogenic differentiation (Ross et al., 2000). Similarly, Indian Hedgehog signalling (Ihh) is necessary for initiating the osteoblastic differentiation of perichondrial cells and endochondral bone formation (Long et al., 2004; St-Jacques et al., 1999).

2.12 Osteoblast maturation and extracellular matrix formation

Runx2 induces the expression of another crucial transcription factor Osterix/Sp7. Originally discovered as a BMP2-induced gene in C2C12 cells, targeted deletion of osterix led to complete ablation of osteoblasts in mouse embryos in spite of normal Runx2 expression (Nakashima et al., 2002). Osterix is also indispensable for post-natal osteoblast differentiation and osteocyte function (Zhou et al., 2010). Runx2 also regulates the expression of several functional genes viz. alkaline phosphatase (ALP), type I collagen, osteocalcin and bone-sialoprotein (Ducy et

al., 1997; Harada et al., 1999; Javed et al., 2001; Kern et al., 2001). Mature osteoblasts (Runx2+ Osx+) synthesize and secrete extracellular matrix, which is primarily made up of type I collagen and small amounts of non-collagenous matrix proteins such as osteonectin, osteocalcin and bone-sialoprotein. The stages of osteoblast differentiation have been depicted in Fig. 5 below.

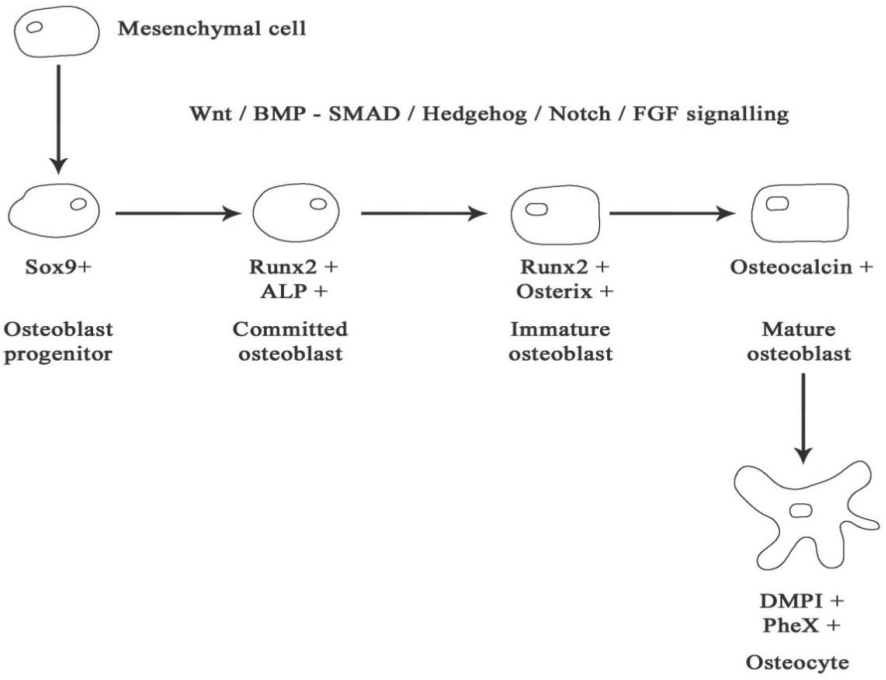

Figure 5. Stages of osteoblast differentiation. (Modified from Long F, Nature Reviews Molecular Cell Biology, 2012). Sox9 expression marks all osteoblast progenitors. Expression of Runx2 results in the commitment towards osteogenic lineage. Runx2 activates the transcription of several osteoblast related genes, such as ALP, type I collagen and osteocalcin and osterix Mature osteoblasts (Runx2+ Osterix+) synthesize and secrete extra-cellular matrix. After mineralization, some osteoblasts get buried in the mineralized matrix and differentiate into osteocytes.

2.13 Osteoblast to osteocyte transition and mineralization

An osteoblast is entitled to undergo one of three potential fates post the ceasing of organic matrix production. It can become a quiescent flat bone-lining cell, it can undergo apoptosis or it can get embedded in its own matrix and differentiate to an osteocyte (Dallas et al., 2013). Mineralization is the process by which the mineral calcium hydroxyapatite gets deposited in the organic matrix secreted by the osteoblasts. Mineralization/calcification of bone is regulated by local and systemic factors. A mature osteocyte is completely buried inside mineralized matrix and forms projections called as dendrites, that via the distinct lacuno-canalicular

system make contact with the osteoblasts/bone lining cells on the non-mineralized surface and with other osteocytes.

Based on morphological observations, some studies have reported the presence of transitional cell stages between osteoblast and osteocytes. Authors have termed the immediate/transitional stages between osteoblasts and osteocytes as osteocytic osteoblasts (Nijweide et al., 1981), osteoid-osteocytes (Palumbo, 1986) or pre-osteocytes (Holtrop, 1990). It remains a question whether the embedding of the osteoblast within its own matrix is a passive process or not. It was initially thought that this was a passive process wherein designated osteoblasts slow down their matrix production, thus getting buried under the osteoid of neighbouring osteoblasts (Franz-Odendaal et al., 2006; Nefussi et al., 1991). However, there also has been evidence to the notion that the embedding of the osteoblast might be a relatively active process wherein matrix breakdown would be required for formation of osteocyte lacuna and canaliculi (Holmbeck et al., 2005; Zhao et al., 2000).

The chemical mechanism of mineralization comprises the precipitation of hydroxyapatite from calcium, phosphate and water at a slightly alkaline pH (Neuman, 1958).

$$6\ HPO_4^{2-} + 2\ H_2O + 10\ Ca^{2+} \rightleftharpoons Ca_{10}(PO_4)_6(OH)_2 + 8\ H^+$$

Calcium and inorganic phosphate ions play a key role in the mineralization process. Calcium transport occurs through calcium channels which are expressed by osteoblasts (Blair et al., 2007), whereas inorganic phosphate (Pi) is actively produced from inorganic pyrophosphate (PPi) by ALP and indirectly by phosphatase orphan 1 (PHOSPHO1) (Hessle et al., 2002; Houston et al., 2004; Stewart et al., 2006). Inorganic PPi is generated by the enzyme NPP1 from nucleoside triphosphates (ATP/AMP). ALP, located on the osteoblast membrane surface hydrolyses PPi to yield inorganic phosphate ions (Pi, $HPO4^-/H_2PO4^-$). Crystalline hydroxyapatite formation initiates in osteoblastic membrane bound organelles termed as matrix vesicles. Hydroxyapatite ruptures the vesicle membranes and is released into the extracellular matrix, where their propagation occurs in the matrix (Anderson et al., 2005). The hydrolysis of PPi to Pi is a crucial step in mineralization process since PPi binds to the mineral calcium hydroxyapatite and blocks mineral apposition. PPi also inhibits ALP to further regulate the production of Pi (Addison et al., 2007). Ratio of Pi/PPi is therefore a critical regulator of mineralization in bone.

2.14 Osteocyte differentiation markers

In the last couple of decades, delineating the molecular gene expression changes that occur during osteocyte differentiation has been possible due to the discovery

of osteocyte specific markers and transgenic Green fluorescent protein (GFP) variant mouse lines (Dallas et al., 2013). Transition towards the osteocytic fate entails down-regulation of ALP and type I collagen and expression of proteins such as Dentin matrix protein1 (DMP1), E11/podoplanin, FGF23, Matrix extracellular phosphoglycoprotein (MEPE) and Phosphate regulating nuclear endopeptidase, X-linked (PHEX) (Franz-Odendaal et al., 2006; Schulze et al., 1999; Toyosawa et al., 2001; Ubaidus et al., 2009). Sclerostin is expressed at a more mature stage of the osteocyte differentiation (Winkler et al., 2003). E11 protein is expressed during initial stages of osteocyte differentiation (Schulze et al., 1999; Wetterwald et al., 1996). Osteocytes also express proteins involved in cytoskeletal arrangement and function viz. Macrophage-capping protein (CapG), Destrin and CD44 (Guo et al., 2010; Hughes et al., 1994).

2.15 Runx2

Runt-related transcription factor 2 (Runx2) is a transcription factor belonging to the family of Runt-related transcription factors (Runx). These transcription factors are heterodimeric proteins, comprised of alpha and beta sub-units. The alpha subunit comprises of evolutionary conserved 'runt-domain' which binds to the DNA as well as to the beta subunit for hetero-dimerization. The beta subunit is non-DNA binding but its hetero-dimerization with the alpha subunit stabilizes the interaction of the latter with DNA and, thus enhances its DNA binding capacity. In mammals, three genes code for the DNA binding alpha subunits of the Runx transcription factors namely, Runx1(Cbfa2/AML1/Pebp2αB), Runx2(Cbfa1/AML3/Pebp2αA) and Runx3 (Cbfa3/AML2/Pebp2αC). The names in the parentheses are synonyms of the respective genes. The core binding factor beta (CBFβ) gene encodes the non-DNA binding beta subunit which heterodimerizes with the alpha subunits to form Runx1/Runx2/Runx3 transcription factors, respectively (Bruderer et al., 2014).

Runx2/Cbfa1 was first identified to be involved in bone formation by two different groups (Komori et al., 1997; Otto et al., 1997). Homozygous mutant mice exhibited complete lack of bone formation and died shortly after birth due to breathing failure. Both intramembranous and endochondral ossification were affected in the mutant mice, giving rise to absence of ossification in the complete skeleton. Failure of osteoblast differentiation was evident in the mutant mice, with low expression of osteoblast marker proteins such as ALP, osteonectin and barely detectable levels of osteocalcin and osteopontin. Failure of osteoblast differentiation and subsequent mineralization was evident in the mutants, owing to maturational arrest in the early differentiation stage, and lack of vascular invasion. In the wild type embryos, Runx2/Cbfa1 was expressed in osteoblasts but not in osteoclasts. Significant Runx2 expression was identified in skeletal

mesenchymal condensations, and skeletal condensations of the developing limbs. Weak expression was also observed in the notochord E9.5, which later extended into the trunk region (Komori et al., 1997; Otto et al., 1997).

2.15.1 Molecular regulation and transcriptional activity of Runx2

Human Runx2 gene is transcribed from two different promoters P1 and P2. The promoters contain binding sites, AP1 (activator protein 1), OSE2 (Osteocalcin specific element – 2) and NF1 (Nuclear factor – 1) for binding of different transcription factors (Bruderer et al., 2014). Runx2 has also been shown to auto-regulate its own expression by binding to an OSE2 elements in its promoter (Tou et al., 2003). Many other transcription factors have been reported to activate Runx2 without binding to the Runx2 promoter viz. HOXA2 (Homeobox A2), BAPX1 (Bagpipe homeobox homolog 1), MSX-2 (Msh homeobox 2), PPArγ2 (Peroxisome proliferator-activated receptor gamma 2) (Kanzler et al., 1998; Satokata et al., 2000; Tribioli and Lufkin, 1999). Both Runx2 transcription and transcriptional activity are regulated by TGF-β/Smad3 signaling (Alliston et al., 2001). Similarly, Runx2 was shown to be induced by BMP-2 /Smad5 signalling and BMP4/7 signalling (Lee et al., 2001; Tsuji et al., 1998). Fibroblast growth factors (FGF2 and FGF8) also induce Runx2 expression (Zhou, 2000). All trans retinoic acid was also shown to induce Runx2 expression (Jiménez et al., 2001). Runx2 is negatively regulated by tumor necrosis factor – alpha (TNFα) and by vitamin D3 (Drissi et al., 2002; Gilbert et al., 2002). The synthetic glucocorticoid dexamethasone was also showed to induce Runx2 expression in human osteoblast cell lines (Prince et al., 2000).

Runx2 phosphorylation was first reported to be modulated by Mitogen activated protein kinase (MAPK) signalling (Xiao et al., 2002). MAPK mediated phosphorylation has been shown to be both positive and negative regulator of Runx2 expression (Ge et al., 2009; Huang et al., 2012; Li et al., 2012; Xiao et al., 2002). Other kinases have also been shown to regulate phosphorylation of Runx2, namely protein kinase A (PKA), protein kinase c delta (PKcδ), protein kinase B (Akt), homeodomain interacting protein kinase 3 (HIPK-3), Cyclin-dependent kinase 1 (CDK1), Cyclin-dependent kinase 4 (CDK4) and Glycogen synthase kinase 3 beta (GSK-3β) (B. G. Kim et al., 2006; Kugimiya et al., 2007; Pande et al., 2013; Pierce et al., 2012; Qiao et al., 2006; Selvamurugan et al., 2000; Shen et al., 2006; Sierra and Towler, 2010). Phosphorylation at several different residues regulates the expression of Runx2 differentially. Runx2 acetylation was shown to be regulated by the E1A-associated 300 kDa protein (p300) (Jeon et al., 2006), which enhanced its transcriptional activity. Ubiquitination of Runx2 and subsequent proteasomal degradation is known to be controlled by an E3 ubiquitin ligase SMAD ubiquitin regulatory factor -1 (Smurf1) (Zhao et al., 2003).

Additionally, two other E3 ubiquitin ligases promote Runx2 ubiquitination/ degradation and thereby negatively regulate osteoblast differentiation. They are C terminus of Hsc 70-interacting protein (CHIP) and WW domain containing E3 ligase (WWP1) with the adaptor protein Schnurri-3 (Jones et al., 2006; Li et al., 2008). Several other proteins are known to interact with Runx2. The most classical interaction is the interaction of the beta subunit (core binding factor beta) which is the non-DNA binding subunit of the Runx2 heterodimer. Binding of the beta subunit enhances the transcriptional activity of Runx2 by stabilizing the binding of Runx2 to DNA (Kagoshima et al., 1993; Ogawa et al., 1993). Many other proteins also bind to Runx2 and modulate its activity. These are summarized in table 2.

2.15.2 Runx2 target genes and biological functions

Runx2 regulates the expression of several osteoblastic genes which are required for osteoblast differentiation namely, osteocalcin, type I collagen, bone sialoprotein, osteopontin (Bruderer et al., 2014). Runx2 also regulates the expression of the osteoblast transcription factor Osterix, which is also necessary for normal osteoblast differentiation and mineralization (Nishio et al., 2006). In addition to regulating osteoblast specific genes, Runx2 also negatively regulates osteoclast differentiation by inducing the expression of osteoprotegerin (OPG), which in turn inhibits osteoclast differentiation (Thirunavukkarasu et al., 2000). Conversely, Runx2 over-expression stimulated osteoclastic bone resorption by up-regulating RANKL (Geoffroy et al., 2002). In addition to being the master transcriptional regulator of the osteogenic differentiation program, Runx2 has been shown to regulate other biological functions. Runx2 was shown to be involved in tooth development (Gaikwad et al., 2001), in chondrocyte hypertrophy and maturation (Takeda et al., 2001), as well as in angiogenesis (Namba et al., 2000; Sun et al., 2001).

2.16 Osterix/Sp7

Osterix was first identified in 2002 as a zinc-finger domain containing transcription factor necessary for osteoblast differentiation and bone formation (Nakashima et al., 2002). It is 45 kDa protein with three C2H2 type zinc-finger DNA binding domains at C-terminus, and a proline rich transcription activation domain near the N-terminus. The zinc finger motif shows strong sequence homology to similar motifs present in transcription factors Sp1, Sp3 and Sp4, which led to an alternative name Sp7. During mouse embryonic development, osterix was found to be expressed in differentiating chondrocytes (E13.5), but not in the condensed mesenchyme. Later on (E15.5), osterix was expressed in cells associated with bone trabeculae and also in pre-hypertrophic chondrocytes, albeit in lesser amounts. Post-natally, it was strongly expressed in bone trabeculae, secondary ossification centers, endosteal and

perosteal surfaces, as well as in the formed bone matrix. In Osx-null embryos, no mineralization takes place, in the skeletal elements formed by intramembranous as well as endochondral ossification leading to absence of cortical bone and bone trabeculae and therefore giving rise to deformed bones. Osteogenic differentiation in the Osx-null cells was found to be obstructed due to absence of bone matrix formation and subsequent mineralization. Expression of type I collagen was severely decreased in Osx-null mutants, whereas levels of other osteoblast marker proteins such as osteonectin, osteopontin and bone-sialoprotein were not detected. During osteogenic differentiation, osterix is expressed downstream of Runx2 (Nakashima et al., 2002).

2.16.1 Molecular regulation and transcriptional activity of osterix

Runx2 directly regulates expression of osterix during osteoblast differentiation by binding to a Runx2 binding element in the promoter region. Osterix promoter analysis showed that the promoter contains binding sites for several different transcription factors including SOX9, vitamin D response element (VDRE), Sp1 and Dlx5 in addition to the Runx2 binding site (Nishio et al., 2006). Moreover, expression of osterix is also controlled independently of Runx2. In Runx2 null calvarial osteoblasts, osterix is not normally expressed. However, stimulation of the cells by BMP2 activated osterix through the up-regulation of the transcription factor Dlx5. Dlx5 binds to its binding element in the osterix promoter and gets phosphorylated subsequently, leading to the transcriptional activation of osterix (Lee et al., 2003). Osterix regulates its own expression through a positive feedback loop by binding to its own promoter (Yoshida et al., 2012). Both BMP2 signalling pathway and Insulin-like growth factor (IGF1) pathway induce the expression of osterix (Huang et al., 2007; Jadlowiec et al., 2004). Synergistically, BMP2/IGF1 induce greater expression of osterix than IGF1 alone. This is due to the concurrent activation of MAPK and PKD pathways which are activated by BMP2/IGF1 (Celil and Campbell, 2005). The tumor suppressor protein p53 was also shown to regulate osterix expression (Wang et al., 2006). Epidermal growth factor receptor (EGFR) and TNFα negatively regulate osterix expression. EGFR down-regulates osterix expression through inhibition of Runx2 (Zhu et al., 2011), whereas TNFα induces Prx1 (paired-related homeodomain) expression which further interacts with osterix promoter to suppress osterix expression (Lu et al., 2011). Some miRNAs have been reported to regulate osterix function viz. miR-135, miR-93, miR-138 (Eskildsen et al., 2011; Schaap-Oziemlak et al., 2010; Yang et al., 2012). The evolutionary conserved histone demethylase NO66 was reported to negatively regulate osterix (Sinha et al., 2010). Post-translational phosphorylation of osterix by p38 MAPK at serine residues 73 and 77 and by Akt at threonine residues near the C terminus, regulates its transcriptional activity (Choi et al., 2011; Ortuño et al., 2010).

2.16.2 Functional roles during osteoblast differentiation

Osterix interacts with nuclear factor of activated T cells (NFATc1) transcription factor and regulates the expression of type 1 collagen during osteoblast differentiation. NFATc1 directly binds to osterix and enhances its transcriptional activity (Koga et al., 2005). Osterix also suppresses osteoblast proliferation by inhibiting Wnt signalling. It induces the expression of Wnt antagonist DKK1 (Dickkopf 1) and it also disrupts the transcription activity of β-catenin/ T-cell specific transcription factor 1 complex (TCF-1) (Zhang et al., 2008). Osterix was recently shown to regulate the expression of bone sialoprotein in osteoblasts (Yang et al., 2016). Osterix was also suggested to activate the sclerostin gene (sost) as it was able to bind its gene promoter. Similarly, osterix regulates the expression of osteocyte specific genes DMP1 and PHEX (phosphate regulating neutral endopeptidase-X linked). Osx-null mutants displayed deformed osteocytes accompanied by decreased number of dendritic processes, as compared to the wild-type embryos. Osterix is also essential for post-natal skeletal development and maintenance of bone homeostasis (Zhou et al., 2010).

2.17 ATF4

Activating transcription factor 4 (ATF4) is a member of the basic leucine zipper family (bZIP) of transcription factors. ATF4 was first identified as a substrate of the growth factor- regulated Ribosomal S6 kinase 2 (RSK2) and was implicated to play a role in osteoblast biology. Additionally, dysregulation of ATF4 expression and activity was suggested to contribute to the skeletal phenotype manifested in Coffin-Lowry syndrome, in which RSK2 is mutated (Yang et al., 2004). Osteocalcin gene expression is directly regulated by ATF4. ATF4 also plays a role in osteoclast differentiation by regulating the expression of NFATc1 and RANKL, the latter through the induction of M-CSF (Cao et al., 2010). Factor inhibiting ATF4 mediated transcription (FIAT) binds to ATF4 directly and inhibits its transcriptional activity (Yu et al., 2005). ATF4 also facilitates protein synthesis in osteoblasts, by regulating import of amino acids. FOXO1, a transcription factor of the FOXO family promotes bone formation partly by interacting with ATF4 to drive amino acid import and protein synthesis (Rached et al., 2010). Furthermore, ATF4 has also been known to regulate chondrocyte differentiation by activating Indian hedgehog signalling (Wang et al., 2012). ATF4 is therefore an important transcription factor required for osteoblast differentiation.

2.18 AP-1 family transcription factors

AP-1 family of transcription factors comprises of proteins from JUN (c-Jun proto-oncogene, AP-1 transcription factor subunit), FOS (c-Fos proto-oncogene, AP-1

transcription factor subunit), ATF (activating transcription factor) and MAF (musculo-aponeurotic fibrosarcoma) families, which form homodimers/heterodimers through their respective leucine zipper domains (Eferl and Wagner, 2003). Different AP-1 factors have been attributed to have differential effects on osteoblast differentiation and bone formation. Embryo-specific deletion of AP-1 family protein Fra-1 (FOS- related antigen 1) lead to lowered synthesis of bone matrix proteins and subsequent loss of bone mass (Eferl et al., 2004). Overexpression of Fra-1 resulted in gradual accrual of bone mass owing to increased number of mature osteoblasts. Osteoblast differentiation, and not osteoblast proliferation was enhanced in the transgenic mice over-expressing Fra-1 (Jochum et al., 2000). In another study, transgenic overexpression of Delta FosB (ΔFosB), which is a truncated form of FosB arising from alternative splicing and which is expressed in osteoblasts, led to progressive increase of bone mass throughout the skeleton resulting in osteosclerosis (Sabatakos et al., 2000). Genetic deletion of AP1 factor JunB engendered osteopenia in mice (Kenner et al., 2004).

Table 2. Proteins regulating osteoblast differentiation by interacting with Runx2, Osterix, and ATF-4. (Modified from Long et al., Nature Reviews Molecular Cell Biology, 2012)

Factor	Features	Mechanism of action	Function	References
SATB2	Nuclear matrix protein	Enhances Runx2 and ATF4 activities	Stimulation of osteoblast differentiation	(Dobreva et al., 2006)
DLX5	Homeodomain transcription factor	Stimulates Runx2 expression	Stimulation of osteoblast differentiation in craniofacial skeleton	(Lee et al., 2005; Robledo et al., 2002)
MSX2	Homeodomain transcription factor	Stimulates Runx2 expression	Stimulation of osteoblast differentiation in skull	(Satokata et al., 2000)
HOXA2	Homeodomain transcription factor	Down-regulates Runx2 expression	Suppresses osteoblast differentiation in craniofacial skeleton	(Kanzler et al., 1998)
BAPX1	Homeodomain-containing transcription factor	Stimulates Runx2 expression	Stimulation of osteoblast differentiation in axial skeleton	(Tribioli and Lufkin, 1999)
TWIST	bHLH transcription factor	Suppresses Runx2 activity	Inhibits osteoblast differentiation	(Bialek et al., 2004)
HAND2	bHLH transcription factor	Suppresses Runx2 activity	Inhibits intramembranous ossification in jaw bones	(Funato et al., 2009)

Factor	Features	Mechanism of action	Function	References
HES and HEY	bHLH transcription factors	Inhibit Runx2 activity	Inhibit osteoblast differentiation	(Garg et al., 2005; Hilton et al., 2008)
p53	tumor suppressor protein	Reduces osterix expression	Inhibits osteoblast differentiation	(Wang et al., 2006)
Rb	tumor suppressor protein	Stimulates Runx2 activity	Enhances osteoblast differentiation	(Calo et al., 2010; Thomas et al., 2001)
ZFP521	Zinc finger protein	Recruits HDAC3 and suppresses Runx2 activity	Inhibits osteoblast differentiation	(Hesse et al., 2010)
Schnurri 3	Zinc finger protein	Aids in Runx2 degradation	Inhibits bone matrix deposition	(Jones et al., 2006)
GLI3	Zinc finger transcription factor	Suppresses Runx2 activity	Inhibits osteoblast differentiation	(Ohba et al., 2008)
MAF	basic Leu zipper transcription factor	Stimulates Runx2 activity	Enhances osteoblast differentiation	(Nishikawa et al., 2010)
STAT1	STAT family transcription factor	Prevents nuclear translocation of Runx2	Inhibits osteoblast differentiation	(Kim et al., 2003)
Smurf1	E3 ubiquitin ligase	Promotes Runx2 degradation	Suppresses osteoblast differentiation	(Zhao et al., 2003)
CHIP/STUB 1	E3 ubiquitin ligase	Promotes Runx2 degradation	Suppresses osteoblast differentiation	(Xueni Li et al., 2008)
Pin1	Peptidyl-prolyl isomerase	Binds to and stabilizes osterix	Regulates osteoblast differentiation	(Lee et al., 2015)
CyclinD1/ cdk4	multi-protein complex	Induces Runx2 phosphorylation and subsequent proteasomal degradation	Suppresses osteoblast differentiation	(Shen et al., 2006)
TLE	transcriptional co-repressor	Inhibit Runx2 activity	Suppresses osteoblast differentiation	(Javed et al., 2000)
p300	Histone acetyltransferase	Stabilizes and increases Runx2 transcriptional activity	Stimulates osteoblast differentiation	(Jeon et al., 2006)
Protein kinase C delta	Serine- Threonine protein kinase	Runx2 phosphorylation and activation	Acts downstream of FGF2 stimulation	(Kim et al., 2006)
Homeodomain interacting protein kinase 3	Serine-Threonine protein kinase	Runx2 phosphorylation and activation	Stimulates osteoblast differentiation	(Sierra and Towler, 2010)
GSK3Beta	Serine-Threonine protein kinase	Runx2 phosphorylation and inhibition	Inhibits osteoblast differentiation	(Kugimiya et al., 2007)

2.19 Signalling pathways essential for skeletal development

2.19.1 Hedgehog signalling

Hedgehog signalling pathway, promptly named after the 'Hedgehog' ligand proteins that activate it, is an important signal transduction pathway involved in embryonic development, morphogenesis and patterning. There are three hedgehog ligand proteins found in mammals namely, sonic hedgehog (Shh), Indian hedgehog (Ihh) and desert hedgehog (Dhh). Hedgehog ligands, on binding to their receptor Patched homolog 1 (PTCH1), induce the de-repression of a seven pass transmembrane protein called Smoothened (SMO) which further activates transcription factors of the Gli family (Gli1, Gli2 and Gli3) to control gene expression (Ingham and McMahon, 2001).

In a study reported in 1997, Gli2 and Gli3 were shown to be crucial for skeletal development and patterning, with individual mutant mice exhibiting defects in craniofacial, axial and appendicular skeleton (Mo et al., 1997). Indian hedgehog protein was found to be essential for chondrocyte proliferation and maturation during endochondral ossification. Genetic ablation of Ihh in mice resulted in a severely skeleton phenotype displaying shortened limbs, rounded head and tail in the mutant embryos. Lack of Ihh led to a complete absence of mature osteoblasts in the endochondral skeleton, although intramembranous osteoblasts were present (St-Jacques et al., 1999). Indian hedgehog signalling was shown to be essential for perichondrial cells to initiate osteoblast differentiation during endochondral bone formation. Indian hedgehog-deficient perichondrial precursors were unable to express the critical transcription factors Runx2 and osterix, necessary for osteoblast differentiation (Long et al., 2004). Gli2 and Gli3 are the major effectors of Ihh signalling during osteoblast differentiation and skeletal development (Hilton et al., 2005; Joeng and Long, 2009). Intramembranous ossification on the contrary, does not seem to have an essential role of Ihh signalling. The role of hedgehog signalling in post-natal life is incompletely understood. Indian hedgehog deletion in growth plate chondrocytes led to defective growth plate and trabecular bone loss in older mice (Maeda et al., 2007). In another study, hedgehog signalling ablation in mature osteoblasts led to decreased osteoclast differentiation and subsequent increase in bone formation (Mak et al., 2008).

Genetic mutations of Ihh cause Brachydactyly type A-1 (Gao et al., 2001), which is characterized by shortened/missing middle phalanges. Gli3 mutations were reported to cause Grieg cephalopolysyndactyly, Pallister–Hall syndrome and postaxial polydactyly type 3 which are characterized by multiple skeletal deformities (Kang et al., 1997; Vortkamp et al., 1991). Mutations of the receptor PTCH1 cause Gorlin syndrome, also known as nevoid basal cell carcinoma syndrome, which manifests several skeletal abnormalities such as ectopic

ossification, rib anomalies, spina bifida in addition to occurrence of basal cell carcinomas (Johnson et al., 1996). Hedgehog signalling plays an important role in endochondral bone formation, skeletal development and postnatal bone homeostasis.

2.19.2 Notch Signalling

Notch signalling is an evolutionary conserved intracellular signalling pathway which is crucial for embryogenesis and cell fate specification. Notch signalling is also involved in a number of different biological processes which range from cell differentiation, tissue morphogenesis and homeostasis to diseases such as cancer and cardiomyopathies (Artavanis-Tsakonas et al., 1999; Bray, 2006). Notch signalling has a rather simple operating mechanism. Ligand-receptor interaction leads to the release of Notch-intracellular domain (NICD) which translocate to the nucleus, interacts with transcription factor CBF1/RBP-J and co-activator mastermind (MAML1) and activates transcription of target genes namely, Hairy and enhancer of split-1 (HES) and Hairy/enhancer of split related with YRPW motif protein 1 (HEY) transcription factors. HES and HEY transcription factors regulate the expression of several other target genes (Bray, 2006; Long, 2011). There are four types of Notch receptors in mammals (NOTCH1, NOTCH2, NOTCH3 and NOTCH4). These Notch receptors are present on cell surface where they interact with notch ligands (JAG1, JAG2, DLL1, DLL3 and DLL4) present on the surface of the neighbouring cell/cells. Receptor-ligand interaction triggers the proteolytic cleavage of Notch intracellular domain which then activates transcription in the cell nucleus.

Genetic deletion of Notch signalling proteins gamma secretase, Notch receptors 1 and 2 in limb mesenchyme resulted in increase of bone mass in adult mice whereas it led to elongation of hypertrophic cartilage and increased number of trabecular osteoblasts in the embryos analysed. Moreover, decreased number of mesenchymal progenitors was observed in the mutant mice, pointing towards the possibility that Notch signalling suppresses bone formation through acting on the mesenchymal progenitors. Further investigation of the mechanism unveiled the role of HES1 and HEYL (HEY-like) transcription factors in suppressing osteoblast differentiation by physically interacting with transcription factor Runx2 and regulating its transcriptional activity (Hilton et al., 2008). In a recent study, endothelial cell specific genetic disruption of Notch signalling was shown to regulate osteogenesis, chondrocyte differentiation and bone mass, which was due to defective release of Notch regulated Noggin (secreted antagonist of BMP signalling) from endothelial cells (Ramasamy et al., 2014).

Loss of function mutations in NOTCH1 results in aortic valve disease (Garg et al., 2005), whereas gain of function mutations of NOTCH2 caused Hajdu-Cheney

syndrome, an extremely rare skeletal disorder with progressive bone loss and osteoporosis (Isidor et al., 2011; Simpson et al., 2011). Osteoblast specific gain of Notch function led to the development of severe osteosclerosis (Engin et al., 2008). Overexpression of the notch downstream HEY1 transcription factor led to development of osteopenia (Salie et al., 2010). Notch signalling was also implicated to play a role in the development of osteosarcomas (Engin et al., 2009).

2.20 Signalling pathways involved in osteoblast differentiation and skeletal homeostasis

2.20.1 BMP/SMAD signalling

Bone morphogenetic proteins are a class of proteins belonging to the TGF-β superfamily. Originally identified to play a role in bone and cartilage development, they are involved in a wide array of biological processes including cell growth, apoptosis and differentiation (Kobayashi et al., 2005; Zou and Niswander, 1996). BMPs bind to hetero-tetrameric complexes composed of type II or type I serine/threonine kinase receptors. Trans-phosphorylation of type I (BMPR1) receptor by constitutively active type II receptor (BMPRII) follows and leads to the recruitment and phosphorylation of receptor regulated SMAD proteins (R-SMADs: SMAD1, SMAD5, SMAD8/9). Phosphorylated R-SMAD's form a complex with a co-SMAD (SMAD4) and this complex translocates to the nucleus to regulate gene expression. There are three types of type I BMP receptors viz. ALK-2, ALK3/BMPR1A and ALK6/BMPR1B and only one type II BMP receptor, BMPR2 (R. N. Wang et al., 2014; Zhang and Li, 2005). BMP signalling has also been reported to function via SMAD independent, non-canonical mechanism. BMP4 was found to activate Transforming growth factor β – activated kinase 1 (TAK1), a serine threonine kinase of MAPK family, whereas activation of Phosphoinositide-3-kinase (PI3K)/Akt, Protein kinase C, Rho-GTPases by BMP signalling have also been reported (Yamaguchi et al., 1995; Zhang, 2009).

Mice lacking both BMP2 and BMP4 displayed impaired bone formation (Bandyopadhyay et al., 2006). BMP2,4,5,7 and 9 can stimulate osteogenic differentiation (Luu et al., 2007; Wu et al., 2016; Yeh et al., 2002; Zhu et al., 2015). BMP4 is involved in the regulation of limb development (Selever et al., 2004). BMP2 has been shown to be critical to initiate fracture healing response and is essential for chondrocyte proliferation and differentiation during endochondral bone formation (Shu et al., 2011; Tsuji et al., 2006). Osteoblast specific inactivation of BMPR1A resulted in increased trabecular bone mass and also decreased osteoclastogenesis. Moreover BMPR1A ablation led to the down-regulation of SOST gene, which is a negative regulator of Wnt signalling (N.

Kamiya et al., 2008; Nobuhiro Kamiya et al., 2008). Post-natally, BMPR1A deletion in osteoblasts resulted in their decreased function, particularly the ability to form mineralizing nodules was severely affected (Mishina et al., 2004). BMP3 was recently shown to negatively regulate the activities of BMP2, BMP4 (Kokabu et al., 2012). Dynamic signalling crosstalk between BMP, Wnt and TGF-β signalling evident during different stages of osteoblast differentiation (Wu et al., 2016).

2.20.2 Wnt signalling

Wnt signalling pathway is an evolutionary conserved signalling pathway which has crucial roles in many biological processes including but not limited to embryonic development, cell proliferation, axis patterning and cell fate specification. Wnt ligands bind to cell surface receptors and activate the pathway, which ultimately leads to regulation of gene expression via β-catenin dependent (canonical signalling) and independent (non-canonical signalling) modalities. Canonical Wnt β-catenin signalling pathway has been the most studied pathway with regard to skeletal development and homeostasis. Binding of Wnt ligands to the transmembrane receptor Frizzled (FZD) and to the co-receptor Low density lipoprotein related protein 5/6 (LRP5/6), leads to the inhibition of GSK-3β kinase through signals mediated by proteins Dishevelled (DVL), Axin, Adenomatous polyposis coli (APC) and Fra-1. Inhibition of GSK-3β kinase results in the hypo-phosphorylation and stabilization of β- catenin. Stabilized β-catenin translocates to the nucleus and activates gene transcription by interacting with transcription factor TCF/LEF. Wnt signalling is intricately regulated by secreted Wnt antagonist proteins viz. secreted frizzled related proteins (SFRPs), Wnt inhibitory factor 1 (WIF-1), SOST and DKK, which compete with Wnt ligands for binding to their receptors and block the signal transduction. Members of the SFRP family of proteins and WIF-1 bind to Frizzled receptors whereas members of the DKK family of proteins and sclerostin (Sost) bind to the co-receptor LRP5/6. There are ten known members of the Frizzled receptor family in humans (FZD1 to FZD10), more than 15 members of Wnt ligands and sub-types (Wnt 1 to Wnt 16), and five members of the sFRP family of glycoproteins, sFRP1 to sFRP5 (Krishnan et al., 2006; MacDonald et al., 2009; Regard et al., 2012).

Canonical Wnt signalling is essential for the cells of the osteoblastic lineage (Baron and Kneissel, 2013). Wnt signalling suppresses adipogenic and chondrogenic differentiation of mesenchymal stem cells and promotes osteoblast differentiation (Day et al., 2005; Hill et al., 2005; Kennell and MacDougald, 2005). Osteoblastic and osteocytic Wnt signalling negatively regulates osteoclast differentiation by regulating the production of OPG, the decoy receptor for RANKL (Glass et al., 2005; Kramer et al., 2010). In humans, loss of function

mutations in LRP5 Wnt receptor cause osteoporosis-pseudoglioma syndrome, which is a form of juvenile-onset osteoporosis (Gong et al., 2001). Another set of mutations in the co-receptor LRP5 gave rise to high bone mass disorder (Boyden et al., 2002; Little et al., 2002). Deletion of LRP5 or Wnt10b in mice resulted in post-natal loss of bone mass (Bennett et al., 2005; Kato et al., 2002). β-catenin has also suggested to play a role in the transition from Runx2+ cells to Runx2+ Osx+ cells during osteoblast differentiation (Hu et al., 2005; Rodda and McMahon, 2006).

Osteocyte secreted SOST and DKK1 were found to affect bone formation and bone mass accrual by negatively regulating Wnt signalling (Li et al., 2006; Xiaodong Li et al., 2008; Morvan et al., 2006; Winkler et al., 2003). On the contrary, the Wnt antagonist DKK2 which binds to LRP6, has been shown to regulate terminal osteoblast differentiation and mineralization (Li et al., 2005). Loss of function mutations in the SOST gene were identified to be the cause of Sclerosteosis or van Buchem's disease (Balemans et al., 2002, 2001).Wnt signalling has also been implicated to play a role in osteocyte mechanosensing. Osteocytes are the main source of SOST, which is a Wnt signalling inhibitor and whose expression is regulated by mechanical loading (Turner et al., 2009). Gain of function mutations in LRP5, to which sclerostin binds and blocks Wnt signalling, resulted in increased responses to mechanical stimuli whereas deleterious mutations in LRP5 reduced them (Robinson et al., 2006; Saxon et al., 2011)

Wnt signalling in bone has been shown to be interacting with other signalling pathways involved during osteoblast differentiation and bone formation. Particularly, Wnt signalling is involved in inter-pathway crosstalk with PTH signalling as well as BMP signalling. Multiple lines of evidence indicate the presence of molecular crosstalk of Wnt signalling with PTH signalling. PTH negatively regulates the expression of Wnt antagonists, SOST and DKK1 (Guo et al., 2010; Keller and Kneissel, 2005). Regulation of SOST by PTH has also been shown in humans (Costa et al., 2011). Activation of PTH signalling leads to β-catenin phosphorylation and subsequent stabilization (Guo et al., 2010). Similarly, in vitro studies have shown that PTH-PTH1R (Parathyroid hormone receptor 1) complex can trigger Wnt signalling by interacting with the Wnt co-receptor LRP6 (Wan et al., 2008). BMPR1A mediated BMP signalling in osteoblasts induces the transcription of the Wnt inhibitors DKK1 and SOST (Kamiya, 2012). BMP2 modulates osteoblast Wnt β-catenin signalling (Bain et al., 2003). Moreover, BMP signalling antagonist protein Wise has been shown to negatively regulate Wnt signalling by acting as Wnt antagonist (Lintern et al., 2009). β-catenin also interacts with cell adhesion facilitating cadherin proteins, which are also shown to be involved in bone homeostasis (Mbalaviele et al., 2006).

2.20.3 FGF signalling

Fibroblast growth factors and their receptors (FGFs) are a large family of proteins that are involved in diverse array of biological functions. Until now, 22 members of FGF proteins have been identified in humans and mice. Many of the FGF proteins bind to specific cell surface tyrosine kinase receptors called as fibroblast growth factor receptors (FGFRs) thereby initiating signal transduction to carry out their biological functions. There are four different FGFR's in mammals (FGFR1-4). Heparin and heparan sulphate proteoglycans (HPSG) facilitate the binding of FGF ligands to their FGF receptors. Binding of FGF and Heparan sulphate proteoglycans to its receptor leads to the dimerization of the receptor and subsequent auto-phosphorylation of multiple tyrosine residues in its intracellular domain. Multiple intermediate/docking proteins are activated in response to the activation of FGF receptor, such as Fibroblast growth factor receptor substrate 2a (FRS2a), Fibroblast growth factor receptor substrate 2b (FRS2b), GRB2-associated binding protein 1(Gab1), SHC-transforming protein (SHC) and Phospholipase C gamma (PLCγ) that in turn facilitate the activation of MAPK, PI3K, Akt, protein kinase C signalling pathways, which further modulate cellular responses (Eswarakumar et al., 2005; Ornitz and Itoh, 2001; Ornitz and Marie, 2015).

Mice lacking FGF2 showed reduced bone mass due to decreased osteoblast differentiation (Montero et al., 2000). Similarly, FGF18 null embryos displayed defects in chondrogenesis as well as osteogenesis (Ohbayashi et al., 2002). Conditional ablation of FGF receptor FGFR2 led to the development of skeletal abnormalities and decreased bone density (Yu et al., 2003). Mice lacking FGFR3 displayed osteopenia and defective mineralization (Valverde-Franco et al., 2004). Mutations in FGFRs 1-3 have been identified in multiple skeletal disorders of different etiologies viz. dwarfism syndromes, such as achondroplasia (FGFR3), hypo-chondroplasia (FGFR3), craniosynostosis syndromes such as Apert syndrome (FGFR2), Crouzon syndrome (FGFR2), Pfeiffer syndrome (FGFR1), Jackson-Weiss syndrome (FGFR2) and non-syndromic craniosynostosis (FGFR3) (Ornitz and Marie, 2002).

2.21 Insulin and Insulin-like growth factor (IGF1)

Insulin, a hormone secreted by pancreatic beta cells is the central regulator of glucose metabolism in the human body. Insulin acts via signalling through the insulin receptor which is expressed in a wide range of cells, including osteoblasts. Binding of insulin to its receptor leads to auto-phosphorylation of the tyrosine residues in the receptor subunit and subsequently leads to the activation insulin receptor substrates 1-3 (IRS1-IRS3), which further activate PI3K and protein

kinase B/Akt and ultimately lead to regulation of gene expression (Boucher et al., 2014; Siddle, 2011). Two independent studies provided compelling evidence that insulin signalling modulates bone remodeling and acts as a link between skeletal homeostasis and glucose homeostasis. Insulin signalling in osteoblasts regulates the expression and activation of hormone osteocalcin, which in turn mediates glucose homeostasis by acting on pancreas and adipose tissue. Furthermore, insulin signalling stimulates bone formation by suppressing the Runx2 inhibitory factor Twist 2 (Ferron et al., 2010; Fulzele et al., 2010).

Along with TGFβ1, Insulin like growth factor 1 (IGF1) is one of the most abundant growth factors deposited in the bone matrix and plays a role in coupling of bone formation to resorption (Bautista et al., 1990; Hayden et al., 1995; Pfeilschifter et al., 1995). IGF1 binds to IGF1 receptor (IGF1-R), leading to the activation of substrate proteins IRS1 and IRS2, which further activate MAPK,PI3K and subsequently Akt (Guntur and Rosen, 2013). IGF1 was reported to induce osteoblast differentiation of mesenchymal stem cells through activation of mTOR (mammalian target of rapamycin) via PI3K-Akt signalling and control bone mass (Xian et al., 2012). Different mouse models in which IGF1 or IGF1-R were conditionally deleted showed that IGF1 regulates bone mass accrual (Fulzele et al., 2007; Govoni et al., 2007; Jiang et al., 2006; Xian et al., 2012). Osteocyte specific deletion of IGF1 resulted in abnormal skeletal growth development in mice (Sheng et al., 2013).

2.22 Transforming growth factor β (TGF-β)

Transforming growth factor β1 (TGF-β1), is a growth factor belonging to the transforming growth factor superfamily, which is involved in multiple biological processes, such cell proliferation, apoptosis, growth and differentiation. Three isoforms of TGF-β exist in mammals, namely TGF-β1, TGF-β2 and TGF-β3. TGF-β1 is the most widely expressed isoform and one of the most abundant cytokines present in the mammalian skeleton (Hering et al., 2001). TGF-β1 is synthesized as a pre-pro-peptide and is composed of the mature TGF-β1 peptide accompanied by a latency associated peptide (LAP). Mature TGF-β1 with LAP is secreted by bone cells and gets embedded in the bone matrix. The Latency associated peptide blocks the epitopes which are necessary for binding of the TGF-β1 peptide to its cellular receptors. During bone remodelling, osteoclasts cleave the latency associated peptide (LAP) and activate the mature TGF-β1 peptide (Dallas, 2002; Oreffo et al., 1989). Members of the TGFβ subfamily signal through type I and type II serine/threonine kinase receptors. Binding of TGF-β1 protein to homodimeric Type II TGF-β receptor (TGFβR-II) leads to the recruitment of homodimeric type I TGFβ receptor (TGFβR-I) to form a hetero-tetramer complex (TGFβR II-TGFβ RI). Trans-phosphorylation of TGFβ-RI by constitutively active

TGFβ-RII follows and leads to the recruitment and phosphorylation of receptor regulated SMAD proteins (R-SMADs SMAD1-3, SMAD 5, SMAD8/9). Phosphorylated R-SMAD's form a complex with a co-SMAD (SMAD4) and this complex translocates to the nucleus to regulate gene expression (Crane and Cao, 2014; Janssens et al., 2005). Studies have also reported TGFβ signal transduction by Smad independent pathways (Itoh et al., 2003; Yu et al., 2002).

Osteoclast activated TGF-β1 induces the migration of perivascular mesenchymal stem cells to the resorption site to initiate osteoblast differentiation (Tang et al., 2009). TGF-β1 stimulates osteogenic differentiation in osteoprogenitors, but inhibits terminal osteoblast differentiation and mineralization. TGF-β1 increases the pool of osteoprogenitor cells by stimulating their proliferation (Lucas, 1989; Pfeilschifter et al., 1990). Many studies have reported the differential regulation of osteoblast marker genes such as type I collagen, ALP, osteopontin, osteonectin and osteocalcin by TGF-β1, without any clear consensus (Chen and Bates, 1993; Kassem et al., 2000; Noda and Rodan, 1987; Yamada et al., 1999). TGF-β1 was reported to inhibit terminal stages of osteoblast differentiation and mineralization by directly inhibiting the transcription factor Runx2 through SMAD3 and therefore the transcription of its target genes Alkaline phosphatase, Type I collagen and osteocalcin (Alliston et al., 2001; Kang et al., 2005). TGF-β signaling was also reported to influence mechanical and mineral properties of the bone matrix (Balooch et al., 2005). Mutations in TGF-β1 which lead to premature activation of TGF-β1 cause Camurati-Engelmann syndrome (Janssens et al., 2000; Kinoshita et al., 2000). Inactivating mutations in TGFβR-I and II cause Loeys-Dietz syndrome (Loeys et al., 2005). Elevated TGF-β signaling is also evident in many other disorders viz. Marfan syndrome, neurofibromatosis type I and Shprintzen-Goldberg syndrome (Doyle et al., 2012; Neptune et al., 2003; Rhodes et al., 2013). Increased TGF-β1 activity has also been implicated in the onset of osteoarthritis (Xu et al., 2015). TGF-β has also been reported to stimulate osteoclastogenesis (Fuller et al., 2000; Karsdal et al., 2003).

2.23 Osteocalcin

Osteocalcin, also known as bone gamma carboxyglutamic acid-containing protein, is a relatively small non-collagenous protein (46 aa in mice and 49 aa in humans), specifically produced and secreted by osteoblasts. Osteocalcin undergoes gamma-carboxylation, a post translational modification on 3 glutamic acid residues (Glu 17, 21 and 24 in humans), which is vitamin K dependent. The gamma carboxylation confers osteocalcin a greater affinity to bind calcium ions found in the mineral hydroxyapatite. Osteocalcin is released into the circulation during bone turnover (leaked into circulation during bone formation as well as released during osteoclast mediated bone resorption) (Ivaska et al., 2004). Fragments of mid-molecular region

of osteocalcin are also found to be secreted in circulation (Ivaska et al., 2005, 2004). Serum osteocalcin is widely considered as a marker for bone formation and turnover (Booth et al., 2013). Osteocalcin was suggested to regulate insulin sensitivity and therefore modulate glucose homeostasis. Osteocalcin promoted B-cell proliferation, insulin secretion and adiponectin expression, and improved glucose tolerance in-vivo (Lee et al., 2007). Insulin signalling in bone cells was shown to activate osteocalcin expression and thereby regulate glucose homeostasis (Ferron et al., 2010). Thus, there exists a positive feedback regulation loop between insulin and osteocalcin, in which uncarboxylated osteocalcin regulates insulin secretion and sensitivity, and, secreted insulin acts on bone cells to induce the expression of osteocalcin. Leptin, a hormone secreted by adipose cells, functions as a negative regulator of the osteocalcin-insulin regulatory loop. Leptin suppresses insulin secretion by decreasing osteocalcin activity (Hinoi et al., 2008). Uncarboxylated form of osteocalcin is thought to be biologically active and therefore responsible for the regulation of its endocrine functions, such as regulation of glucose homeostasis and energy metabolism (Booth et al., 2013; Lee et al., 2007).

2.24 Parathyroid hormone (PTH) and Calcitriol

PTH is secreted from parathyroid glands as a metabolically active 84 amino acid peptide. Low serum calcium level is the major inducer of PTH secretion. In addition to regulating calcium level, PTH also regulates serum phosphorus levels by its concerted actions on bone, kidney and gastro-intestinal tract. In bone, PTH indirectly stimulates osteoclastic bone resorption and the release of calcium into the blood. In kidney, PTH increases calcium reabsorption (promotes calcium retention) and decreases phosphorus reabsorption, thereby increasing phosphorus excretion. PTH also stimulates the activity of 1-alpha hydroxylase enzyme, which converts 25-hydroxycholecalciferol (circulating inactive form of vitamin D) to 1,25-dihydroxycholecalciferol (metabolically active for of vitamin D). PTH induced renal formation of vitamin D3 increases the intestinal absorption of calcium and phosphate (Penido and Alon, 2012).

Calcitriol, also known as 1,25 dihydroxy vitamin D3 is the metabolically active form of vitamin D. Cholecalciferol (endogenous vitamin D3) is produced in the skin from 7-dehydrocholesterol when subjected to ultraviolet radiation. In the liver, cholecalciferol is converted into 25-hydroxy vitamin D (25-D), which is the principle fat storage form of vitamin D. 25-D is further converted to 1,25 – dihydroxy vitamin D3 (1,25-D) by the enzyme 1-alpha hydroxylase in the proximal tubule of nephrons (Bergwitz and Jüppner, 2010). In the intestine, 1-25D stimulates absorption of calcium into blood by activating transcription of calcium transport proteins (calbindins and TRPV6) (Van Cromphaut et al., 2001; Wood et al., 2001). It also stimulates absorption of phosphate in the intestine (Fukumoto,

2014; Xu et al., 2003). Vitamin D receptor (VDR) signalling in osteoblasts activates osteoclastic bone resorption indirectly through production of RANKL (Kim et al., 2006). Furthermore, 1,25-D suppresses bone mineralization by increasing pyrophosphate (PPi) levels (Lieben et al., 2012). VDR signalling in osteocytes induces the production of FGF23, which consequently suppresses 1,25-D activation in the kidneys, adding another level of fine tuning to the overall bone mineral homeostasis (Lieben and Carmeliet, 2013).

2.25 Fibroblast Growth Factor 23

Fibroblast growth factor (FGF23) was initially characterized as the cause of autosomal dominant hypophosphatemic rickets (White et al., 2000). Later studies established the role of FGF-23 as a key player in phosphate and vitamin D metabolism (Shimada et al., 2004b; Sitara et al., 2004). FGF23 is mainly secreted by osteocytes and osteoblasts in bone (Guo and Yuan, 2015; Yoshiko et al., 2007). FGF23 acts in kidney where it inhibits phosphate reabsorption via suppression the activity of sodium-phosphate co transporters NPT2a and NPT2c in the proximal renal tubules, thus increasing phosphate excretion through urine (Gattineni et al., 2009; Shimada et al., 2004c). FGF23 also suppresses expression of the enzyme 1-alpha hydroxylase, which converts 25-hydroxy vitamin D3 to its metabolically active form 1,25-dihydroxy vitamin D3 (Perwad et al., 2007; Shimada et al., 2004a). Both 1,25-D and PTH can induce the expression of FGF23 (Lavi-Moshayoff et al., 2010; Lieben and Carmeliet, 2013). FGF-23 has also been shown to inhibit mineralization (Shalhoub et al., 2011; Wang et al., 2008).

2.26 Calcitonin

Calcitonin hormone is synthesized and secreted by the C-cells of the thyroid gland as the physiological response to increased serum calcium levels. Calcitonin binds to calcitonin receptor (CTR) which is predominantly present on osteoclasts and nephrons in the kidney, whereas it is also present in some regions of the brain. Calcitonin acts on osteoclasts to inhibit bone resorption. It also increases calcium excretion by inhibiting renal tubular calcium reabsorption (Masi and Brandi, 2007; Pondel, 2000). Recently, calcitonin has also been implicated to play an indirect role in the regulation of bone formation, through the regulation of sphingosine 1-phosphate expression (Keller et al., 2014).

2.27 Fam3c

Fam3c (family with sequence similarity 3, member C) is a protein belonging to Fam3 family of proteins. It was first identified as a candidate gene for non-

syndromic hearing loss (Greinwald et al., 1998). Many classical cytokines, such as erythropoietin, interleukins, growth hormone, macrophage colony stimulating factor to name a few, possess a common four helix bundle structure in spite of no sequence similarity. In order to search for new cytokines, a computational program called as ostensible recognition of folds was developed and used to identify new families based on similarities in predicted secondary structures. By using this program, proteins that could possibly form four helix bundle structures were searched. This search yielded the identification of four proteins comprising a novel cytokine-like family. These proteins had high sequence homology and were named as Fam3A, Fam3B, Fam3C and Fam3D. Fam3A and Fam3c were expressed ubiquitously in almost all tissues examined. Fam3B was predominantly found to be expressed in pancreas, and Fam3D in the placenta (Zhu et al., 2002).

In a separate study, the mouse ortholog was also found to be expressed ubiquitously in most of the tissues tested. Genomic organization revealed that the murine Fam3c gene is present on chromosome 6 and encompasses approximately 50 kb. The gene was found to be comprised of 10 exons and 9 introns. Two alternative transcripts were reported, with both of them using different polyadenylation sites to make the same protein product. This study also found Fam3c to be expressed in the semicircular canals during the embryonic development of inner-ear in mouse. Furthermore, analysis of gene sequence revealed binding sites for multiple transcription factors (Sp1, Ap1, Ap2, GC-box). Binding sites for transcription factors of the homeobox family (NKX5.1and En1) were also found. It was also hypothesized that Fam3c might be a secreted protein, according to the predicted presence of a transmembrane domain and a cleavable signal peptide in the N-terminus. The study also reported conserved structural similarities between human Fam3c protein and its mouse, rat and zebrafish orthologs (Pilipenko et al., 2004).

More recently, the 3D structure of murine Fam3b was reported. Unlike the earlier predicted four helix bundle structure, murine Fam3b exhibited a globular β-β-α fold with two anti-parallel β sheets and one layer of 3 short α helices. Furthermore, a homology model of Fam3c based on the sequence similarity to Fam3b revealed that Fam3c also exhibits similar β-β-α fold secondary structure. This study suggested that Fam3c/Fam3b have different structures in as compared to traditional four helix bundle cytokines and, therefore, could have a different signalling mechanism (Johansson et al., 2013).

2.28 Fam3c and cancer

Fam3c was found to be secreted by human pancreatic cancer cell line suit2 (Mauri et al., 2005) and the finding was further corroborated by another study, although a

different pancreatic cancer cell line Panc1 was used (Grønborg et al., 2006). In another study, Fam3c was shown to be essential for epithelial to mesenchymal transition (EMT), tumor formation and metastatic cancer progression in mammary gland epithelial cancer cells. In this study the authors renamed Fam3c as Interleukin-like EMT inducer ILEI (Waerner et al., 2006). Fam3c/ILEI was also found to induce EMT in hepatocytes. However, in this case, expression of oncogenic Ras was shown to be essential for ILEI to induce EMT (Lahsnig et al., 2009).

2.29 Suggested role of Fam3c in the regulation of bone homeostasis

In 2009, in a large genome wide association study (GWAS) on a Korean population cohort, single nucleotide polymorphism rs7776725 (within first intron of Fam3c gene) was found to be associated with bone mineral density at multiple sites including radius, tibia and heel (Cho et al., 2009). rs7776725 was similarly found to be associated with wrist, spine and whole body bone mineral density (BMD) in a caucasian population cohort. Furthermore, several other single nucleotide polymorphisms (SNPs) associated with Fam3c gene were found to be associated with bone mineral density (Zhang et al., 2012). SNP rs7776725 was also associated with increased forearm fracture risk in a separate study (Zheng et al., 2012).

A few other independent GWASs studies on different population cohorts also verified the association of Fam3c with BMD and bone mineral content (Chesi et al., 2015; Zhang et al., 2014). Table 3 summarizes the details of the different genome-wide studies implicating Fam3c gene locus to be involved in the regulation of bone mineral density and fracture risk. In a clinical case, duplication of Fam3c, Wnt16 and cadherin-like and PC-esterase domain containing 1 (CPED1) was reported in an obese female exhibiting features resembling Coffin-Siris syndrome, which is a rare genetic disorders that causes developmental delays (Zhu et al., 2012). With these preliminary studies, Fam3c was predicted to have a role in the regulation of bone homeostasis.

Table 3. List of genome-wide studies associating Fam3c gene locus with bone mineral density and fracture risk.

GWAS Study	Association of Fam3c with
Cho YS et.al 2009	BMD at radius, tibia and heel
Zheng HF et.al 2012	Forearm BMD and fracture risk
Zhang L et.al 2014	Hip BMD
Zhang LS et.al 2012	Hip, spine and whole body BMD
Chesi A et.al 2015	Pediatric areal BMD and bone mineral content

2.30 Introduction to microRNAs

MicroRNAs (miRNAs) are short (approximately 22 nucleotides), non-coding, single stranded RNA molecules that negatively regulate gene expression. These miRNAs bind to complementary sequences present in the 3'-untranslated region (3'-UTR) of mature mRNA transcripts to block mRNA translation or affect mRNA stability leading to subsequent degradation. MicroRNAs are known to play crucial roles in many biological processes, such as embryonic development, apoptosis, morphogenesis and cell growth and differentiation (Ambros, 2003; Carthew, 2006). To this date, there are over 2500 miRNAs that have been annotated in humans (miRBase database version 21). A single miRNA can repress many target genes and a cluster of microRNAs can regulate a single target gene (Bracken et al., 2016; Selbach et al., 2008).

Majority of miRNAs in humans are encoded by introns of protein coding or non-coding genes, but some miRNAs are transcribed from exonic regions. Often, multiple miRNA loci are located in propinquity to each other, giving rise to a 'polycistronic transcription unit' and therefore are co-transcribed simultaneously. The nucleotide sequence at the 5' region of the mature miRNA (nucleotide 2 to 7) is considered to be critical for identification of the target gene and is termed as the 'miRNA seed'. MicroRNAs with identical sequences at nucleotides (2-7) belong to the same miRNA family. For example, there are 14 members of the let-7 microRNA family in the human genome (Ha and Kim, 2014; Lee et al., 2002). Many miRNA genes often have multiple transcription start sites and further, promoters of the miRNA might be different from the promoters of the protein coding host gene (Monteys et al., 2010; Ozsolak et al., 2008). The first three letters represent the organism from which the microRNA has been characterized. For example, hsa-miR-15 implies the organism to be homo sapiens (humans) whereas mmu-miR-216 conveys the organism be *Mus musculus* (house mouse). The presence of a capital 'R' denotes that the microRNA is processed and therefore mature, whereas small 'r' indicates the miRNA gene, as well as to the stem-loop precursor which precedes the processing step. Individual miRNAs from the same family are distinguished with a letter suffix, for example, miR-34a, miR-34b. If the same miRNA is transcribed from two separate genomic locations then they are identified by a number suffix. For example, hsa-miR-16-1 (Chromosome 13), hsa-miR-16-2 (Chromosome 3). Two mature miRNAs arise from each miRNA locus, one from the 5' strand of the precursor miRNA and the other from the 3' strand, and they are suffixed 5p/3p respectively. The lesser dominant miRNA from the two is sometimes denoted with an asterisk (*). For example, hsa-miR-21-5p, hsa-miR-21-3p/hsa-miR*-21 (Ambros et al., 2003).

Transcription of miRNA genes is executed by RNA polymerase II and its associated transcription factors and other regulator proteins. Some transcription

factors are also known to regulate miRNA gene expression viz. p53, c-Myc, E2F7, MyoD1 regulate the expression of many individual miRNAs (Chang et al., 2007; Mitxelena et al., 2016; O'Donnell et al., 2005; Zhao et al., 2005). Epigenetically, promoter DNA methylation and histone modifications are also known to regulate miRNA gene expression (Au et al., 2012; Brueckner et al., 2007; Buurman et al., 2012; Liu et al., 2013; Sato et al., 2011).

2.31 Nuclear processing by the microprocessor complex (Drosha/DGCR8)

After the transcription by RNA polymerase II, the initial transcript generated is termed as 'pri-miRNA'. The pri-miRNA is long (>1 kb) and consists of three structural parts viz. a stem loop structure (with to-be mature miRNA sequences interred), a terminal loop structure and single stranded RNA fragments at 5' and 3' ends. The pri-miRNA is then processed to pre-miRNA (pre-cursor miRNA) by a protein complex called as 'microprocessor'. The microprocessor complex consists of two proteins viz. endo-ribonuclease Drosha and Di-George syndrome critical region 8 (DGCR8) also known as Pasha (in Drosophila melanogaster) and PASH-1 (in *C. elegans*). The microprocessor complex identifies the pri-miRNA, and Drosha cleaves part of the pri-miRNA to generate an intermediate form called as the pre-miRNA. The pre-miRNA is approx.70 nt long and is subsequently exported from the nucleus to the cytoplasm for further processing (Lee et al., 2002; Y. Lee et al., 2003).

Drosha is a nuclear protein of approx.160 kDa molecular mass and belongs to the double stranded RNA (dsRNA) cleaving RNAse III family of endonucleases. Structurally, Drosha has two RNAse III domains and a dsRNA binding domain. The two RNAse III domains dimerize to form a single catalytic activity center, under which one RNAse III domain cleaves 5' end of the stem loop whereas the other RNAseIII domain cleaves the 3' end. Drosha makes this incision at approximately 11 nucleotide (nt) preceding the 'basal junction' between dsRNA and single stranded RNA (ssRNA) at the 3' end and at approximately 22 nt preceding the apical junction, thus releasing the pre-miRNA (approx. 70 nt) out of the pri-miRNA fragment. The dsRNA binding domain of Drosha is necessary for the identification of the dsRNA domain (Ha and Kim, 2014; J. Han et al., 2004; Zeng et al., 2005).

The second protein constituting the microprocessor complex is DGCR8. It is a nuclear protein of approx. 90 kDa molecular mass with two dsRNA binding domains. The two dsRNA binding domains interact with the dsRNA components of the pri-miRNA whereas the C-terminal domain associates with Drosha (Shiohama et al., 2007; Yeom et al., 2006). DGCR8 and Drosha interact with each other to locate the cleavage site for Drosha, which is approximately 11 nt away from the ssRNA-

dsRNA junction at both 5' end and 3' end (Han et al., 2006). Post translational modifications of both Drosha and DGCR8 regulate their activity (Herbert et al., 2013; Tang et al., 2010; Wada et al., 2012). Furthermore, certain RNA binding proteins bind to Drosha or to regions of the pri-miRNA to modulate Drosha mediated pri-miRNA processing (Davis et al., 2008; Guil and Cáceres, 2007).

2.32 Nuclear export and cytoplasmic processing by Dicer

After the processing of pri-miRNA to pre-miRNA by the microprocessor complex, the pre-miRNA is exported out of the nucleus into the cytoplasm for subsequent processing. The protein exportin 5 (XPO5) is involved in this process. Exportin 5 forms a complex with Ran-GTP (GTP binding nuclear protein Ran) and the pre-miRNA. After the translocation of the pre-miRNA from the nucleus to the cytosol, the GTP is hydrolysed to GDP and the complex is disassembled to release the pre-miRNA in the cytosol for further processing (Bohnsack et al., 2004; Lund et al., 2004; Yi et al., 2003).

The next step after the export of the pre-miRNA into the cytoplasm is the processing of the pre-miRNA by the RNAse III endonuclease enzyme Dicer. Dicer cleavage removes the 'terminal loop' fragment from the pre-miRNA to generate a small dsRNA duplex. The human dicer has two C terminal RNAse III domains, which dimerize to form the catalytic center similar to Drosha. It also has a N-terminal helicase domain which interacts with the terminal loop of the pri-miRNA. A PAZ (PIWI-AGO-ZWILLE) domain lies between the N- terminal helicase domain and the two RNAse III domains. The PAZ domain recognizes the termini of the pre-miRNA (Ha and Kim, 2014; Tian et al., 2014). The recognition of the exact cleavage sites by dicer has been attributed to two independent principles (the 3'-counting rule and the 5'-counting rule). Dicer cleaves at a fixed length of approximately 22-25 nt from the 3' end of the dsRNA terminus (3'-counting rule). Dicer also interacts with the 5' end of the dsRNA terminus and cleaves at a distance of 22 nt from it (5'-counting rule) (Macrae et al., 2006; MacRae et al., 2007).

Dicer mRNA contains sites that allow binding of let-7 miRNA, resulting in a negative feedback regulatory loop between let-7 miRNA and the Dicer transcript, thus controlling the homeostatic regulation of Dicer activity (Forman et al., 2008; Tokumaru et al., 2008).

2.33 RNA induced silencing complex (RISC) formation and terminal processing

Once Dicer generates a small miRNA duplex, it is then loaded onto argonaute proteins (AGO1-4 in humans) to form RNA induced silencing complex (RISC

complex). The argonaute proteins (AGO1-AGO4) are ubiquitously expressed and associate with miRNAs and small interfering RNAs (siRNAs) (Azuma-Mukai et al., 2008; Dueck et al., 2012; Su et al., 2009). Argonaute proteins have two conserved domains viz. PAZ domain and PIWI domain, which bind to the 3' and 5' ends of the guide strand of the miRNA (Elkayam et al., 2012; Nakanishi et al., 2012).

After loading onto the argonaute protein, the miRNA duplex is unwound, and one strand (passenger strand) is discarded, whereas the other strand (guide strand) functions as the mature miRNA. However, the less dominant miRNA strand (passenger strand) has also been found to be differentially expressed in tissues. For example, miR-142-5p (guide strand) is expressed in brain, ovaries and testes, whereas miR-142-3p (passenger strand) is expressed in embryonic stages (Chiang et al., 2010). The loading of small miRNA duplexes onto argonaute proteins requires ATP but the excision of the passenger strand does not (Kawamata et al., 2009; Yoda et al., 2010).

RISC-complex, along with the bound guide strand miRNA directs the miRNA to its complimentary sequence mRNA where it represses the mRNA either by degradation or by inhibition of translation (Filipowicz et al., 2008). The process of microRNA biogenesis is depicted in Fig. 6 below.

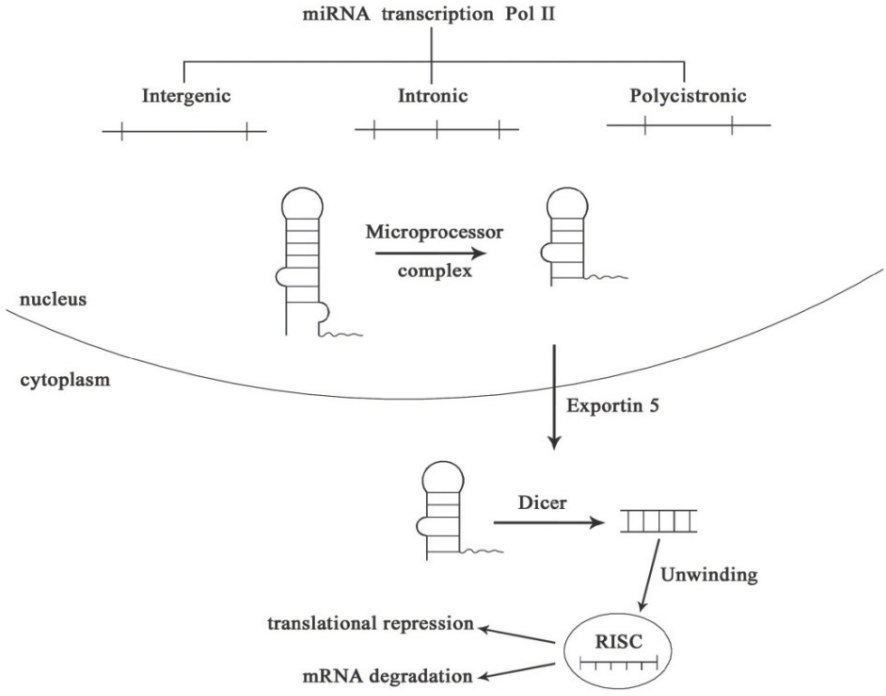

Figure 6. MicroRNA biogenesis. (Modified from Qi et.al, Biomed Research International, 2013)

2.34 Role of Dicer in skeletal development and homeostasis

The RNAse III endonuclease Dicer is essential for the generation of small regulatory RNAs (miRNAs and siRNAs). Mutations of Dicer in both mice and zebrafish lead to embryonic lethality, suggesting that Dicer gene function is crucial for embryonic development and patterning (Bernstein et al., 2003; Giraldez et al., 2005; Wienholds et al., 2003). To overcome the problem of embryonic lethality, a Cre recombinase conditional allele was created and it was subsequently used to conditionally inactivate Dicer in limb mesoderm. This Cre recombinase mediated Dicer deletion revealed that Dicer was indeed required for proper development of the limb but the differences seen in the mutant animals were not exceptionally severe (Harfe et al., 2005). Cartilage specific inactivation of Dicer, by employing Cre recombinase expressed under collagen type II alpha 1 (Col2a1) promoter in mice resulted in several skeletal defects, early death and deficient chondrocyte proliferation and differentiation (Kobayashi et al., 2008).

Dicer deletion was also studied in osteoprogenitor cells and mature osteoblasts by using a Cre recombinase driven by collagen type I alpha 1 (Col1a1) promoter and osteocalcin promoter respectively. Col1a1 is expressed in osteoprogenitor cells, osteoblasts and hypertrophic chondrocytes, whereas osteocalcin is expressed in mature osteoblasts. Cre recombinase mediated deletion of Dicer in procollagen alpha 1(II) expressing cells gave rise to embryonic lethality, whereas its deletion in mature osteoblasts by osteocalcin driven Cre recombinase resulted in viable mice with increased bone mass phenotype (Gaur et al., 2010). Osteoclast specific Dicer deletion suppressed bone resorption and increased trabecular bone mass in vivo. Further, Dicer was also found to be essential for modulating osteoclastogenesis in vitro (Mizoguchi et al., 2010).

Many individual miRNAs are involved in regulating osteogenic as well as chondrogenic differentiation (Lian et al., 2012). Many different miRNAs have been found to be involved in the pathogenesis of skeletal diseases (Gennari et al., 2016). MicroRNAs miR-34c and miR-2861 have been suggested to play a role in the development of osteoporosis (Bae et al., 2012; H. Li et al., 2009). miR-214 was found to suppress bone formation, and its elevated levels were found to be associated with decreased bone formation in osteoporotic patients (Wang et al., 2013). Multiple studies have reported the detection of circulating miRNAs in serum of patients affected with osteoporotic fractures (Gennari et al., 2016; Seeliger et al., 2014). MicroRNAs that are involved in the regulation of osteoblast differentiation and skeletal homeostasis have been listed in table 4. However, the requirement of Dicer dependent miRNA biogenesis during post-natal skeletal homeostasis has not been extensively studied.

Table 4. List of miRNAs regulating osteoblast differentiation and bone homeostasis. (Modified from Lian et.al, Nature Reviews Endocrinology, 2012)

MicroRNA	Target gene	Function	Reference
miR-29a	Osteonectin,Dkk-1,Kremen-2, sFRP2	Promotes osteoblast differentiation by activating Wnt signaling	(Kapinas et al., 2010, 2009)
miR-29b	HDAC4, TGFb3,ACVR2A, DUSP2,CTNNBIP1	Promotes osteoblast differentiation by down-regulating its inhibitory factors	(Li et al., 2009)
miR-29c	Dkk-1, Kremen, sFRP2, Osteonectin	Promotes osteoblast differentiation	(Kapinas et al., 2009)
miR-138	Focal Adhesion Kinase (FAK)	Inhibits osteoblast differentiation	(Eskildsen et al., 2011)
miR-34	Jagged 1, SATB2, Cyclin D1, CDK4, CDK6	Inhibits osteoblast differentiaton	(Bae et al., 2012; Chen et al., 2014; Wei et al., 2012)
miR-143	Osterix	Suppresses osteoblast differentiation	(Li et al., 2014)
miR-141, miR-200a	Dlx5	Suppresses osteoblast differentiation	(Itoh et al., 2009)
10 individual microRNAs	Runx2	Suppresses osteoblast differentiation	(Zhang et al., 2011d)
miR-20a	BAMB1,CRIM,PPARγ	Promotes osteogenic differentiation	(Zhang et al., 2011a)
miR-214	ATF4	Suppresses osteoblast differentiation	(Wang et al., 2013)
miR-210	Acvr1b	Promotes osteoblast differentiation	(Mizuno et al., 2009)
miR-335-5p	DKK1	Promotes osteoblast differentiation	(Zhang et al., 2011c)
miR-27	APC	Promotes osteoblast differentiation	(Wang and Xu, 2010)
miR-2861	Hdac5	Promote osteoblast differentiation	(Li et al., 2009)
miR-26a	SMAD1	Suppresses osteoblast differentiation	(Luzi et al., 2008)
miR-637	Osterix	Suppresses osteoblast differentiation	(Zhang et al., 2011b)
miR-196a	Hoxc8	Promotes osteoblast differentiation	(Kim et al., 2009)
miR-206	Connexin 43	Negatively regulates osteoblast differentiation	(Inose et al., 2009)
miR-21	Smad7, Pdcd4	Promotes osteoblast differentiation and osteoclast differentiation	(Li et al., 2015; Sugatani et al., 2011)
miR-17-92 cluster	multiple genes regulating cell cycle, proliferation and apoptosis	Regulates osteoblast differentiation	(de Pontual et al., 2011;Zhou et al., 2014a)
miR-132-3p	Ep300	Inhibits osteoblast differentiation in simulated microgravity	(Hu et al., 2015)
miR-100	BMPR2	Regulates osteoblast differentiation	(Zeng et al., 2012)

MicroRNA	Target gene	Function	Reference
miR-182	FOXO1	Negatively regulates osteoblast differentiation	(Kim et al., 2012)
miR-199a, miR-346	Leukemia inhibitory factor (LIF)	Regulation of osteoblast differentiation	(Oskowitz et al., 2008)
miR-208	Ets1	Negatively regulates osteoblast differentiation	(Itoh et al., 2010)
miR-370	Ets1, BMP-2	Negatively regulates osteoblast differentiation	(Itoh et al., 2012)
miR-378	Nephronectin GalNT-7	Promotes osteoblast differentiation	(Kahai et al., 2009)
miR-183	Heme-oxygenase 1	Promotes osteoclast differentiation	(Ke et al., 2015)
miR-155	Mitf, Socs1	Inhibits osteoclast differentiation	(Zhang et al., 2012)
miR-335	Runx2	Suppresses osteoblast differentiation	(Tomé et al., 2011)
miR-23a/27a/24-2	Runx2/Satb2	Inhibits osteoblast differentiation	(Hassan et al., 2010)
miR-146a	TRAF6	Inhibits osteoclast differentiation	(Nakasa et al., 2011; Taganov et al., 2006)
miR-223	NFI-A	Promotes osteoclast differentiation	(Sugatani and Hruska, 2007)
miR-93	Osterix	Suppresses osteoblast differentiation	(Yang et al., 2012)

2.35 Summary of the literature review

Skeletal disorders are commonly seen in aging populations. Moreover, trauma injuries cause skeletal deformations in millions of people worldwide. Development of novel therapeutic targets and approaches are therefore a constant need. Bone formation and post natal skeletal homeostasis are stringently regulated processes in vivo. Studying the role of new players involved in osteoblast differentiation and bone formation is therefore important.

Fam3c was predicted to be an osteoblast-expressed protein and was found to be associated with BMD and fractures in GWASs studies on independent population cohorts. However, there were no existing studies on the in vivo involvement of Fam3c in the regulation of bone formation and homeostasis prior to the commencement of this work. In a different aspect looking at the regulation of post-natal skeletal homeostasis, the role of Dicer1 is incompletely understood especially in the context of cortical bone formation and homeostasis. This thesis work addresses the aforementioned questions.

3. AIMS OF THE STUDY

The lack of information for the role of Fam3c in bone biology and the role of osteoblast specific Dicer1 in the regulation of post-natal skeletal homeostasis engendered the basis of my doctoral research work. Although these questions address separate aspects of the overall field of bone biology, their role in cortical bone formation and homeostasis was utilized as a unifying platform for the purpose of this thesis.

The overall aims of my doctoral research work were as follows:

1. To determine the function of Fam3c during bone formation and osteoblast differentiation.

2. To determine the role of osteoblastic Dicer1 during post-natal skeletal homeostasis.

4. MATERIALS AND METHODS

4.1 Ethical approval and permissions for animal experimentation

All the animal experimentation studies were approved by the Finnish animal ethics committee, in accordance with international guidelines regarding use and care of laboratory animals. The licences obtained from the National Animal experiment board and the state provincial office were #2009-06915 and #2165/04.10.07 respectively.

4.2 Antibodies and reagents

Table 5. Antibodies and reagents used in the studies.

Reagent name	Source	Catalogue number	used in study
Recombinant Fam3c	Custom made	-----	I
Fam3c antibody	Custom made	-----	I, II
Runx2 antibody	Cell Signaling Technology	8486S	II
Beta Actin antibody	Thermo Fisher Scientific	MA515739HRP	II
Secondary anti rabbit HRP	Jackson Immunoresearch	111-035-003	I, II
Alexa flour 594 goat anti-rabbit (H+L)	Abcam	ab150080	II
HiPerfect transfection reagent	Qiagen	301704	II
Alpha MEM	Thermo Fisher Scientific	41061029	I, II, III
Alpha MEM without ascorbic acid	Thermo Fisher Scientific	A1049001	II
Tamoxifen	Sigma-Aldrich	T5648	III
Dexamethasone	Sigma-Aldrich	D4902	I, II, III
Natrium-β-glycerophosphate	Sigma-Aldrich	50020	I, II, III
Fam3c plasmid	Origene Technologies	MR202685	II
Recombinant mouse TGF-β1	Cell Signalling technology	5231	II
Heat-inactivated fetal bovine serum (FBS)	Gibco		I, II, III
Opti-MEM	Thermo Fisher Scientific	31985-062	II
Lipofectamine 2000 transfection reagent	Thermo Fisher Scientific	11668027	II

4.3 Cell lines and primary cells

Table 6. Cell lines and primary cells utilized in the studies.

Cells/Cell line	Source/obtained from	used in study
Bone marrow mesenchymal stromal cells	Femur and tibia of mice (males and females)	I, II, III
MC3T3-E1 pre-osteoblasts	Dr. Kati Tarkkonen, Institute of Biomedicine, University of Turku.	II
C3H10T1/2 mesenchymal cells	Fan Wang, Institute of Biomedicine, University of Turku.	II

4.4 Western blotting and agarose gel electrophoresis

Table 7. Reagents and consumables used for western blotting and agarose electrophoresis experiments.

Reagent/consumables	Source	Catalogue number	used in study
TEMED	Fisher chemicals	T/P190/04	I, II
Ammonium persulphate	Biorad	161-0700	I, II
Acrylamide-bisacrylamide mix	Biorad	161-0158	I, II
TBS tablets	Medicago AB	09-7500-100	I, II
Protease inhibitor cocktail tablets	Thermo Fisher Scientific	#88666	I, II
Immobilon-P PVDF membrane 0.45um	Millipore	IPVH00010	I, II
Tween-20	Fluka	93773	I, II
Super signal west pico chemiluminescence detection kit	Thermo Fisher scientific	#34087	I, II
Amersham ECL rainbow protein marker	GE healthcare sciences	RPN-800E	II
All Blue protein standards	Biorad	161-0373	I
Laemmli sample buffer	Biorad	161-0747	I, II
Agarose	Cambrex Bioscience	50004	III
Midori Green advance DNA stain	Nippon Genetics	MGO4	III

4.5 RNA extraction and quantitative RT-PCR

Table 8. Reagents, kits and consumables used for RNA purification and quantitative RT-PCR analysis.

Reagent/kit/consumable	Source	Catalogue number	used in study
Nucleospin RNA isolation kit	Macherey-Negel	740955.250	I, II
Dynamo cDNA synthesis kit	Thermo Fisher Scientific	#F-470L	I, II
iTaq Universal SYBR Green	Biorad	172-5121	I, II
96-well plates (white)	Applied Biosystems	AB-0700/W	I, II

4.6 Methods

Table 9. Methods utilized in respective studies.

Methods	Described in Study
RNA extraction and cDNA synthesis	I, II, III
Quantitative RT-PCR	I, II, III
Western Blotting and densitometry	I, II
Alkaline phosphatase staining and activity	II, III
Immunofluorescence	II
von-Kossa staining	I
Transfection	II
Acetone precipitation	II
Bone Histology	I, III
Immunohistochemistry	I
Bone break point analysis	I
Three-point bending biomechanical test	III
Histomorphometry	I, III
Micro-CT analysis	I, III
Blood analysis	I
Statistical analysis	I, II

4.7 Selected methods are described in the following sections:

4.7.1 Construction of the Fam3c gene trap allele

The Fam3c KO mice strain was generated by employing a gene trap methodology. The gene trap clone LST057 obtained contained the trap allele between the exons 3 and 4 of Fam3c gene. The trap allele consisted of two selectable markers; β-galactosidase fused to neomycin resistance (Geo) sequence and placental alkaline phosphatase (PLAP). An intracistronic ribosomal entry site (iRes) was located in between the two markers whereas a transcription termination sequence was located in the terminal part of the trap allele. Expression of this trap allele resulted in the transcription of a truncated Fam3c mRNA with the first three exons, followed by two markers β-Geo and PLAP.

4.7.2 Generation of Dicerosx strain

Dicerosx mouse line was generated by crossing Dicer (Flox/Flox) strain (Harfe et al., 2005) with OsxCreERt2 strain (Maes et al., 2010). Animals were genotyped for both Cre recombinase and Dicer (Flox/Flox). Animals were fed soya-free diet and were housed at University of Turku central animal laboratory. Appropriate ethical permissions were obtained prior to commencing the studies. Tamoxifen induced Cre recombination was verified in vitro. Primary bone marrow stromal cells were isolated from 10 weeks old Cre+ and Cre- animals and were induced to

undergo osteogenic differentiation in the presence of Tamoxifen (1 µg/ml). DNA extraction was carried out. Recombination was verified by performing PCR. *Mus musculus* dicer Exon 23 specific primers were designed for the purpose. Primer sequences are described in the original publication (III).

4.7.3 Dicerosx animal experiments

To study the role of osteoblast-specific Dicer1 in the regulation of post-natal bone homeostasis, two age groups of animals were selected; young mice (3 weeks old) and older mice (10 weeks old). The reason behind selection of these two age groups was that the 3 week old mice are approaching puberty and, therefore, this period shows rapid skeletal growth. The 10 week old mice are fully grown fertile adults and contrary to the young mice, exhibit lesser skeletal growth. Both males and females from the two age groups were utilized for the experiments. Body weight of the animals was noted prior to the beginning of the experiment. The animals were administered Tamoxifen (0.5 mg/10 g of body weight) intra-peritoneally for 3 days and the animals were then followed for 5 weeks under normal housing conditions. After 5 weeks, animals were euthanized and the bones were collected for micro- CT and histological analysis.

4.7.4 Cell Culture

Primary cells from the long bones of mice were cultured according to a protocol described previously (Morko et al., 2009). Primary bone marrow stromal cells were isolated from the diaphysis of 10 week old mouse tibiae and femora, counted and plated in culture medium (Alpha MEM, 15% inactivated FBS, HEPES buffer, Glutamax and penicillin-streptomycin and 10 nM dexamethasone). After two days of culture, unattached cells were removed by aspiration and fresh medium was added. After one week of culture, cells were detached with trypsin-EDTA, counted and plated onto 12-well plates in osteogenic culture medium (culture medium supplemented with 10 mM Na-β-glycerophosphate, 70 µg/ml ascorbic acid and 10 nM dexamethasone). Dexamethasone was removed after initial three days of osteogenic differentiation.

MC3T3-E1 cells and C3H10T1/2 cells were cultured in Alpha MEM without ascorbic acid, supplemented with 10% inactivated FBS. Osteogenic differentiation was carried out by plating equal number of cells in 12 well-plates containing osteogenic differentiation medium (culture medium with added 5 mM Na-β-glycerophosphate, 50 µg/ml Ascorbic acid and 10 nM dexamethasone).

Table 10. Cell culture specifics for osteogenic differentiation.

Ingredient/variable	Primary bone marrow stromal cells (mouse)	MC3T3-E1/ C3H10T1/2 cell lines
Cell density (cells/cm^2)	10 000	20 000
Culture medium	Alpha MEM	Alpha MEM without ascorbic acid
FBS	15%	10 %
Penicillin-Streptomycin	100U/ml	100 U/ml
Na-β-glycerophosphate	10mM	5 mM
HEPES buffer	10mM	10 mM
Ascorbic acid 2-phosphate	70ug/ml	50 µg/ml
Dexamethasone	10nM	10 nM

MEM: minimum essential medium; FBS: fetal bovine serum

5. RESULTS AND DISCUSSION

5.1 Phenotype characterization of Fam3c knockout (KO) mouse model (I)

Fam3c KO mice were seemingly normal, fertile with no significant differences in body weight gain as compared to their wild-type (WT) counterparts over the analysed time span. No abnormality was detected during the histological analysis of soft tissues analysed which included esophagus, jejunum, ileum, stomach, colon, rectum, pancreas, kidney, adrenal gland, trachea, lung, heart, cerebellum, cerebrum, pituitary gland, eye, salivary gland, thyroid gland, spleen, lymph node, thymus, skeletal muscle, testicles, epididymis, bladder, prostate, seminal vesicle, ovary and uterus. Blood composition analysis revealed increased numbers of polymorphonuclear neutrophils and red blood cells in male knockout mice as compared to their WT counterparts. Number of lymphocytes was reduced in male knockout mice. The mean corpuscular volume as well as the mean corpuscular hemoglobin concentration was reduced in knockout male mice. However, the total hemoglobin did not differ significantly between the WT and the KO mice. Bone marrow niche comprises of many other cell populations such as hematopoietic cells, osteoclasts, endothelial cells, immune cells etc wherein the niche microenvironment plays a crucial role in the proliferation and differentiation of these cell populations (Kusumbe et al., 2014; Méndez-Ferrer et al., 2010). It is a possibility that the small differences seen in the blood cell numbers in the knockout mice could arise because of the disturbed Fam3c regulation in bone cell populations, thereby making a small impact in the regulation of blood cell proliferation in the bone marrow niche. Reporter analysis showed Fam3c expression in all bone cell populations. Positive β-gal staining was seen in growth plate chondrocytes, osteoblasts, osteoclasts as well as periosteal cells. Articular chondrocytes at the synovial surface stained positive for Fam3c. Additionally, the fibroblastic population of cells at the region of ligament attachment also showed strong staining for the reporter.

5.2 Enhanced osteoblast differentiation and accelerated mineralization in Fam3c knockout mice (I)

In vitro osteogenic differentiation of primary bone marrow stromal cells isolated from Fam3c KO animals exhibited accelerated mineralization. Primary cells from KO male mice showed positive staining for von Kossa as early as day 14 of differentiation, whereas WT cells were only beginning to stain positive for von Kossa at day 21 of differentiation. KO cells from the female mice similarly showed increased staining for von Kossa at day 21 of differentiation whereas WT female

cells showed only weak staining at the corresponding day. Expression of osteoblast-related genes ALP and procollagen alpha 1(I) were highly elevated at day 9 and day 15 of in vitro osteogenic differentiation in the KO cells as compared to their WT counterparts. Similarly, expression level of Runx2, which is the master transcriptional regulator of osteoblast differentiation, was significantly increased in the KO cells. Additionally, osteocalcin expression peaked early at d15 in the KO cells whereas WT cells expressed osteocalcin at weak levels on day 21 of differentiation. At day 21, KO cells displayed corresponding increased levels in osteocalcin as compared with WT cells. This drastic differentiation phenotype seen in the KO cells was however surprising. In vitro osteogenic differentiation is a rather simplified process whereas in vivo osteogenic differentiation is an enormously intricate process involving myriad facets of regulation at different levels by different factors. Due to the simplified conditions involved in in vitro osteogenic differentiation, the effect of Fam3c ablation might have manifested more prominently thus leading to the exhibition of the strong phenotype. It might also be possible that in Fam3c knockout mice in vivo loss of Fam3c function may have been compensated by some other proteins/factors in the bone marrow niche which may or may not form a part of the Fam3c regulatory network in bone cell populations and which remain to be identified and investigated.

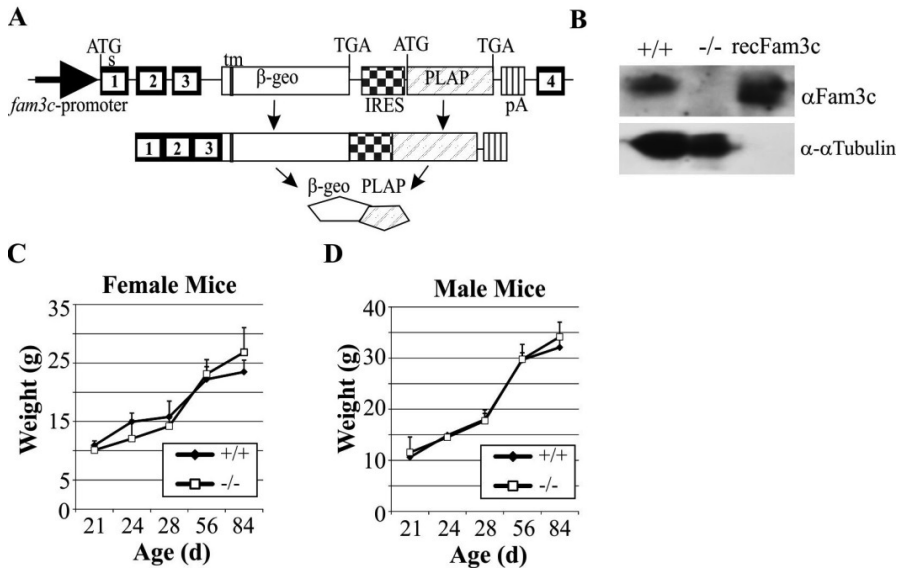

Figure 7. Fam3c knockout mouse strain. A] Gene-trap construct with β-geo and PLAP as reporters. **B]** validation of the knockout by western blot. **C]** graph showing body weight pattern of Fam3c knockout female and **D]** male mice. (Adapted from: Määttä et.al, BoneKey Reports, 2016)

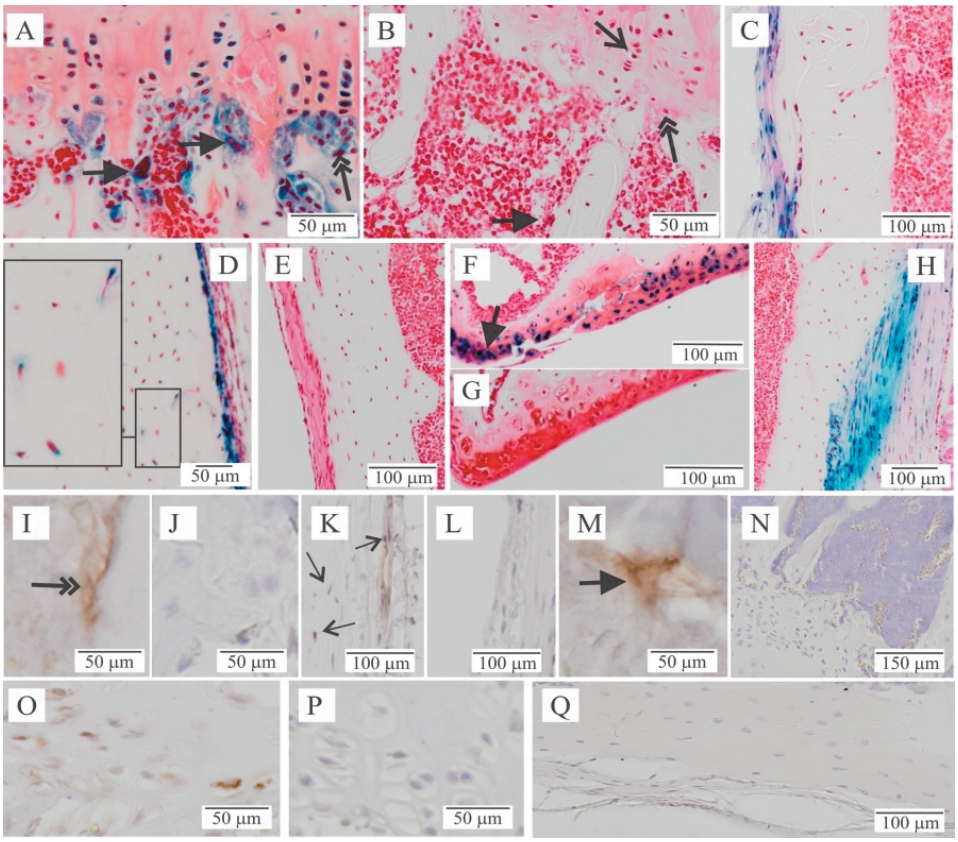

Figure 8. Reporter gene activity in bone. A] osteoclasts (thick arrows), osteoblasts (double arrows) and proliferating growth-plate chondrocytes in knockout (-/-) mice. **B]** corresponding wild-type (+/+) controls. **C]** (-/- male mouse) and **D]** (-/- female mouse) periosteal cells were positive for the reporter with occasional positive osteocytes (inset, D). **E]** corresponding control (+/+ female mouse) for (D). **F]** chondrocytes of articular cartilage were positive for the reporter in (-/- male mouse). **G]** corresponding control (+/+ male mouse). **H]** attachment point for ligaments were also positive for the reporter. **[I-Q]** Immuno-histochemical staining images. Images I,K,M and O represent WT mice whereas images J, L and P represent knockout mice. Images N and Q are conjugate controls. (Adapted from: Määttä et.al, BoneKey Reports, 2016.)

5.3 Analysis of bone phenotype (I)

Previously, a study had reported Fam3c KO mice but they did not find any differences in the bone phenotype of wild-type and Fam3c KO male mice (Zheng et al., 2012). However, we found significant changes affecting bone morphology in both Fam3c KO male and female mice.

Tibiae from 3 month old and 7 month old Fam3c WT and KO animals were scanned by micro-CT analysis and the underlying bone phenotype was studied. In 3 month old female mice, trabecular tissue volume of the tibial metaphysis was increased in KO animals as compared to WT counterparts. Furthermore, increased

trabecular separation and decreased trabecular number was also seen in the KO mice. However, trabecular bone volume did not differ significantly between the two groups therefore suggesting that the same volume of bone is disseminated to a larger tissue volume. In 7 month old KO females, trabecular bone volume was significantly reduced besides increased trabecular separation and decreased trabecular number. Interestingly, differences in trabecular bone phenotype were not prominent in male KO mice. The trabecular tissue volume was sparsely elevated in 3 month old KO mice as compared to their WT counterparts. On the contrary, in 7 month old KO mice, the trabecular tissue volume was found to be decreased.

Three month old female KO mice exhibited an increase in cortical tissue area, endocortical area and overall cortical tissue volume as compared to matched WT females. Increase in cortical tissue volume also manifested in decreased BV/TV ratio of cortical bone. In 7 month old Fam3c KO females, the cortical tissue volume was similarly increased, giving rise to a significant decrease in BV/TV ratio. Corresponding increases in cortical tissue area, endocortical area and bone perimeter were also observed. Cortical bone mineral density was significantly increased in the 7 month old KO females. In 3-month old male mice, endocortical area was increased in the KO animals whereas the overall cortical BV/TV ratio was decreased. Cortical BMD was increased in the 3 month old KO males. However in 7 month old males, BV/TV ratio was slightly increased in the KO animals as compared to their WT counterparts with corresponding decrease in endocortical area. Mean cortical BMD was similarly increased in the 7 month old KO males as it was seen in 3 month old males. However, this increase was found to be statistically insignificant.

Sex hormone mediated signaling has been well known to have an important role in the regulation of bone growth and homeostasis (Almeida et al., 2017; Maatta et al., 2013a; Määttä et al., 2013b; Syed and Khosla, 2005). The gender-specific differences seen between the bone phenotype of Fam3c KO females and males could imply sex-specific regulation of Fam3c during bone formation and homeostasis. It is a therefore a possibility that biological effects of Fam3c might be manifested by sex hormone receptor-mediated signalling.

In spite of the increased cortical BMD, the breaking strength was reduced in KO animals of both sexes. Altered bone geometry (narrower tibiae in lateral direction) especially in males, might explain this dissimilarity. It will be interesting to investigate whether Fam3c does have a function in regulating the mechanical load dependent bone remodelling. Furthermore, Fam3c might also manifest changes in the collagen containing bone matrix, thereby affecting its mechanical properties. However, further studies are needed to address these implications.

5.4 Dysregulation of proportionality between bone mass and body weight (I)

Allometry is generally defined as the relationship of body size to anatomical, physiological, behavioural and ecological characteristics of animals (Damuth, 2001). In mammals, as body size increases, bones become more robust to counter the corresponding increase in mechanical load (Alexander et al., 1979; Barak et al., 2013; Doube et al., 2011). However, allometric correlation between body weight and bone volume in rodents has not been extensively studied. We examined the allometric relationship between body weight and bone volume in Fam3c WT and Fam3c KO animals and found the existence of a negative co-relation between body weight and bone volume in Fam3c knockout animals. Similar relationships between body weight and BMD were, however, not seen in our studies.

In 7 month old Fam3c WT animals, both trabecular and cortical bone volume increased along with an increase in body weight. In Fam3c KO females, trabecular bone volume was decreased with corresponding increase in body weight. Similarly, in Fam3c KO males, both cortical bone volume and trabecular bone volume were decreased with corresponding increases in body weights. Moreover, the variation of body weight was more in KO animals as compared to WT animals, leading to the speculation that the regulation of body weight might be inadequate or inefficiently implemented in the knockout animals. However, the relationship between cortical bone volume and body weight in KO female mice did not show any irregularities.

5.5 Characterization of Fam3c expression during osteogenic differentiation (II)

Our previous findings suggested that Fam3c was involved in the regulation of osteoblast differentiation and cortical bone volume. To ascertain whether Fam3c is indeed expressed during osteoblast differentiation, primary bone marrow stromal cells from Fam3c WT animals and MC3T3-E1 cells were utilized. Primary cells were isolated from both male and female WT mice. Both primary cells and MC3T3-E1 cells were differentiated into the osteogenic lineage for 3 weeks and the expression of Fam3c was studied.

Fam3c was expressed at both mRNA and protein levels during osteogenic differentiation of MC3T3-E1 cells. In earlier studies, Fam3c was initially identified as a secreted protein which was released into the culture supernatant of pancreatic cancer cells (Grønborg et al., 2006; Mauri et al., 2005). In order to verify whether Fam3c is secreted into the culture medium during osteogenic differentiation, protein extracts from culture medium of MC3T3-E1 cells undergoing differentiation were analysed for Fam3c expression. However, Fam3c was not found to be secreted into the culture medium. This prompted us to study the localisation of Fam3c in MC3T3-

E1 osteoblasts. Immunofluorescence experiments with Fam3c specific antibody revealed cytoplasmic localisation of Fam3c, with prominent staining in the vicinity of the nuclei. Regions near to the plasma membrane also showed Fam3c expression, albeit to a lesser extent. Endogenous Fam3c expression was earlier shown to be localised to granules/vesicular structures in salivary gland, mammary gland and duodenum. Carcinomas exhibit enhanced cytoplasmic expression of Fam3c (Lahsnig et al., 2009; Waerner et al., 2006). In a recent study carried out on neuronal cells, Fam3c expression was localized to perinuclear vesicular structures and overlapped strongly with Trans-Golgi marker protein TGN46, and weakly with cis-golgi marker protein GM130, ER marker protein GRP78 and Endosome marker protein EEA1 (Liu et al., 2016). It is possible that the nuclear vicinity region which showed positive staining for Fam3c might represent trans golgi network or vesicular structures whereas the small areas near the cell membrane which exhibited positive staining for Fam3c might represent secretory granules. To rule out the possibility that Fam3c is indeed secreted extracellularly, immunoprecipitation studies were carried out by using Fam3c specific antibody. However, they did not show any evidence of secreted Fam3c. Finally, administration of recombinant Fam3c to MC3T3-E1 cells did not result in any stark differences in the osteogenic differentiation. Both the control and treated cells exhibited comparable differentiation.

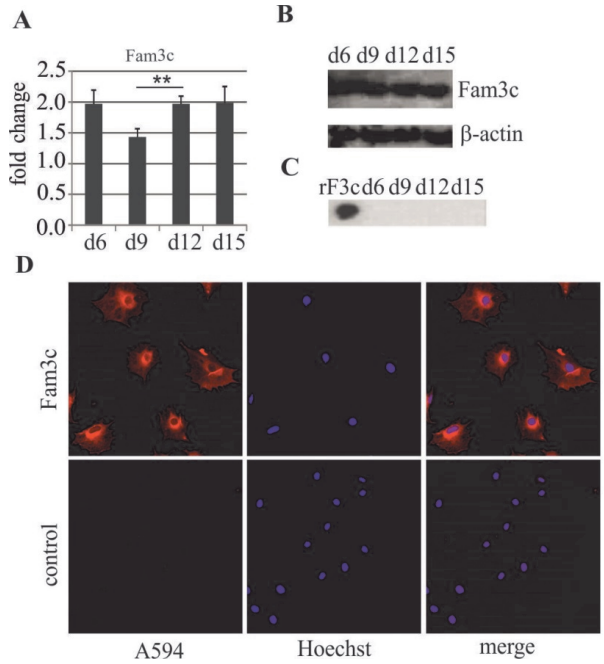

Figure 9. Fam3c expression during osteoblast differentiation. A] Fam3c mRNA during day 6 to day 15 of osteogenic differentiation. **B]** Protein expression analysed by western blot. **C]** Western blot of protein extract obtained from conditioned medium with recombinant Fam3c as a positive control. **D]** Fam3c localization analysed by immunofluorescence. (Adapted from: Bendre et.al, Differentiation, 2017.)

Primary bone marrow stromal cells also expressed Fam3c during in vitro osteogenic differentiation. In cells from both sexes, Fam3c mRNA expression was significantly increased at day 12 of osteogenic differentiation as compared to the corresponding Fam3c expression at day 9 of osteogenic differentiation. However, there was no difference in Fam3c expression at day 15 of differentiation as compared to day 12 in both males and females. Moreover, statistically significant difference was seen in the expression of Fam3c when compared between males and females (interaction of sex and differentiation time points). At day 6 and day 9 of differentiation, Fam3c expression was lesser in cells derived from males as compared to females. At day 15 of differentiation, Fam3c expression was lower in females than males. The differences in the expression levels of Fam3c between males and females suggest that there might be sex-specific differences in the regulation of Fam3c.

5.6 Reciprocal feedback regulation between Fam3c and TGF-β1 (II)

TGF-β1 is one of the most abundant cytokines present in bone and plays a crucial role in the regulation of osteoblast differentiation and skeletal homeostasis (Crane and Cao, 2014; Janssens et al., 2005). TGF-β1 is known to stimulate early steps of osteogenic differentiation whereas it inhibits terminal osteoblast differentiation (Bonewald and Dallas, 1994; Centrella et al., 1994). Furthermore, TGF-β1 plays an important role in the regulation of bone matrix mechanical properties (Balooch et al., 2005). Moreover, TGF-β1 was shown to induce the expression of Fam3c in cancer cells (Chaudhury et al., 2010). We looked at the possibility whether TGF-β1 could also induce Fam3c expression during osteogenic differentiation.

Administration of mouse recombinant TGF-β1 to MC3T3-E1 cells undergoing osteogenic differentiation led to the induction of Fam3c expression at both mRNA and protein levels. Moreover, TGF-β1 treatment also recovered Fam3c mRNA levels post siRNA mediated knockdown. TGF-β1 also led to reduced ALP expression at the mRNA level and also diminished its specific activity. Knockdown of Fam3c by Fam3c specific siRNA in differentiating MC3T3-E1 cells led to slight increase in ALP expression and its specific activity. Furthermore, the increase in ALP expression manifested upon Fam3c knockdown was nullified by subsequent TGF-β1 administration. From these sets of observations, we concluded that Fam3c expression was indeed regulated by TGF-β1 during osteogenic differentiation.

Finally, we asked the question whether Fam3c could regulate TGF-β1 expression in differentiating osteoblasts. In cancer cells, TGF-β1 induced Fam3c expression engenders epithelial-to-mesenchymal transition and subsequent metastatic

progression whereas attenuation of Fam3c expression suppresses the metastatic capacity of the cancer cells (Lahsnig et al., 2009; Sun et al., 2017; Waerner et al., 2006). Recently, it has been shown in melanoma that phenotype switching alters Fam3c expression and conversely, Fam3c regulates phenotype switching partially, thus creating a feedback regulation loop (Noguchi et al.,2017). We asked the question whether Fam3c could regulate TGF-β1 expression in differentiating osteoblasts, generating a similar feedback circuit. Surprisingly, TGF-β1 mRNA levels were significantly elevated in Fam3c siRNA treated MC3T3-E1 cells undergoing osteogenic differentiation. On the contrary, cells overexpressing Fam3c exhibited reduced levels of TGF-β1 mRNA. These findings point towards the possibility that Fam3c and TGF-β1 could be a part of negative feedback loop and could regulate each other's expression during osteogenic differentiation.

5.7 Fam3c mediates Runx2 down-regulation (II)

Earlier results showed a slight increase in ALP expression and activity post siRNA mediated knockdown of Fam3c. To counter-verify this observation, we overexpressed Fam3c in MC3T3-E1 cells undergoing osteogenic differentiation by using a cDNA plasmid and looked at ALP expression. As expected, Fam3c overexpressing cells displayed reduction in ALP expression as seen by quantitative PCR and by ALP staining. Additionally, transcript levels of type procollagen alpha 1 (I) (type I collagen) were also reduced in the Fam3c overexpressing cells.

Runx2 is the master regulatory transcription factor of the osteogenic lineage. Mice lacking Runx2 exhibit a complete lack of bone formation (Komori et al., 1997). Runx2 has also been shown to regulate the expression of ALP and type I collagen genes during bone formation (Harada et al., 1999; Kern et al., 2001; Weng and Su, 2013). We determined Runx2 expression in our set of experiments. In MC3T3-E1 cells undergoing differentiation, overexpression of Fam3c led to reduction of Runx2 at both mRNA and protein levels. We also verified the Fam3c mediated down-regulation of Runx2 in C3H10T1/2 cells. C3H10T1/2 cells are of mesenchymal origin and have also been utilized to study in vitro osteogenic differentiation in addition to MC3T3-E1 cells (Shea et al., 2003; Zhao et al., 2009). Overexpression of Fam3c in differentiating C3H10T1/2 cells resulted in similar decline of Runx2 at both mRNA and protein levels, thus supporting our findings on MC3T3-E1 cells.

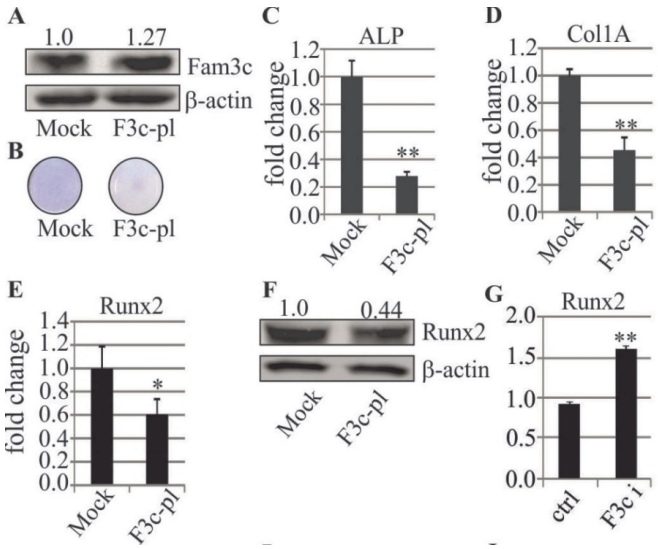

Figure 10. Fam3c mediated down-regulation of Runx2. A] Western blot for the validation of Fam3c overexpression; F3c-pl is Fam3c plasmid. B] Alkaline phosphatase staining. C] mRNA quantification of ALP and D] Type 1 Collagen. E] Runx2 mRNA and F] protein expression analysis. G] Runx2 mRNA quantification in Fam3c knockdown experiments. (Adapted from: Bendre et.al, Differentiation, 2017.)

Runx2 expression during osteogenic differentiation is stringently controlled by a wide range of factors including transcription factors, cytokines and post-translational modifications (Bruderer et al., 2014). TGF-β1 regulates Runx2 via signaling mediated by SMAD proteins (Alliston et al., 2001). In our experiments, TGF-β1 treatment led to down-regulation of Runx2 in differentiating MC3T3-E1 cells, supporting the earlier finding as well. However, TGF-β1 levels were reduced in Fam3c overexpressing cells. Our results therefore suggest that Fam3c can regulate Runx2 expression independently of TGF-β1 during osteoblast differentiation.

5.8 Possible intracellular mechanism of action of Fam3c (II)

In addition to immunofluorescence studies which localized Fam3c to the cytosol, we did not find any evidence of secreted Fam3c in the culture medium of differentiating MC3T3-E1 cells. The Fam3 protein family consists of four proteins, namely Fam3a, Fam3b, Fam3c and Fam3d. Traditionally thought to be secreted proteins, members of Fam3 protein family have been shown to function intra-cellularly. Recent studies have reported Fam3a to be involved in the regulation of hepatic glucose and lipid metabolism. Fam3a was also reported to be expressed in vascular smooth muscle cells and was found to be involved in their proliferation and migration. These studies suggested that Fam3a is expressed intra-cellularly and activates the kinase Akt in a PI3K-dependent manner (Jia et al., 2014; Wang

et al., 2014). Another study reported mitochondrial expression of Fam3A in 3T3-L1 adipocytes (Chi et al., 2017). On the contrary, Fam3d has been shown to function as a secreted protein. Fam3D is known to be expressed and secreted by intestinal enterocytes (de Wit et al., 2012). More recently, the receptor of Fam3d was identified. Fam3d was shown to bind formyl peptide receptors FPR1/FPR2 and regulate gastrointestinal homeostasis (Peng et al., 2016). 3D crystal structure analysis of murine Fam3b determined the presence of a distinct β-β-α fold, which was also found to be present in the structure of murine Fam3c based on construction of a sequence based homology model. Contrary to earlier predictions, Fam3b and Fam3c did not possess a traditional four helix bundle secondary structure, which most cytokines exhibit. This study therefore suggested Fam3b and Fam3c to be a novel class of cytokines with distinct signaling mechanisms (Johansson et al., 2013). Therefore, it is a possibility that in osteoblasts, Fam3c could function intra-cellularly through a different mechanism.

5.9 Verification of Tamoxifen mediated recombination in Dicerosx strain (III)

In order to verify Tamoxifen induced recombination in osterix positive cells, we isolated primary bone marrow stromal cells from long bones of Cre+ and Cre- animals and induced them to undergo osteogenic differentiation. Tamoxifen was supplemented in the culture medium to promote Cre mediated recombination in osterix positive cells undergoing differentiation. Recombination was verified by PCR results with no amplification of desired exonic region in cells from Cre+ animals whereas cells from Cre- animals amplified the desired region as expected.

5.10 Role of osteoblast specific Dicer1 during cortical bone formation (III)

In one of the first studies looking at the role of Dicer1 in skeletal development, a Cre-inducible Dicer1 conditional allele was generated and was utilized to study the role of Dicer1 in mouse limb development. Dicer1 was shown to be essential for proper morphogenesis of mouse limb (Harfe et al., 2005). In a later study, Dicer1 was specifically ablated in two distinct cellular populations during mouse embryonic development. Inactivation of Dicer1 in osteoprogenitor cells by using a Cola1-Cre driver compromised fetal survival post E14.5, whereas its deletion in osteocalcin expressing mature osteoblasts resulted in an increased bone mass phenotype (Gaur et al., 2010). Osteoclast-specific Dicer1 deletion led to suppression of bone resorption (Mizoguchi et al., 2010). Prior to our work, there was limited information existing which looked at the role of Dicer1 in the regulation of post-natal skeletal homeostasis. By employing our novel Dicerosx

strain, we were able to specifically ablate Dicer1 gene function in osterix-positive osteoblasts, thereby enabling us to address its role in the regulation of post-natal skeletal homeostasis.

Micro-CT analysis of Dicerosx femora from Cre- and Cre+ animals (males and females) revealed a prominent role for Dicer1 dependent microRNA biogenesis in the post-natal cortical bone formation. Two groups of animals were chosen for the experiment, 3 week old mice and 10 week old mice. The reason for choosing animals at two separate ages was that regulation of microRNA biogenesis could be studied at two different developmental stages. 3 week old mice are approaching puberty and therefore exhibit a period of swift bone growth. 10 week old mice on the other hand are sexually mature and therefore skeletal development is complete. Thus, analysis of post natal Dicer1 ablation was possible at two different developmental stages giving a complete picture of post-natal role of Dicer1 in skeletal homeostasis.

In the group of younger mice (3-8 weeks) old males and females, cortical tissue volume and bone volume were significantly decreased in the Cre+ animals as compared to their Cre- counterparts. Similarly, total cross-sectional bone area, cross sectional tissue area as well as cross-sectional thickness were reduced in Cre+ mice. BV/TV ratio correspondingly lowered in Cre+ animals. However, this decrease was statistically significant only in 3 month old male Cre+ mice and not in Cre+ females. As expected, cortical BMD was slightly decreased in Cre+ animals. In the group of older mice (10-15 weeks), an equivalent cortical bone phenotype was observed. In both sexes, cortical tissue volume and cortical bone volume were significantly reduced in Cre+ animals as compared to their Cre- counter pairs. Additionally, in both sexes, cross-sectional tissue area, bone area and cross-sectional thickness were significantly decreased in the Cre+ animals. The BV/TV ratio was decreased in both sexes but was statistically significant only in females. In older males, endocortical area and the total cross sectional bone perimeter was also reduced in Cre+ animals. Cortical BMD was also significantly reduced in Cre+ females. In males this decrease was close to statistical significance. Additionally, histomorphometric analysis revealed decreased number of endosteal osteoblasts in 15 week old Cre+ females. However, this was not seen in males.

Analysis of trabecular bone did not reveal any significant changes in the Cre+ bones as compared to their Cre- counterparts. We also measured the thickness of Cre+ and Cre- femora from 15 week old male and female animals and found a striking reduction in thickness (both lateral and anterior-posterior) in the Cre+ bones than the Cre- bones, further strengthening our results. Our findings have therefore revealed a role for osteoblast specific microRNAs during post-natal regulation of cortical bone formation.

5.11 Biomechanical testing and analysis of gene expression (III)

The strength/stiffness of a bone defined as its ability to resist deformation. It primarily depends upon bone mass, bone microstructure and intrinsic properties of the bone matrix. Changes in the bone microstructure or the matrix properties often lead to a perceptible decline in the overall bone strength (Bouxsein, 2013). The apparent differences in cortical bone structure in the Cre+(test) group bones prompted us to look at the biomechanical properties of the bones from the Cre+ (test) group and the Cre-(control) group.

3 point bending test was performed on older Dicerosx males (Cre+ and Cre-; tibiae). Cre+ (test) samples exhibited significant differences in two of the biomechanical parameters analysed, namely stiffness and work from preload to upper yield. These two parameters are related to the load displacement curves obtained for the Cre+ and Cre- samples. Cre+ (test) bones showed decreased stiffness as compared to their Cre- bones (controls). On the contrary, Cre+(test) samples showed increased work from preload to upper yield. The bones in the test group had larger plastic regions and smaller elastic regions when compared to the bones in the control group. In general, it could be said that the bones in the test group (Cre+) displayed increased ductility than the control bones.

The increased ductility might also indicate aberrant mineralization in the Cre+ (test) bones. It is also possible that the Dicer1 ablated bones have defective collagen matrix formation. To address these questions, more studies are needed.

5.12 Main results of the work

The main results of this work are summarized as follows:

- Fam3c is not essential for normal mouse embryonic development.
- Fam3c is involved in the regulation of osteoblast differentiation and bone homeostasis.
- Fam3c is expressed during osteoblast differentiation, but not secreted extra-cellularly.
- Fam3c down-regulates Runx2 during osteoblast differentiation.
- TGF-β1 and Fam3c regulate each other reciprocally.
- Tamoxifen induced Dicer1 inactivation in bone forming osteoblasts leads to deficient cortical bone homeostasis in osteoblasts.

6. SUMMARY AND CONCLUSIONS

The regulation of bone homeostasis is a complex process regulated by different proteins and microRNAs. Multiple signalling pathways co-ordinate this process. Recently, genome-wide studies have implicated the possible involvement of novel genes in the regulation of osteogenic differentiation and bone remodelling. Many of these novel genes have not been thoroughly investigated for their role in the regulation of skeletal homeostasis. Exploring the function of these novel genes would facilitate the development of novel therapeutic options for the treatment of skeletal disorders such as osteoporosis. This work characterized the function of Fam3c in bone homeostasis.

MicroRNAs are important regulators of bone formation and remodelling. Prior to this study, their role in the regulation of post-natal skeletal homeostasis was poorly characterized. This work studied the role of osteoblast-specific Dicer1 in the regulation of post-natal skeletal homeostasis. In this study the role of Fam3c in bone formation and homeostasis was studied at the tissue level and its function in osteoblasts was probed at the cellular level. Fam3c was not found to be essential for the normal skeletal development or any critical physiological function in mouse. However, several cellular events concerning bone formation, osteogenic differentiation and skeletal homeostasis were affected. Based on the studies carried out, the following conclusions can be made regarding the role of Fam3c:

1. Fam3c is expressed at both mRNA and protein levels during osteoblast differentiation and it regulates cortical bone volume and cortical BMD. Additionally, smaller changes were observed in the trabecular bone volume.

2. In osteoblasts undergoing differentiation, Fam3c down-regulates Runx2. Fam3c and TGF-β1 regulate each other reciprocally.

In the second part of the study, the role of osteoblast-specific Dicer1 was studied in the regulation of post-natal skeletal homeostasis. It was possible to delete Dicer1 gene function in osteoblasts without any potential adverse effects. The model allowed us to address the role of Dicer1 in bone forming cells, during bone homeostasis. Based on the studies carried out, the following conclusions can be made regarding the role of osteoblast-specific Dicer1:

1. Inactivation of Dicer1 gene function in bone forming cells leads to deficient cortical bone formation.

2. Osteoblastic microRNAs might be essential for maintenance of normal post-natal cortical bone homeostasis.

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