Role of MicroRNA-29a,b,c in Bone Healing of Laser Additive Manufactured Commercially Pure Titanium Implants in Rabbit Model

A DISSERTATION SUBMITTED

BY

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DECLARATION

I, **Sheenu A S**, hereby declare that I had personally carried out the work depicted in the thesis entitled, "**Role of MicroRNA-29a,b,c in Bone Healing of Laser Additive Manufactured Commercially Pure Titanium Implants in Rabbit Model**" under the direct supervision of **Dr. A.Sabareeswaran, Scientist E, Histopathology lab**, Division of Experimental Pathology, Department of Applied Biology, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India.

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CERTIFICATE

This is to certify that the dissertation entitled "**Role of MicroRNA-29a,b,c in Bone Healing of Laser Additive Manufactured Commercially Pure Titanium Implants in Rabbit Model**" is a bonafide work done by **Ms. Sheenu A S** in partial fulfilment for the degree of **Master in Philosophy** under my supervision and guidance at **Histopathology lab**, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India.

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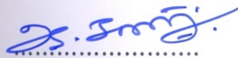

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ABBREVIATIONS

µL	micro litre
miRNA	microRNA
pri-miRNA	Primary microRNA
qPCR	Quantitative polymerase chain reaction
RISC	RNA induced silencing complex
UTR	Untranslated region
PMMA	Polymethyl methacrylate
Pre-miRNA	Precursor-miRNA
RNA	Ribonucleic acid
MSC	Mesenchymal stem cells
HSC	Haematopoietic stem cells
ECM	Extra cellular matrix
Runx2	Runt-related transcription factor 2
SPARC	Secreted protein acidic and rich in cysteine
MiR-29	microRNA-29
dsRBD	double stranded RNA binding domain
LAM-Ti	Laser additive manufactured titanium
Cp-Ti	Commercially pure titanium

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SYNOPSIS

For last few decades, researchers in biomedical field are trying to establish a unity between implant and tissue. Titanium is one of the extensively used biomaterial for orthopedic treatment. In the present study, osteointegration ability of laser additive manufactured commercially pure Titanium (LAM-Cp-Ti) was evaluated in a rabbit model. This work mainly focuses on the molecular mechanisms which regulate osteogenesis during the early period of bone healing. MicroRNA 29a,b,c expression pattern of the bone tissue at the bone-implant interface has been studied and correlated with the mRNA expression pattern of osteogenic associated genes namely Runx2, Collagen I and SPARC.

Chapter One: Introduces the back ground and review of literature on previous studies conducted. It concentrates on bone, bone defects and tissue response, role of microRNA in the bone healing. Bone, a dynamic endoskeletal tissue which is always prone to damages caused by accidents and bone loss disorders. For the fixation of these fractures, different types and design of metal implants can be used including Titanium plates, intramedullary pins and rods, screws etc. The success of prosthesis depends on the interaction maintained between the prosthesis and surrounding tissue. It facilitates mechanical support, homeostasis, cell formation, calcium metabolism, mineral storage and haematopoiesis. Various signaling molecules accelerate bone formation by acting on transcriptional as well as translational level. The key signaling molecules in bone metabolism include Runx2, Collagen I and SPARC. Among the post transcriptional silencing mechanisms, microRNA provides a novel pathway in osteogenesis. These small noncoding RNA molecules functions by inhibiting

translation of different mRNAs. MicroRNAs have been identified to be involved in the process of bone healing and bone loss disorders. From microRNA family, microRNA-29 plays an important role in osteogenesis. They regulate bone metabolism by maintaining the balance between osteoblast, bone forming cells and osteoclasts, bone resorbing cells. Therefore it is necessary to study the role of microRNA in bone healing to evaluate osteogenesis.

Chapter Two: Describes the materials and methods used to conduct the experiment. Approval has been obtained from Institutional Animal Ethics Committee and following CPCSEA guidelines, conducted the experiments in animals. Animal implantation studies were conducted in five New Zealand White rabbit as per ISO 10993-6 standard. In one femur bone LAM-Cp-Ti were implanted and in another femur Cp-Ti were implanted. Animals were sacrificed post one month of implantation. For histology, bone tissue with implant was harvested, fixed in 10% neutral buffer formalin and embedded in PMMA. Sections were cut using high precision saw microtome, ground and polished. Sections were stained using Stevenel's blue and Van Gieson picrofuchsin stain and evaluated microscopically. For molecular studies, bone samples were crushed in liquid nitrogen and stored in trizol reagent. RNA was isolated following guanidium-phenol method for both microRNA and osteogenic associated gene expression studies. For microRNA experiments, the EXIQON kit has been used and the expression of miR-29a,b,c were studied using real time PCR technique. For osteogenic associated mRNA expression namely Runx2, Collagen I and SPARC specific primers were used to synthesis the cDNA based on TAKARA kit protocol and real time PCR was carried out.

Chapter Three: Analyses the results and briefly discuss the same with reference to already published data. Results of animal experiments, gross and histological features were presented. Histology revealed new bone formation at the interface. Molecular studies post implantation confirmed the expression of microRNA 29a,b,c. MiR-29 family of microRNAs influence the expression of osteogenic factors Runx2, Collagen I and osteonectin. Results obtained from comparison of LAM-Cp-Ti and Cp-Ti indicate that the microRNA 29a,b,c regulate osteogenesis in tissue surrounding both implant materials. Upregulation in the mRNA expression of Collagen I and SPARC around the implant area depicts that the bone tissue is at mineralization phase which is an important phenomenon of bone healing. Reduced miR-29b expression was observed in the bone tissue around the implant area in test group when compared to the control group, while the expression of 29c was higher in both groups. Increased osteogenic gene expression is associated with lower miR-29b expression. Collagen I and SPARC expression was upregulated in the implanted tissue of the LAM-Cp-Ti which is associated with higher 29c expression.

Histological analysis showed osteointegration and new bone growth in both LAM-Cp-Ti and Cp-Ti. New bone formation at the implant-bone interface can be easily distinguished from old bone which supports osteointegration and osteogenesis.

Chapter four: Summarizes and concludes the present study briefly and gives an outline on future aspects. Animal experiments and the histological analysis revealed bone healing and new bone formation at the interface. MicroRNA-29a,b,c influence osteogenic associated gene expression during early phase of bone healing in LAM-Cp-Ti and Cp-Ti. Results showed the expression pattern of miR-29a,b,c is correlated with the expression of osteogenic associated genes Runx2, Collagen I and SPARC.

Other genes involved in osteogenesis has to be studied which will pave way to use this miRNA molecules as potential therapeutic agent.

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

An implant may be defined as an object made from a non-living material that is inserted into the body, where it has to remain for a significant period of time. When a foreign material is inserted into the body there will be some interaction in the implant-tissue interface. A prerequisite for the long term success of an implant is biocompatibility, which is provided by the proper remodelling of tissue around implant. The success of an implant depends on the bond obtained and maintained between the implant and surrounding tissue. Furthermore, long term success of implant material is orchestrated by a cohort of cellular activities and genetic mechanisms that can maintain the stress condition due to implantation.

Majority of early work on implants was focused within the field of orthopaedics. In the recent years a tremendous strides forward in the application of implants and in the evaluation of implant materials. In orthopaedics, attention is focused on bone defects or damages and treatment of bone diseases. Bone defects are mainly caused by breakage or fracture. For the fixation of these fractures, different types and design of implant can be used such as metal plates, intramedullary pins and rods, screws etc. The immense variety of fractures paved the way for varying shape, consistency and design of implants.

Titanium is the main metallic implant material in orthopaedic surgery and has conventionally been used in commercially pure form. They are available in a range of

commercially pure grades depending on the amount of impurity. Pure titanium is intrinsically very reactive and the thin tenacious film of the oxide that forms the surface, protects the material from wide range of corrosive media. Fabrication of titanium is generally a little more difficult than other material such as stainless steel.

Bone is a dynamic tissue, a specialized hard connective tissue which undergoes constant remodelling throughout life helping in regeneration of bone. It facilitates most prominent functions of body including mechanical support, homeostasis, cell formation, calcium metabolism, mineral storage and haematopoiesis. Moreover bone protects vital organs from external shock. Composition of bone includes both organic (extracellular matrix) and inorganic (hydroxyapatite) molecules. Based on the variation in structure and function bone can be classified into trabecular and cortical bone. Increased porosity of cortical bone leads to reduced strength and accompany fracture risk. Trabecular bone provides increased surface area for vascular tissue and connective tissues. During aging trabecular bone is lost earlier and leads to bone diseases such as osteoporosis.

This endoskeleton comprises of four types of cells namely osteoprogenitor cells, osteoblasts, osteocytes and osteoclasts. Of these osteoprogenitors are mesenchymal cells and osteocytes are most abundant. Osteoblasts are bone forming cells that located near the bone surface and functions in making osteoid and hormones such as prostaglandins. Osteocytes are produced from osteoblasts and helps in bone formation, calcium homeostasis and matrix maintenance. They regulate the bone response during damage conditions. Osteoclasts are bone resorbing cells which are multinucleated and located on bone surface called Howships's lacunae. The process of bone resorption releases stored calcium into circulation and bone formation

actively fixes released calcium in mineral form. These bone remodelling units are coupled together by paracrine cell signalling pathways such as Wnt family. In a nutshell, osteoblasts add new bone to the outer surface and osteoclasts remove bone in the inner surface which is called as bone remodelling or turn over. Remodelling prevents the accumulation of damaged bone and maintains mineral homeostasis.

During aging, bone resorption may exceed and bone formation decreases and leads to decrease in bone mass. Osteoblasts secrete mediators of remodelling such as SPARC, osteopontin, growth factors such as bone morphogenic proteins and proteoglycans.

Nowadays, most clinical observations indicate bone defect and damage due to fracture. Most prominent solution to this is acceleration of bone formation. When implants are placed in bone acute and chronic inflammation occurs followed by healing with deposition of new bone around the implant. By the entry of inflammatory cells, osteoblasts get activated and converts into new bone. The newly deposited bone is called woven bone and it is remodelled by matrix deposition and resorption. Evaluation of biocompatibility of implants in new bone requires processing of bone with implant-bone interface.

Bone formation depends on the balance between bone forming cells called osteoblasts and bone resorbing cells called osteoclasts. Researchers have unveiled lot of signalling molecules and genetic mechanisms that promote osteogenesis. Genetic mechanisms activate bone forming genes and promote biogenesis of bone. During bone formation, central role is played by Runx2, a member of runt related transcription factor family. They play role numerous vertebrate animals. They regulate a broad spectrum of osteogenic signalling pathways.

Collagen I is an important component of bone extra-cellular matrix, forming connections with cell surface integrins and other extra cellular matrix proteins. The protein cannot be considered bone specific because it has been identified in numerous cell types. Collagen is required for the deposition of matrix during bone formation so that it can promote neoformation and prevent fibrosis. This protein play important role in cell adhesion, proliferation and differentiation. Upregulation of Collagen is observed during bone formation. These are only a part this complex regulatory complex. Runx2 is the transcription factor that induces the commitment of mesenchymal stem cells to osteogenic lineage and acts upstream from the other osteoblast-specific transcription factors. They are key transcription factors associated with osteoblast phenotype. It acts as a scaffold for regulatory factors in skeletal gene expression which is essential for the intramembranous and endochondral ossification. It binds to the promoters of Collagen I and activate transcription. SPARC (Secreted Protein Acidic and Rich in Cysteine) also known as Osteonectin, a bone glycoprotein which binds to calcium and promote mineralization during bone formation.

Several factors control the signalling molecules in genetic mechanisms. From genetic mechanisms microRNA (miRNA) provide a novel direction to bone remodelling by post transcriptional regulation together with expression of osteogenesis associated genes. They control the complex process of bone formation.

MiRNAs are small noncoding single stranded RNA molecules composed of approximately 20-24 nucleotides that mediate numerous cellular processes. They are found to be involved in lot of physiological and biological process by post-transcriptional modifications that regulate gene expression. The complementarity of 3’UTR of miRNA with the target mRNA results in inhibition of gene expression by

mRNA break down or by inhibition of translation. Thus they silence the expression of mRNA and attenuate protein synthesis. Various miRNAs regulate cell differentiation and proliferation, thus play prominent role in regulatory mechanisms. Manipulation of miRNAs can promote osteogenesis by osteoblast differentiation and can be proposed as a strategy for the treatment of different bone related diseases.

Targets of microRNA in osteogenesis include bone matrix proteins, regulators of metabolic activities and components of osteogenic signalling pathways. The inactivation of Dicer, a prerequisite for the manufacture of canonical miRNAs leads to bone defects and impaired mineralization during embryonic stage. They can bind to target sequences in the UTR of mRNAs leading to translational arrest or causing the RNA induced silencing complex (RISC) to degrade the message. Several miRNAs are identified that regulate osteogenesis such as miR-26, miR-133, miR-223, miR-20, miR-17 etc.

MicroRNAs play key roles in many cellular processes together with transcription factors and signalling molecules. Many miRNA activities remain poorly understood. Among this group of miRNAs recent studies provide significant evidence on miR-29 family which regulate different activities including osteogenesis. It consists of miR-29a, miR-29b and miR-29c. They have similar expression patterns and functions. MiR-29 play relevant role in adipogenesis, osteogenesis, tumorigenesis and remaining biological activities. According to research they are conserved in mouse, rat and human. miR-29 family share a common seed sequence and overlap functions. They act as both positive and negative regulators of bone formation.

In conclusion molecular mechanisms play a prominent role in regulation of bone healing and metabolism. They provide environment for the differentiation of cells into osteoblast lineage. The biocompatibility of biomaterials is determined by their integration with host tissue and their structural modifications. Together both molecular mechanism and tissue-implant interaction activates the osteogenesis associated genes and provide a platform for the normal development of bone tissue. The growth of tissue around implant material increases the longevity and stability of patient life.

1.2 REVIEW OF LITERATURE

Bone is a metabolically active tissue that is constantly formed and resorbed in response to changes in mechanical loading, altered serum calcium levels and in response to a wide range of paracrine and endocrine factors (1). It is necessary to maintain the metabolic functions and structural integrity. Bone damage or breakage affects the homeostasis of body. Bone can heal properly in its original position. There are several treatments for broken bone. To fix broken bone metal screws, pins, rods, and plates can be used to hold the bone in place. Most commonly used is metallic implants. Successful integration of an implant is generally accepted to rely on its surface characteristics such as chemical composition, morphology, and energy. Various metallic materials have been used for orthopaedic replacements i.e., knees, shoulders and damages. The list of materials includes stainless steel, Co –Cr –Mo alloys, titanium alloys and other more specialized alloys, e.g., Au –Pd(2). Of these titanium alloys offers several benefits, including lower elastic modulus, excellent corrosion resistance and enhanced biocompatibility.

1.2.1 Titanium

Titanium (Ti) is considered as the most frequently used material for orthopaedic implants due to their high degree of biocompatibility and good mechanical properties. They are extensively used in many industries due to their low density, high corrosion resistance and oxidation resistance (3). The mechanical characteristics include very light weight, excellent resistance to traction and breaking. They have the greatest corrosion resistance. It has no allergic or immunological reactions.

1.2.2 Commercially pure titanium

Titanium is recognized as a strong metal with low density ($4.51 g/cm^3$). It is considered to be physiologically inert. Because of its biocompatibility titanium is used in medical applications including surgical implements and implants, such as hip and sockets that can stay in place for up to 20 year.

It has an inherent property to osteointegrate, enabling use in dental implants that can remain in place for over 30 years. This property is also useful for orthopaedic implant applications. These benefit from titanium's lower modulus of elasticity to more closely match that of the bone that such devices are intended to repair. As a result, skeletal loads are evenly shared between bone and implant leading to lower incidence of bone degradation. Since titanium is non ferromagnetic, patients with titanium implants can be safely examined with magnetic resonance imaging. Titanium is also used for making surgical instruments used in image-guided surgery as well as wheelchairs, crutches and any other products where high strength and low weight are desirable.

1.2.3 Laser additive manufacturing of titanium

The surface properties of titanium determine the cell-surface interactions. This enhances the process of osteointegration and act as a good candidate for bone remodelling after damage. The advantage of porous materials is their ability to provide biological anchorage for the surrounding bone tissue via the ingrowth of mineralized tissue into the pore spaces. Various production methods are reviewed for additive manufacturing like sintering and bubble expansion method. Laser sintering (LS), laser melting (LM), and laser metal deposition (LMD) are presently regarded as

the three most versatile laser-based additive manufacturing (AM) processes. Laser-based AM processes generally have a complex physical and chemical metallurgical nature, which is material and process dependant. Each AM process has its specific characteristics in terms of usable materials, processing procedures, and applicable situations (4). It consists of a laser, an automatic powder layering apparatus, a computer system for process control, and some accessorial mechanisms.

The functional principle behind additive manufacturing is the layer by layer shaping and consolidation of powdered material using a computer controlled laser beam or electron beam as an energy source.

The system starts by applying a thin layer of the powder material to the building platform. A powerful laser beam then fuses the powder at exactly the points defined by the computer generated component design data. Platform is then lowered and another layer of powder is applied. Once again the material is fused so as to bond with the layer below at the predefined points. The major advantages include freedom of design. The structure can be manipulated to bone defect. It can be used to fabricate tissue scaffolds that are biocompatible, biodegradable and bio-absorbable.

1.2.4 Titanium and its biomedical applications

Titanium alloys, because of their excellent mechanical, physical and biological performance, are finding ever-increasing application in biomedical devices (2). Commercial purity titanium has long been used for biomedical devices for a long time. The most important property of an implant material is biocompatibility and corrosion resistance. In biomedical field they are used for replacing failed hard tissues such as knee and hip joints. Most attractive property of titanium implants are they did

not interfere with the bone healing process (5). Titanium alloys made of 6% aluminum and 4% vanadium are most common type of titanium used in medicine. The greatest benefit of this implant material is there features including strong, durable, light weight, low cost and flexibility. Osteointegration firmly attaches the titanium material around the damaged tissue. They are used in producing pins, bone plates, rods, wires, rib cages and finger and toe replacement materials. Because of its great properties it is used to create a large number of surgical devices. Properties can be modified by fabricating the surface; these modifications increase its applications. Modifications on the implant surface with the purpose of encouraging and improving bone growth, provide better fixation of the implant to the bone, greater bone-to-implant contact at the interface and earlier bone formation (6).

1.2.5 Tissue response to implantation

The host response after implantation is designed by the characteristics and stability of implant. An implant is considered as osteointegrated when there is no progressive relative movement between the implant and the bone with which it has direct contact (7). Bone healing around implants involves a cascade of cellular and extracellular biological events that take place in the implant-tissue interface (8). These biological events include the activation of osteogenic processes and host response with respect to implant surface properties. The biological events during osteointegration is regulated by growth and differentiation factors released by the blood cells at the implant-bone interface (9).

Major stages of skeletal response to implantation-related injury and key histological events as related to the host response after insertion and mechanical fixation of

cementless implants include hematoma formation and mesenchymal tissue development, woven bone formation through the intramembranous pathway, and lamellar bone formation on the spicules of woven bone (7). Blood is the primary components which come in contact with endosseous implant. The components of blood cells such as platelets, granulocytes such as polymorphonuclear cells migrate to the tissue surrounding the implant. The blood cells entrapped at the implant interface are activated and release cytokines and other soluble, growth and differentiation factors (9). Initial response due to interaction of blood cells is clot formation. In response to the foreign surface, platelets undergo changes including adhesion, spreading, aggregation, and intracellular biochemical changes such as induction of phosphotyrosine, intracellular calcium increase, and hydrolysis of phospholipids (10). Fibrin acts as a scaffold for osteoconduction by osteogenic cells and further healing of cells takes place. These osteogenic cells from the osteoid tissue and new trabecular bone remodels into lamellar bone on implant surface. From the day one post implantation osteoblasts and mesenchymal cells migrate and attach to the implant surface which deposits bone related proteins and matrix layer for regulating cell adhesion (11).

Osteogenesis, new bone formation is a tightly regulated process orchestrated by numerous molecular determinants and cellular activities (12). This dynamic process is carried by osteoblast (bone forming cells) and osteoclast (bone resorbing cells) cells. In these co-ordinated actions, the amount of bone resorbed by osteoclasts is equal to amount of bone formed by osteoblasts. These responses occur in both cortical and trabecular bone. Bone formation occurs in the absence of osteoclasts and bone resorption in the absence of osteoblasts which indicate that they act independently and

other cell type is required for normal bone metabolism. Remodelling commences with the initiation of osteoclast formation, osteoclast-mediated bone resorption, a reversal period and then a long period of bone matrix formation mediated by osteoblasts, followed by mineralisation of the matrix (1). The regulators of the bone modelling include growth factors, cytokines and bone matrix proteins.

Modelling is the process by which bone change their overall shape in response to physiologic influences or mechanical forces, leading to gradual adjustment of the skeleton to the forces that it encounters. This remodelling is necessary to maintain the structural integrity of the skeleton and metabolic functions such as store house of calcium and phosphorus. Bones may widen or change axis by removal or addition of bone to the appropriate surfaces by independent action of osteoblasts and osteoclasts (14). Bone contains large number of growth factors. These are polypeptides produced by the bone cells themselves or in extra-osseous tissue and act as modulators of growth, differentiation, and proliferation (13).

Skeletal development and bone remodelling require stringent control of gene expression for osteoprogenitor lineage cells to progress through stages of differentiation (15). In many physiological and pathophysiological processes, miRNAs have been demonstrated to play crucial roles (16).

1.2.6 Signalling pathways in bone remodelling

Cellular damage activates bone remodelling by triggering cellular communication. Remodelling commences with the initiation of osteoclast formation, osteoclast-mediated bone resorption, a reversal period, and then a long period of bone matrix formation mediated by osteoblasts, followed by mineralisation of the matrix (1).

Cellular signalling provokes osteoclast promoting cells and leads to resorption of bone. Resorption leads to the death of osteoclast cells and initiates the differentiation of osteoblast cells. Finally osteoblast formation promotes bone formation or osteogenesis. Thus an old bone area regenerates into new bone (17). Regulatory factors for osteoblastic phenotype include the essential transcription factors, Runx2/Cbfa-1 and osterix/SP7 and major signalling pathways, bone morphogenetic protein (BMP),Wnt and notch as well as other growth factor-mediated kinase signalling pathways(18).RANKL-OPG, Wnt, and BMP pathways have been identified as the classic pathways in the process of bone remodelling (19).

RUNX2 is the transcription factor that induces the commitment of mesenchymal stem cells to osteogenic lineage and acts upstream from the other osteoblast-specific transcription factor OSTERIX and other specific osteoblastic genes such as SPARC (Osteonectin), SPP1 (Osteopontin), and *COL1A1* (Type I Collagen) (20). This prominent pathway is regulated by Wnt signalling. Osteonectin is also known as SPARC (Secreted Protein Acidic and Rich in Cysteine) is produced by osteoblast cells during osteogenesis. It plays a major role in bone development and mineralization (21). It binds with Collagen and promote mineralization (22). Collagen is the main protein component of bone matrix. The reduced level of these proteins may lead to bone lose. So they are required for maintaining structural integrity of bone.

Besides the pathways described above, epigenetic factors, such as DNA methylation, microRNA (miRNA), and chromatin structure modification, regulate osteogenesis (23). In particular, miRNAs, short, non-coding RNAs, may affect both osteoblast lineage/bone formation and osteoclast lineage/bone resorption. Post-transcriptional

regulation of osteoblastogenesis by miRNAs may affect the expression of RUNX2. The expression of Type I Collagen genes may also be affected by miRNAs (24). Even if the involvement of miRNAs in osteogenesis has been poorly investigated so far, it has been reported that microRNAs may affect osteoblast differentiation and maturation by direct or indirect regulation.

1.2.7 MicroRNA

Victor Ambros and colleagues, Rosalind Lee and Rhonda Feinbaum, discovered that lin-4, a gene known to control the timing of C.elegans larval development, does not code for a protein but instead produces a pair of small RNAs (25),called miRNA. MicroRNAs are noncoding, single stranded RNA molecules composed of approximately 20-24 nucleotides that regulate gene expression. miRNAs interact with targets that have similar sequences, which inhibits translation of different genes, although miRNAs are not completely complementary to the mRNA sequence (19). These short RNA molecules bind to complementary sequences in the 3' untranslated region (UTR) of mRNAs to block protein translation or modulate mRNA stability. Depending on their degree of complementarity with the target mRNAs, the binding event induces translational repression of the target gene or stimulates rapid degradation of the target transcript (26). Thus directly inhibits mRNA translation. It is evolutionarily conserved and a key epigenetic mechanism for control of expressed genes. miRNAs contribute to osteogenesis by regulating the growth, differentiation and functional activity of cells that constitute bone tissue.

The founding members of the miRNA class, lin-4 and let-7, were discovered in *Caenorhabditis elegans* to regulate the development timing and progression of the

nematode life cycle (18). Over a hundred miRNA genes have been discovered so far from biochemical and bioinformatic studies of *Caenorhabditis elegans*, *Drosophila melanogaster* and *Homo sapiens* (27). MiRNA genes are found on every chromosome in humans, except for the Y chromosome (28). It has been found to play a crucial role in cell cycle control, apoptosis and other cellular processes including metabolism and differentiation (29). The breadth and importance of miRNA-directed gene regulation are coming into focus as more miRNAs and their regulatory targets and functions are discovered (25). More recently hundreds of miRNAs have been found in many animal species by computational predictions, experimental approaches or combined strategies (30). Numerous studies have shown the importance of these RNAs in all biological processes and become an integral part of gene expression studies.

1.2.8 Biogenesis of microRNA

MiRNAs are processed through a series of post-transcriptional modifications. Few miRNA genes are located in the introns of protein coding genes and remaining are located in the regions distant from annotated genes. MiRNA promoters are regulated by epigenetic and transcription factors like the promoters of protein coding genes.

MiRNAs originate from long primary transcripts called pri-miRNAs(31),which are transcribed by RNA polymerase II and then cut by the ribonuclease III enzyme, with the assistance of Drosha (a member of ribonuclease III family (RNaseIII)) and Dicer(29) in the nucleus from independent miRNA genes or from introns of both coding and non coding genes. Pri-miRNA, capped and polyadenylated, consists of the mature miRNA in an elongated RNA hairpin structure containing a loop structure directing its cleavage and the loop is recognized by Drosha. Drosha has two RNase III

domains and one double stranded RNA binding domain (dsRBD), the two RNase III domains (RIIIDa and RIIIDb) form an intramolecular dimer and cleave the 3' and 5'strands of the stem, respectively (31). Drosha cleaves the flanks of pri-miRNAs to liberate ~ 70 nucleotide stem-loop structures, called precursor miRNAs (pre-miRNAs) (32).

Exportin-5, a Ran-GTPase protein transports the pri-miRNA into the cytoplasm from the nucleus. There endonuclease Dicer cleaves them to obtain imperfect miRNA duplexes. The miRNA duplex is then incorporated into a multicomponent protein complex known as RNA-induced silencing complex (RISC), which contains the Argonuate-2 protein (26). RISC is composed of Dicer, TRBP, Protein activator of PKR (PACT) and Ago proteins. This mature miRNA can regulate its target mRNAs.

The RISC-incorporated miRNA regulates gene expression through sequence-specific interactions of seed region (5' proximal end of a mature miRNA) with its target site, which is typically located within the 3' untranslated region (3'UTR) of an mRNA transcript (33). The miRNA duplex is unwound by helicases into mature strand and complementary strand. Mature strand is determined by the Ago protein in RISC complex.

1.2.9 MicroRNAs in bone healing

Skeletal development requires stringent control of gene activation and suppression. Osteoblasts express many miRNAs, and approximately 36% of the miRNAs were down regulated by BMP-2 treatment, only 4% of them were upregulated. Osteoblasts arise from mesenchymal precursors that undergo a well-defined program of gene

expression as they progress through osteoblastic commitment, proliferation, and terminal differentiation (26).

The maintenance of bone mass mainly depends on the balance between osteoblast-mediated bone formation and osteoclast-mediated bone resorption (19). Different families of miRNAs regulate bone metabolism, such as miR-210, miR-29, miR-148. Thus these short RNA molecules are one of the important modulators in bone metabolism and bone metabolic disorders.

Bone healing involves the replacement of old tissue with new one. It is a continuous process from birth to adulthood as well as during repairing of fracture. Bone healing after implantation leads to bone remodelling which include preservation of bone mechanical strength by replacing older, micro damaged bone with newer, healthier bone and calcium and phosphate homeostasis (13). It involves the production or deposition of bone by osteoblast cells, differentiated from mesenchymal stromal cells (MSCs) and resorption of bone by osteoclast cells, originated from haematopoietic progenitors, which resorbs the old bone. Remodelling help bone to adapt during stress conditions by increasing the strength and thickness after bone healing. During bone formation proliferation and differentiation of MSCs into osteoblasts occurs by the expression of osteogenic genes such as Osteonectin, Type I Collagen etc.

The maintenance of bone mainly depends on osteoblast and osteoclast mediated bone metabolism. Balance between osteoblast and osteoclast cells determines the maintenance of bone tissue. Differentiation of MSCs occurs in a coordinated manner, which involves cellular commitment, proliferation, matrix maturation and

mineralisation (18). It is regulated by growth factors such as BMPs. These regulatory factors trigger osteogenic lineage pathways such as Runx 2.

Osteoclasts are originated from mononuclear precursors through RANKL-OPG signaling pathway (19). Runt-related transcription factor 2 (Runx2), connects many signal pathways in bone remodelling process by enhancing factors that regulate osteoblast differentiation (34).

1.2.10 Functions of microRNA

Wide ranges of cellular processes are regulated by miRNAs. Evidence from studies shows that homozygous deletion of Dicer, disrupts prenatal development of the murine embryo through its role in miRNA biogenesis (35). Post-transcriptional regulation of gene expression, mediated by miRNAs, plays an important role in the control of osteoblastic differentiation (36). Growing evidence shows that miRNAs regulate various developmental and homeostatic events in vertebrates and invertebrates (37).

These noncoding RNA molecules have important roles in many biological processes such as tissue formation, cancer development, diabetes, neurodegenerative diseases, systemic autoimmunity diseases and cardiovascular diseases (19). They have a prominent role in bone remodelling by modulating post transcriptional events.

MicroRNAs mediate MSCs proliferation and differentiation into adipocyte, cardiac, neural and skeletal tissues for example, miR-124 and miR-128 are found to be highly expressed in the adult brain, suggest their importance in neural development (26). miR-196a plays a role in hASC osteogenic differentiation and proliferation, which

may be mediated through its predicted target, *HOXC8* (38). Knockdown of miR-206 expression promoted osteoblast differentiation (37). BMP-4-induced osteoblastic differentiation of bone marrow-derived ST2 stromal cells was promoted and repressed after transfection of sense and antisense miR-210 (39).

Overexpression miR-378, enhances Alp activity, calcium deposition and mRNA expression of osteogenic marker genes in the presence of BMP2(33). In addition, miR-125b, miR-26a, miR-133 and miR-135 have all been implicated in the differentiation of osteoblasts (40).

The miR-29 family plays crucial roles in osteogenesis by suppressing osteonectin expression rather than reducing collagens and extracellular matrix proteins(24). Studies show that miR-29 participates in various physiological processes. miR-26a and miR-29b facilitate osteogenic differentiation of human adipose tissue-derived stem cells (hADSCs), and positively modulate mouse osteoblast differentiation(41). Recent studies have shown that some miRNAs negatively regulate osteoblast differentiation, whereas some others play a positive role in the regulation of osteoblast differentiation (26).

An accurate and deep understanding was provided by recent studies on the biology of miR-29 family. Like most miRNAs and mRNAs, miR-29s are transcribed by RNA polymerase II (42). MiR-29b is a positive regulator of osteoblast differentiation by down-regulating inhibitory factors of osteogenic signaling pathways and controlling expression of collagen in differentiated osteoblasts. Different studies shown that the members of miR-29 family act as tumor suppressors, down regulate myocardial infarct and contributed to fibrosis of tissues (15).

1.2.11 MiR-29 family

MiR-29 family consists of miR-29a, miR-29b and miR-29c. The genomic organization of miR-29 genes in rodents is less well characterized but appears to share many characteristics of the human genes, including the gene clusters (42). Their seed region are identical and have similar expression patterns and functions (43). Act as a key regulator of osteoblast differentiation by directly targeting several inhibitors of osteogenesis, histone deacetylase (HDAC4), TGF-b3, ACVR2 and catenin-β-interacting protein 1 (CTNNBIP1) and thus promoting osteoblast phenotype (18).

miR-29 regulates osteoblast differentiation and function by suppressing inhibitors of cell signaling pathways and/or transcriptional programs required for osteogenesis, as well as by attenuating collagen gene expression during extracellular matrix maturation. miR-29b functions as a positive regulator of osteoblastogenesis. It is predicted to target a large number of collagen genes. Collagen, protein synthesis and secretion is necessary for osteogenesis and occurs in vitro during the proliferation stage of osteoblasts for formation of the ECM (15). miR-29b mediates translational inhibition and alters the levels of critical regulators of biological pathways, thereby providing a mechanism for spatiotemporal control of developmental and homeostatic events during osteogenesis.

miR-29b targets several negative regulators of osteogenic differentiation, including TGF-3, HDAC4, ACTVR2A, CTNNBIP1, and DUSP2, which impinge on signal transduction pathways promoting osteogenesis *in vivo* (*e.g.* including Smad, ERK, p38 MAPK, and Wnt). It also promotes osteoblastic differentiation, by stimulating Wnt signaling and increased levels of osteogenic marker genes; including Runx2 and

Alkaline phosphatase attenuates expression of Collagen genes that encode essential proteins of the bone extracellular matrix.

On the other hand, miR-29b promotes osteoblast differentiation by targeting negative regulators of osteogenic pathways and thereby contributes to establishment of osteoblast differentiation. On the other hand, miR-29b functions as an attenuator of collagen synthesis in mature osteoblasts to maintain the differentiated phenotype (15).

Activation of canonical Wnt signalling is crucial for osteoblast function and induce miR-29 expression, miR-29 is induced by canonical Wnt signaling in osteoblasts. miR-29 targets negative regulators of Wnt signalling: Dkk1, Kremen and sFRP2 (18). miR-29a and -29c are up-regulated during osteoblastic differentiation, implicating these miRNAs in the down-regulation of osteonectin, and likely the regulation of other proteins critical for osteoblast function (44).

miR-29 is found to promote bone matrix collagen Iα1 expression and mineralization reactions and interact with Wnt signaling in osteoblasts. During osteogenesis miR-29 is highly upregulated (26). The three potential binding sites for the miR-29 family of miRNAs are clustered within this highly conserved proximal osteonectin 3 UTR, and clustered miRNA binding sites mediate the most efficient repression of gene expression (44). Maintaining Runx2 acetylation by inhibiting HDAC4 action contributes to the miR-29a reduction of glucocorticoids-induced defective bone acquisition and excessive marrow adipogenesis.

Overexpression of miR-29a interrupted mesenchymal progenitor cell commitment toward adipogenic cell lineage, thereby facilitating osteogenic differentiation and diminishing the deleterious actions of glucocorticoids on bone acquisition. miR-29a

signaling may change metabolic reactions in other tissues that may directly or indirectly regulate bone mass homeostasis(45). Different established targets of miR-29 shows its role in tumor suppression and apoptosis(43) and also found to be have roles in muscle development .

In conclusion, in vitro studies were conducted to analyse the role of microRNA in bone formation. Molecular studies related to microRNA and titanium implant is lacking in the current scenario. So we were trying to elucidate the role of microRNA in new bone formation using LAM-Cp-Ti and Cp-Ti. In our attempt, we are focusing on the in vivo study of miR-29a,b,c microRNAs in osteogenesis.

1.3 HYPOTHESIS

The hypothesis of this research work is that microRNA 29a,b,c have role in influencing the osteogenesis in LAM-Cp-Ti and Cp-Ti implanted in femur cortical bone defects in rabbit model.

1.4 OBJECTIVES

- To evaluate the bone healing ability of LAM-Cp-Ti and Cp-Ti.
- To elucidate the role of miR-29a,b,c in bone healing.

CHAPTER 2

MATERIALS AND METHODS

2.1 STERILIZATION OF IMPLANT MATERIAL

Control and test titanium implant materials (2mm diameter and 6mm length) were washed in alcohol or four to five times. After rinsing in alcohol, the materials were cleaned by Sonication in ultrapure distilled water for 15 minutes by three times. Implants were rinsed in isopropanol for two to three times. Cleaned samples were packed on self-sealed sterilization pouches and sterilized by autoclave.

2.1.1 Animal studies

Approval has been obtained from Institutional Animal Ethics Committee (SCT/IAEC-193/Nov/2016/90 dated 29.12.2016) and following CPCSEA guidelines, conducted the experiments in animals. Animal implantation studies were conducted in New Zealand White rabbit as per ISO 10993-6 standard. Five adult New Zealand white rabbits aged above 9 months, weighing 3-4 kg, male and females with normal limbs comprised the animal model. The rabbits were randomly selected for implantation. All rabbits were housed at the animal house of SCTIMST, Thiruvananthapuram and maintained on standard laboratory water and diet.

The animals were anesthetized with Ketamine and Xylazine via intramuscular administration. Both the legs and back region of animals were shaved before surgery and the surgical field was prepared with betadine. During operation Isoflurane was

provided for maintaining deep anesthesia. In one femur bone three LAM-Cp-Ti (test) pins were implanted (Figure Figure 2.1) and in other femur three Cp-Ti (control) pins were implanted (Figure Figure 2.2). To prevent wound infection and pain all rabbits were intramuscularly injected with Ceftriaxone and Meloxicam.

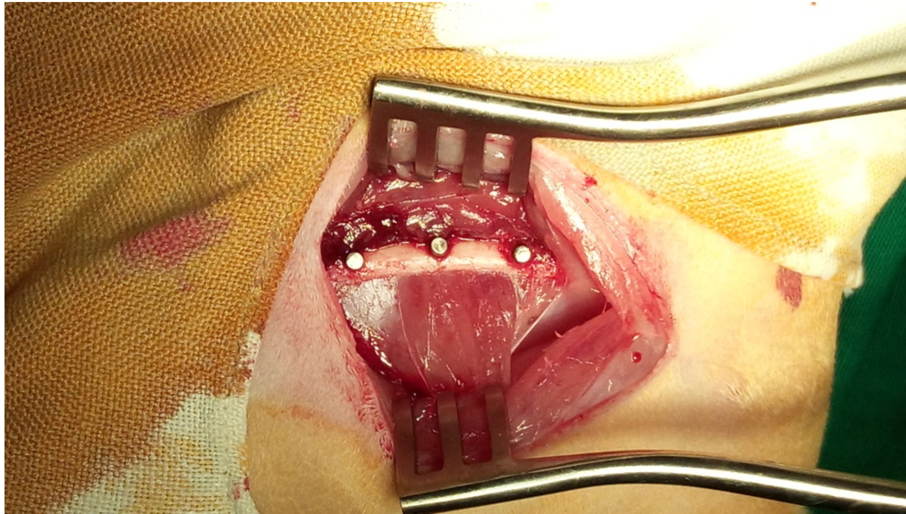

Figure 2.1: Implantation-femur bone with of LAM-Cp-Ti (Test)

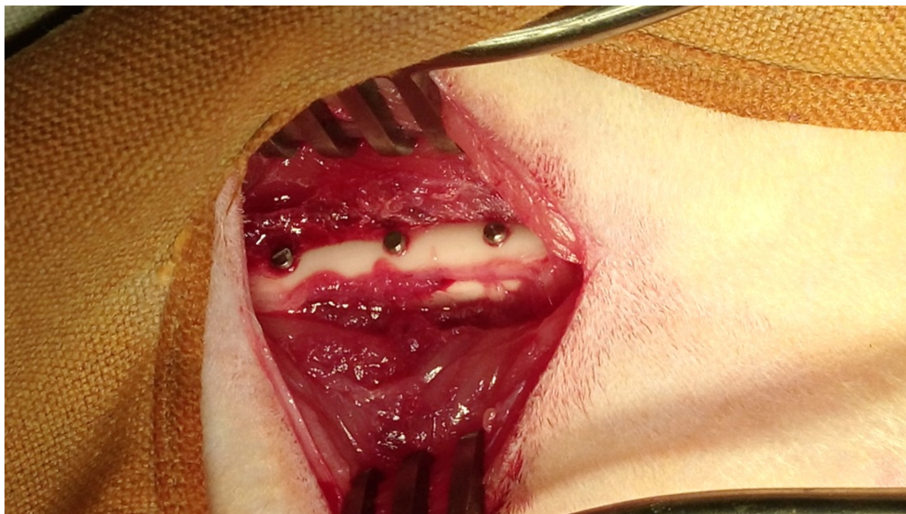

Figure 2.2: Implantation-Femur bone with Cp-Ti (Control)

2.2 EXPLANTATION AND HISTOLOGICAL ANALYSIS

2.2.1 Explantation

Animals were euthanized humanely in CO_2 chamber. Complete post-mortem examination of internal organs was carried out. Femur bones with implant were collected, implant sites were identified. For molecular studies, implant and surrounding bone tissue was cut and stored in RNA later. Any attached soft tissue was quickly removed from the bone using a scalpel before the bone was snap frozen in liquid nitrogen. For histological analysis, femur bones were collected in 10% neutral buffer formalin.

2.2.2 Histological analysis

Materials required

Different grade of alcohol, PMMA, 10% neutral buffer formaldehyde, Washed monomer (PMMA), Benzoyl peroxide, 5% sodium hydroxide, Precision cut-off and grinding machine, Low speed saw microtome, Grinder polisher, Vacuum pump, Stevenel's blue and Van Gieson's Picrofuchsin stain, Distilled water, Embedding bottle, Filter paper

2.2.3 Processing and embedding of hard tissue

Harvested bone tissue were cleaned and fixed in 10% neutral formaldehyde for 1 week. It was processed for embedding in PMMA:-

1. Dehydration of tissue through ascending grades of alcohol at room temperature, duration depending on size of tissues.

- 70% alcohol for 1-4 days
- 80% alcohol for 1-4 days
- 96% alcohol for 1-4 days
- 100% alcohol for 2 days
- Alcohol: acetone (1:1, v/v) mixture for 1 day
- 100% alcohol for 1 day

2. Infiltration of tissue with monomer

- Transfer the tissue into washed monomer and keep for 2-4 days in refrigerator II.
- Keep the tissues into a second change of washed monomer for 2-4 days in refrigerator II.

3. Embedding of tissue in monomer

- To make embedding solution take 1g of recrytallised Benzoyl peroxide in a graduated beaker of 100 ml capacity and add a little washed monomer to dissolve it. Make up to 100 ml with washed monomer. This solution should be made fresh.
- Separate embedding bottle must be used for each tissue. Label each bottle and cap with the identification number using a glass marking pen
- Pour embedding solution into the bottle. Remove tissue from the processing bottle with fine forceps and orient the tissue inside with cutting surface face down.

- Apply the cap loosely and place in the vacuum desiccator for 30 minutes to 1 hour to remove any trapped air bubbles.
- Release the vacuum slowly and tighten cap carefully without displacing the position of tissue.
- Keep in vacuum for 2-3 days to ensure complete evacuation of oxygen in the chamber.
- Release vacuum and check each bottle for polymerization with a needle. If the resin is hard, polymerization is complete
- Remove the bottles, break each bottle with a hammer and write the TSPAT number on the resin block or in the individual bag where each block is kept.

2.2.4 Section cutting of resin embedded tissues

To cut thin sections (100 micron) of resin blocks. Enter blade thickness, section thickness, blade speed, feed rate, cutting length and the number of sections required in the Precision cut-off and grinding machine (ACCUTOM 100, DENMARK). Keep the position/orientation of the resin block. Adjust the block and blade to reach the required starting level of sectioning. Set zero and start the cutting cycles. When cutting has completed, fix each section on clean glass slides using cyano acrylate glue. Store the sections for staining purpose.

2.2.5 Grinding and polishing

Cut sections were stuck to a glass slide and held using slide holder, the sections were ground to reduce the thickness using different grades of sand paper in a grinder cum polisher machine (ECOMET 3, USA).

2.2.6 Staining of resin sections

Resin sections were stained using Stevenel's blue stain for 5 minutes and washed with hot distilled water and placed the sections into Van Gieson picrofuchsin stain for 3 minutes at room temperature then wiped with tissue paper and observed under microscope (NIKON E 600, JAPAN).Microphotographs were captured using the camera attached to the microscope.

Figure 2.3: Resin embedded bone tissue with implant

2.3 RNA ISOLATION FROM FEMUR BONE

2.3.1 Materials required

70% ethanol (filter sterilized), Chloroform, Isopropanol, Distilled water sterile, 1000 µL, 200 µL, 10 µL tips sterile.

2.3.2 Method

Bone tissue surrounding the implant was taken out from RNA later and cleaned in ultra pure water before snap frozen in liquid nitrogen in order to facilitate the grinding process. Keeping mortar and pestle cold on dry ice, empty vial of frozen bones into mortar (weigh the vials before and after to determine weight bones).

- Add liquid nitrogen and crush bones with pestle to a fine powder.
- Keep replenishing liquid nitrogen as needed. Do not allow bone to thaw.
- Pour powder/LN mixture into a 50 ml falcon tube that is propped up on dry ice and quickly put cover on loosely to prevent sample from popping out as N2 evaporates.
- Store at -80°c or continue to next step.
- Add 1ml trizol per 0.1g bone powder.
- To isolate total RNA from the culture previously treated with Trizol reagent. The lysed sample with trizol reagent was stored at -80^{0}C (1 month).
- Homogenize tissue sample in 1 ml of trizol reagent per 50 to 100 mg tissue using a homogenizer (e.g. polytron).
- Incubate the homogenized sample for 5 minutes at room temperature.

- Centrifuged to remove cell debris (1500 rpm).
- Transfer the supernatant to new tube.
- This can be stored at -80^{0}C for 1 month.
- Retrieve the sample from deep freezer and transfer to ice box.
- Keep samples in ice till it thaws.
- Allow to stand for 5 minutes at room temperature, after thawing
- Add 200µl of chloroform to 1 ml of sample.
- Covered the samples tightly and mixed well by gently inverting for 10 to 15 minutes.
- Allow to stand for 5 minutes at room temperature (at this time a transparent phase should be seen above and a slightly pink solution below).
- Centrifuged at 12000 rpm for 15 minutes at 4^{0}C (some nucleoprotein complexes may still remain in solution, hence chloroform washing can be repeated for 1 to 2 times).
- Remove the colourless upper aqueous layer containing RNA and transfer to a fresh tube Added 200µl of chloroform.
- Centrifuged at 12000 rpm for 5 minutes at 4^{0}C.
- Separated aqueous phase leaving the last few micro tubes to avoid pipetting of DNA precipitate
- Added 500µl of isopropanol and mix gently by inverting
- Allow to stand at room temperature for 10 minutes.
- Centrifuged at 12000 rpm for 10 minutes
- Discarded the supernatant by gently tilting the tubes, slowly leaving the RNA pellet at the bottom (do not pipette).

- Place the tubes in tilted position with lids open on a tissue paper
- Gently tap the tubes to remove excess isopropanol
- Pipette out (by keeping tube inverted) excess isopropanol from wall of tube.
- Add 1ml of 70% alcohol.
- Mixed gently by pipetting 1 to 2 times as the pellet, sticking to bottom is found floating.
- Centrifuge the samples at 7500 rpm for 5 minutes at 4^0C.
- Decant 70% ethanol by tilting in tissue paper gently.
- Dry the pellet in orbital shaker incubator for 10 minutes.
- Dissolve the pellet in 25µl of water (sterile).
- To facilitate dissolution, mix by gently pipetting repeatedly.
- Spin for 10-15 minutes and keep in ice.
- After isolation, RNA was quantified using Colibri Microvolume spectrometer.

2.3.3 List of primers for expression studies of miR-29a,b,c

Sl.no	Oligo name	5’----------3’ sequence	length
1	miR-29a	CUAGCACCAUCUGAAAUCGGUU	22
2	miR-29b	UAGCACCAUUUGAAAUCAGUGUU	23
3	miR-29c	UAGCACCAUUUGAAAUCGGUUA	22

2.3.4 List of primers for expression studies of Runx2, Collagen I and SPARC

Sl.no	Oligo name	5’----------3’ sequence	length
1	Collagen I forward	GCAAGAACGGAGATGACGGA	20
	Collagen I reverse	TTGGCACCATCCAAACCACT	20
2	Runx2 forward	ACCAGTCTTACCCCTCTTACCT	22
	Runx2 reverse	AGGTGCTGGGCTCTGAATCTG	21
3	SPARC forward	GAAGTAGTGGCCGAAAACCC	20
	SPARC reverse	TGGGGGTGTTGTTCTCATCC	20
4	GAPDH forward	GGCAAGTTCAACGGCACAGT	20
	GAPDH reverse	GCCAGTAGACTCCACGACAT	20

2.4 cDNA SYNTHESIS FOR miR-29a,b,c

2.4.1 Materials required

Nuclease free water, PCR Master Mix, Primer mix, RNA spike in template (UniSp6), Control primer mix, 5x reaction buffer, Enzyme mix.

2.4.2 Protocol

- Dilute template strand (adjust each of the template RNA samples to a concentration of 5ng/µL using nuclease free water).
- Spin down all reagents.
- Combine reagents.

REAGENT	VOLUME (µL)
5x reaction buffer	2
Nuclease free water	4.5
Enzyme mix	1
Synthetic RNA spike ins, optional replace with H_2O if omitted	0.5
Template total RNA (5ng/µL)	2
Total volume	10

- Mix and spin reagents.
- Incubate and heat inactivate.
- Incubate for 60 minutes at 42^{0}C.
- Heat inactivate the reverse transcriptase for 5 minutes at 95^{0}C.
- Immediately cool at 4^{0}C.
- Store at -20^{0}C for later use.

2.5 REAL-TIME PCR AMPLIFICATION FOR miR-29a,b,c

2.5.1 Materials required

PCR Master Mix, PCR primer set, Diluted cDNA sample

2.5.2 Protocol

- Prepare reagents for real-time PCR.
- Immediately before use, dilute only the amount of cDNA template needed for the planned real time PCR reactions 80x in nuclease free water

- Combine PCR Master Mix, PCR primer set and cDNA according to table.

Reagent	Volume (µL),96/384 – well plate, tubes or strips
PCR Master Mix	5
PCR primer mix	1
Diluted cDNA template	4
Total volume	10

- Mix and spin reagents
- Incubate the reaction mixture under following conditions

  - 95^0C for 10 minutes
  - 95^0C for 10 seconds
  - 60^0C for 1 minute

- Data analysis was performed in Bio-Rad iQ5 system software.

2.6 cDNA SYNTHESIS FOR EXPRESSION OF RUNX2, COLLAGEN I AND SPARC

2.6.1 Materials required

5X prime script buffer, Prime script RT enzyme mix I, Oligo dT primer, Random 6mers, RNase free water.

2.6.2 Protocol

Prepare the reaction mixture on ice according to the following table.

REAGENT	VOLUME (µL)
5X prime script buffer	2
Prime script RT enzyme mix I	0.5
Oligo dT primer	0.5
Random 6 mers	0.5
Total RNA	
RNase free water	
Total volume	10

- Incubate the reaction mixture under the following condition
    - 37^0 for 15 minutes
    - 85^0 for 5 seconds

2.7 REAL-TIME PCR AMPLIFICATION FOR EXPRESSION OF RUNX2, COLLAGEN I AND SPARC

2.7.1 Materials required

PCR grade water, KAPA SYBR FAST qPCR master mix, 10 µM forward primer, 10 µM reverse primer, Template DNA

2.7.2 Protocol

- Mix reagents according to the below table and spin down.

REAGENTS	VOLUME (µL)
PCR grade water	Up to 20 µL
KAPA SYBR FAST qPCR master mix	10
10 µM forward primer	0.4
10 µM reverse primer	0.4
Template DNA	
Total volume	20

- Incubate the reaction mixture under following condition
  - 95^{0}C for 3 minutes
  - 95^{0}C for 1 to 3 seconds
  - 60^{0}C for above 20 seconds
- Data analysis was performed in BioRad iQ5 system software.

2.8 STATISTICAL ANALYSIS

Standard deviation of all quantitative data was calculated based on MS Excel.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 RESULTS

3.1.1 Animal studies

Figure 3.1: Rabbit: Post one month of implantation

All animals (Figure 3.1) were found healthy and no limbing was observed during experimental period. All the rabbits presented satisfactory postoperative results such as wound healing, with no evidence of inflammation or infection at the surgical site. No adverse reaction was observed during this procedure.

3.2 EXPLANTATION AND HISTOLOGICAL ANALYSIS

All implants were found at the implant site. No gross abnormalities were detected at the implant site (Figure 3.2 and 3.3). The titanium pins were intact at the implant site.

Histologically, necrosis and degeneration were absent at implant bone interface. There was no intervening soft tissue between the implant and bone. New bone formation was observed at the interface. New woven bone arising from periosteum and endosoteum of bone was observed which is found filling the interface. Bone tissue was found anchoring the pins all around the implant region. Both in test and control, the new bone formation was observed at the implant-bone interface which indicates osteointegration.

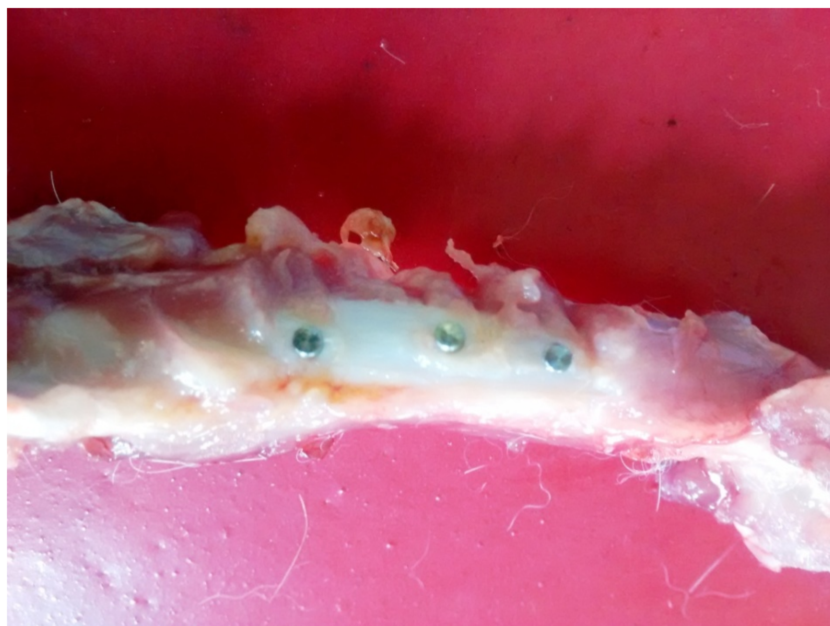

Figure 3.2: Explantation- femur bone with LAM-Cp-Ti (Test)

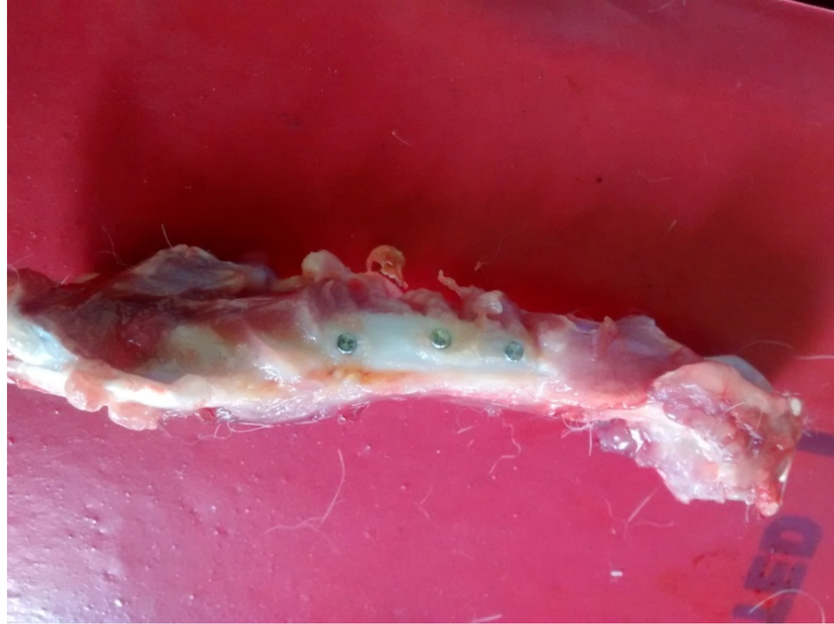

Figure 3.3: Explantation- femur bone with Cp-Ti (control)

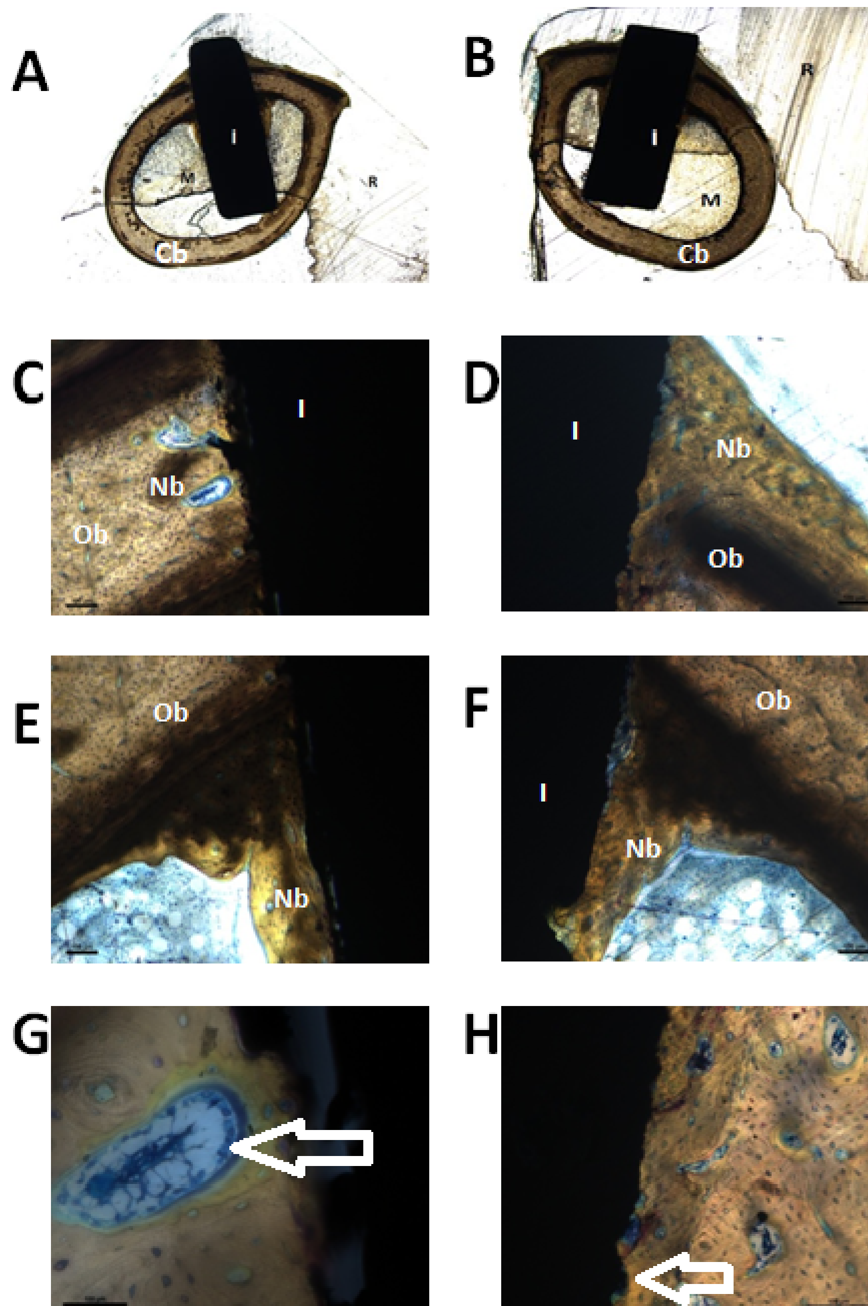

Figure 3.4: Histology images of Cp-Ti implant (A,C,E,G) and LAM-Cp- Ti (B,D,F,H). A, B) Cross section of femur cortical bone (Cb) is seen all around with implant (I) in the implant site and marrow (M) in the centre. C) Implant interface with new bone (Nb) formation and old bone (Ob) in Cp-Ti. D) Implant interface with new bone (Nb) formation and old bone (Ob) in LAM-Cp-Ti. E) New bone (Nb) formation from endosteal surface in Cp-Ti. F) New bone (Nb) formation from endosteal surface in LAM Cp-Ti. G) Interface new bone region with rosette of osteoblast cells activity (arrow) in Cp-Ti. H) New bone formation observed in the grooves (arrow) in LAM Cp-Ti.

3.3 GENE EXPRESSION PATTERN OF RUNX2, COLLAGEN-I AND SPARC IN BONE HEALING

To elucidate the role of osteogenic gene expression in bone healing, real time PCR analysis was conducted. Upregulation of Runx2, Collagen I and SPARC were observed post one month post-implantation in the test group. The graph was prepared after subtracting the normal gene expression in cortical bone (Figure 3.5). In test (LAM-Cp-Ti) group, Runx2 gene was expressed 5 fold higher than the control group (Cp-Ti). In test (LAM-Cp-Ti) group, SPARC gene was expressed 4 fold higher than the control group (Cp-Ti). In test (LAM-Cp-Ti) group, Collagen I gene was expressed 4 fold higher than the control group (Cp-Ti). Difference was observed in the expression of all three genes in both groups. In the present study, the expression of Collagen is highly upregulated in both groups. Recent research (22) suggests that SPARC binds to Collagen and initiate the process of deposition of calcium or mineralization. This study also shows that the expression of Collagen and SPARC are associated. Compared to the expression of Collagen I and SPARC, the expression of Runx2 is decreased. Runx2 is necessary for the differentiation of osteoblast cells.

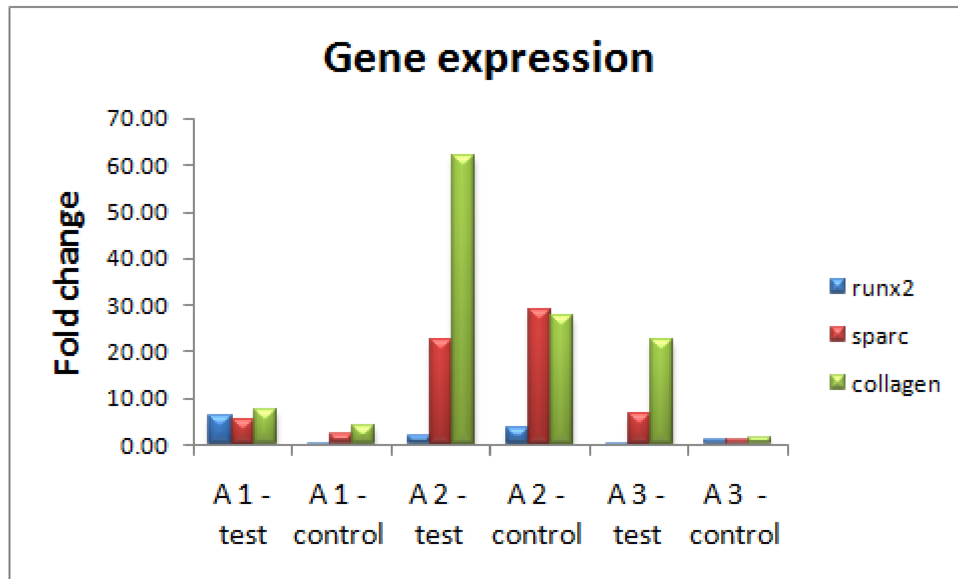

Figure 3.5 Expression of Runx2, SPARC, Collagen I

3.4 EXPRESSION OF miR-29a,b,c

In order to investigate the osteogenic role of miR-29 family, real time PCR analysis was conducted. This family consists of three members expressed together with overlapping functions due to the presence of seed region (Figure 3.6) in the primer. Quantitative PCR analysis confirmed that miR-29a, miR-29b and miR-29c regulated osteogenic genes during bone formation. One month after implantation, miR-29c expression is significantly upregulated. The graph was prepared after subtracting the normal gene expression in cortical bone (Figure 3.7). The expression level of miR-29a,b,c is correlated with the expression of key osteogenic genes namely Runx2, SPARC and Collagen I.

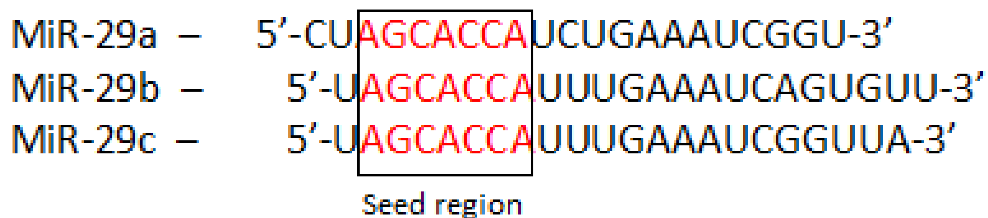

Figure 3.6: Seed region of miR-29 family

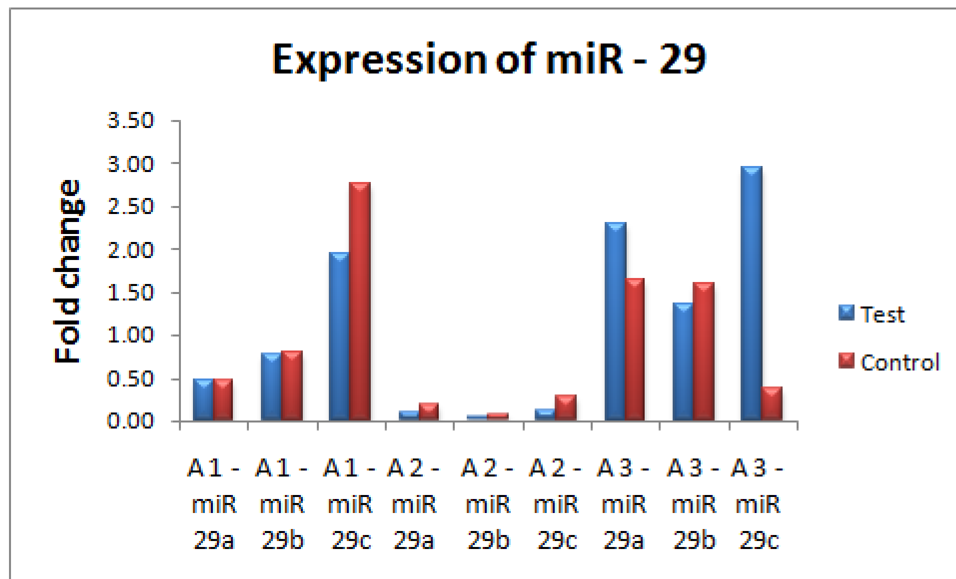

Figure 3.7: Expression of miR 29

Real time PCR analysis indicated an expression pattern for all miR-29 family members and osteogenic genes. miR-29a and b act as a negative regulator of collagen expression and influences collagen deposition and fibrosis (46). Decreased expression of miR-29 family correlated with the up-regulation of Collagen. Correlating the mRNA expression data with microRNA expression profile (Figure: 3.8), Collagen I expression was modestly increased in bone in response to miR-29 inhibition. As the miR-29a,b expression decreases, the expression pattern of Collagen I increases which subsequently influence the expression of SPARC. Simultaneous

expression of

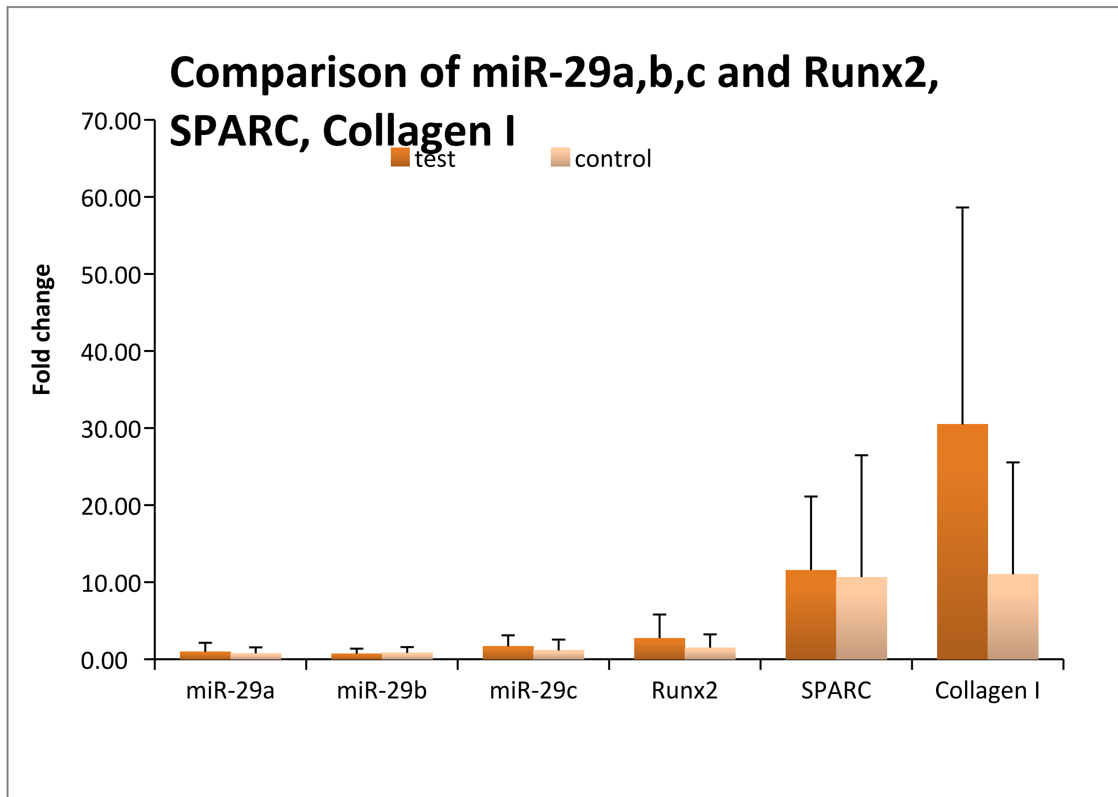

Figure 3.8 Comparison of miR-29a,b,c and Runx2, SPARC, Collagen I

Collagen I and SPARC promotes calcium deposition during mineralisation phase due the binding of Collagen I and SPARC together. The expression of miR 29a,b,c does not affect the level of Runx2. Thus our result shows that the miR-29 family of microRNAs regulate the osteogenic associated genes Runx2, Collagen I and SPARC.

The histological data and gene expression data supported the new bone formation at the implant bone interface.

3.5 DISCUSSION

According to current investigations, histological analysis revealed that bone tissue has increased and accomplished healing. Implant surface properties have a direct role in osteogenesis at the bone-implant interface, influencing a series of coordinated events including protein adsorption, cell proliferation, and bone tissue deposition (47). The advantage of porous materials is their ability to provide biological anchorage for surrounding bone tissues (48) and greater contact area at the implant-bone interface (6). The bone filled porous structures are of great importance due to more osteointegration and tissue growth without any unwanted tissue reactions. The influence of porosity and pore size on the biological behavior of bone has been studied in several studies (49). Changes in pore size rapidly affect bone formation. New bone growth into porous metal implants also depends on the presence of gaps between the implant and bone surface. Data indicate that in LAM-Cp-Ti, more bone in growth has taken place making it more patient friendly. The optimal pore size for bone in growth has been reported to range from 150 to 600µm (50). Bone formation was found on both Cp-Ti and LAM-Cp- Ti.

Bone healing follows a specific and complex signaling pathways and involves changes of the gene expression level (51). It is a highly coordinated process responsible for the repair of damaged bone and maintenance of mineral homeostasis (52). In addition to the traditional bone cells (osteoclasts, osteoblasts, and osteocytes) that are necessary for bone remodeling, genetic mechanisms are also implicated in bone growth. They regulate the signaling molecules and proteins.

The key signaling molecules investigated in our study showed upregulation of Collagen I, SPARC and Runx2. Of these Collagen was highly expressed. Different determinants of bone quality are interrelated, especially the mineral and Collagen(53). Collagen has specific role in mineralization process during new bone formation. In the present study, the expression of collagen indicates the mineralization phase of bone healing. Bone mineralization influences the mechanical strength of bone tissue (54).

SPARC is a bone specific protein which selectively binds to collagen and encoded by SPARC gene. It is secreted by osteoblasts during bone formation. They have affinity for collagen and calcium and also have a vital role in bone mineralization. Its expression is increased in the areas of extracellular matrix and helps in collagen fibril assembly. SPARC binds to ECM and therefore has the potential to contribute to the organization of matrix in connective tissue as well as basement membranes (55). In our study also the expression of SPARC has increased along with Collagen I. Studies suggest that SPARC is a bone tissue-specific protein, linking the bone mineral and collagen phases and initiating active mineralization in normal skeletal tissue (22).

Bone healing is regulated by transcriptional and post transcriptional mechanisms. Of these post transcriptional mechanisms miRNAs play key role in regulation by silencing the mRNA. The expression of osteogenic genes is positively and negatively regulated by miRNAs. From the large group of miRNAs, we studied miR-29 family of microRNAs and its role in regulation of new bone formation in rabbit model. Our studies indicate that miR-29 family directly regulates the target genes for osteogenesis. miR-29 family consists of three members, miR-29a/b/c. They have similar seed region which causes overlap of functions.

The results of this study revealed that the expression of miR-29 family regulate the expression of osteogenesis associated genes studied namely Runx2, Collagen I and SPARC. The miR-29a,b,c are dynamically regulated with respect to osteogenic genes during healing process. miRNAs provide a mechanism for fine tuning of cellular processes in combination with a variety of transcription factors and signaling molecules (36). They recognized as important regulatory factors in organism development and disease expression and regulate the proliferation and differentiation of osteoblasts, osteoclasts influencing metabolism and bone formation (56).

In our studies, the expression of miR-29a was decreased and the level of expression of collagen I has been increased. miR-29a and 29c are upregulated during osteoblast differentiation by reducing the level of Osteonectin expression (44). The miR-29 family together act and regulate functions. They also target other genes for osteoblast function. It is important that miR-29 is not the sole member that regulates gene expression.

The ability of miR-29a,b,c to coordinately regulate mRNAs with role in osteogenesis enhances remodeling by controlling the expression of inhibitors of bone growth. miR-29 appears to regulate multiple gene expression programs (46) during bone formation. miR-29 targets collagen(43) which is essential for bone formation. Collagen I is the primary structural component of the extracellular matrix of skin, tendon, and bone(57). Quantitative defects of collagen expression cause the mild form of osteogenesis imperfecta (OI) or early onset osteoporosis(58). The increased expression of miR-29 family members inhibits collagen I matrix accumulation in systemic sclerosis (59). miR-29 directly targets collagen I, higher levels of miR-29b preferentially negatively regulate collagen genes expressed at mineralization stage

(15). Our study also revealed that miR-29a,b,c directly affect the expression of Collagen I.

Runt-related transcription factor 2 (RUNX2), belonging to Runx transcriptional factors family, is a transcription factor closely associated with the osteoblast phenotype(60). This transcription factor is identified as a master gene required for osteoblastic differentiation process from mesenchymal precursors (61). It connects many signal pathways in bone remodeling by regulating osteoblast differentiation (34). The differentiation of MSCs into the osteogenic lineage and bone formation is done by this essential transcription factor (62). Defects in osteogenic activity occurs in Runx2 knockout mice (63). Runx2 regulate genes related to osteogenesis such as bone sialoprotein (BSP) Decreased Runx2 results in low level of bone formation and changes expression pattern of BSP(64). The transcriptional activity of runx2 is negatively regulated by HDACs. miR-29b directly targets HDACs and regulate osteoblast differentiation (18). Our study showed that miR-29 does not affect Runx2.

miR29a and miR-29c inhibit expression of the extra cellular matrix protein osteonectin(44). Type I Collagen provides the structural framework for mineral deposition (65) and its synthesis is necessary for osteogenesis (66), expression of collagen is supported by SPARC. They bound together to promote mineralization. Our work revealed that miR-29b has no direct effect on SPARC expression. Overall, the expression of miR-29a,b,c affected the process of bone healing by regulating osteogenic genes namely Runx2, Collagen I and SPARC.

CHAPTER 4

SUMMARY AND CONCLUSION

Titanium implant materials are the most attractive material for implantation during bone defects and bone loss disorders. Animal experiments and the histological analysis revealed bone healing and new bone formation at the interface in both LAM-Cp-Ti and Cp-Ti groups. The elevated levels of miR-29a,b,c, has a role in regulating Collagen I and SPARC and subsequently promoting the mineralization in the newly formed bone.

Collagen has specific role in mineralization process during new bone formation. In the present study, the expression of collagen indicates the mineralization phase of bone healing. SPARC binds to collagen and initiate the process of deposition of calcium or mineralization. This study also shows that the expression of collagen and SPARC are associated. According to the results, the expression of Runx2 is independent of miR-29 family expression. Thus the mRNA expression studies showed that the expression of osteogenesis associated genes namely Runx2, Collagen I and SPARC are regulated by miR-29a,b,c family of microRNAs.

In future, other genes like Osteocalcin, Osteopontin etc involved in osteogenesis has to be studied which will pave way to use these miR-29a,b,c family of microRNAs as potential therapeutic agent for treating bone disorders as well as promoting osteointegration with implants.

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