Selective modulation of PPARγ activities in marrow mesenchymal stem cells and their effects on bone

Vipula Petluru

The University of Toledo
A Dissertation

entitled

Selective modulation of PPARγ activities in marrow mesenchymal stem cells and their effects on bone

by

Vipula Petluru

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the Doctor of Philosophy Degree in Biomedical Sciences

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Dr. Beata Lecka-Czernik, Committee Chair

-----------------------------------------------
Dr. Sonia Najjar, Committee Member

-----------------------------------------------
Dr. Edwin R. Sanchez, Committee Member

-----------------------------------------------
Dr. Edith Mensah-Osman, Committee Member

-----------------------------------------------
Dr. Champa Jayasuriya, Committee Member

-----------------------------------------------
Dr. Patricia Komuniecki,
Dean of the College of Graduate Studies

The University of Toledo

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An Abstract of

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Thiazolidinediones (TZDs), a class of oral anti-diabetic agents, sensitize cells to insulin through a specific activation of PPARγ nuclear receptor, however their prolonged use causes bone loss and increases fracture risk. In this study, the effects of three different TZDs (rosiglitazone, pioglitazone, and troglitazone) on osteoblast and adipocyte differentiation, phenotype-specific gene expression, and TGFβ/BMP signaling pathways were evaluated and showed that all tested TZDs simultaneously inhibited osteoblast differentiation and stimulated adipocyte development. They suppressed the expression of osteoblast-specific transcription factors and decreased expression of components of the TGFβ/BMP pathways. In conclusion, TZDs affect an activity of TGFβ/BMP signaling pathways which may account for their inhibitory effect on osteoblast differentiation and bone formation. Microarray analysis done to determine the pathways involved in PPARγ2 mediated pro-adipocytic and anti-osteoblastic activities indicated that among the early responders BMP4 a ligand for the osteoinductive signaling pathway was downregulated
and Tob1, a suppressor of activity of BMP signaling pathway was upregulated. Tob1 upregulation with Rosiglitazone (Rosi) activated PPARγ2 was correlated with the downregulation of members of the TGFβ/BMP pathway, along with downregulation of its target genes. Based on the profile of Tob1 expression and its function as a suppressor of TGFβ/BMP signaling, we hypothesize that Tob1 mediates PPARγ2 anti-osteoblastic activity. To test this hypothesis, we regulated the expression of Tob1 using overexpression vectors or gene silencing techniques. Results indicate that Tob1 doesn’t mimic PPARγ2 anti-osteoblastic activity completely and indeed it inhibits both osteoblast and adipocytic phenotypes. This might suggest that Tob1 may play an important role in maintaining the mesenchymal stem cells (MSCs) and inhibiting its differentiation towards any of the lineages i.e. it protects the “stemness” of MSCs. As there was no single candidate gene which completely mediates the anti-osteoblastic activity of activated PPARγ2, there is a need to identify the PPARγ2 ligands which can selectively activate the beneficial anti-diabetic/pro-adipocytic but not anti-osteoblastic activities. One such selective PPARγ modulator is the Telmisartan (Tel) which is an anti-hypertensive drug inhibiting the activity of angiotensin II receptor. Tel acts as a selective PPARγ agonist and improves insulin resistance and lipid profile in both humans and mice; however no clinical evidence or scientific research has been done to investigate the effects of Tel on bone mass. We hypothesize that Tel does not have any effect on osteoblast phenotype by its own and can protect bone from the negative effects of Rosi by protecting the integrity of TGFβ/BMP pathway. We investigated this, and results indicate that Tel did not affect osteoblast phenotype, however it induced adipocytic phenotype and it protected against the anti-OB effect of Rosi. Tel did not affect the
expression of genes or ALP activity \textit{in vitro} and \textit{in vivo} and even it protected from Rosi’s anti-osteoblastic effect. Unlike Rosi activated $PPAR\gamma 2$ which suppresses the activity of $TGF\beta/BMP$ pathway, Tel did not affect pathway activity and protected from negative effects of Rosi. When administered to hyperglycemic and diabetic Avy/a mice, both drugs had a similar anti-diabetic effect. However, administration of Rosi resulted in 60% decrease in trabecular bone mass, whereas Tel did not have any effect on bone. Moreover, when mice received combination of both drugs, Tel prevented bone loss induced by Rosi. Infact, our lab is the first one to investigate the effects of Tel on bone mass and the mechanisms involved in the protection. Thus, Tel selectively induces $PPAR\gamma 2$ anti-diabetic activity without affecting osteoblastic phenotype in bone MSCs and in mice. Thus, Tel represents a paradigm of multifunctional drug, which can be used alone or in combination with Rosi, for simultaneous treatment of hypertension and diabetes without adverse effects on bone.
Dedication

I dedicate this work to my wonderful family. My dear father, Mr. Sankara Reddy Petluru, my mother, Mrs. Prabhavathi Petluru, for instilling the importance of hard work and higher education, and to my dearest husband Mr. Venkata Phani Kiran Reddy Kolli who have been a source of encouragement and support of my life and who has put up with these many years of research. I must also thank my loving in-laws who have given me their fullest support. Finally, I dedicate this work to the people who taught me first, and from whom I continue to learn each day. I could not have done it without you. This accomplishment is yours as much as it is mine.
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CHAPTER 1

Background Information

Obesity and diabetes are major public health concerns and are widely increasing in sedentary lifestyle all over the world. According to Centers for Disease Control and Prevention and International diabetes Federation, diabetes currently affects 285 million people worldwide and is expected to affect 438 million by 2030. Diabetes is the seventh leading cause of death in the United States, affecting an estimated 25.8 million children and adults. The total annual costs of diabetes in the U.S. is estimated to be $174 billions [1]. The most common form is insulin –independent, or Type 2 Diabetes which is characterized by insulin resistance and glucose intolerance and associated with occurrence of hyperinsulinemia and hyperglycemia.

The Skeleton:

Age-related bone loss otherwise known as Type II osteoporosis occurs both in animals and humans, whereas post-menopausal bone loss or type I osteoporosis, affects individuals regardless of their glucocorticoid status [2] [3]. Bone homeostasis is maintained throughout life by a process called bone remodeling. It is a process where
bone strength and elasticity are maintained by continuously replacing the old and damaged bone with new bone [4]. Two types of cells are involved in this bone remodeling. Osteoblasts, which originate from mesenchymal stem cells (MSCs), are responsible for new bone formation. Osteoclasts, which originate from hematopoietic cells, are responsible for bone resorption (Fig I and IV). Osteoblasts differentiation is initiated by growth factors like bone morphogenetic protein (BMP), wingless-type MMTV integration site family (Wnt) and fibroblast growth factor (FGF). They produce and secrete bone matrix or osteoid, and become embedded in its own matrix and turn into osteocyte; losing its ability to divide but retain some matrix secreting features. Osteoid then turns into hard bone after the deposition of calcium phosphate crystals. Final fate of osteoblasts is either osteocyte or lining cell or undergoes apoptosis. Alkaline phosphatase activity is the cellular marker of osteoblasts. Osteoclast is a member of the
monocyte/macrophage family and a polykaryon which can be generated *in vitro* from mononuclear phagocyte precursors residing in the tissues [5]. Two cytokines are essential for basal osteoclastogenesis, the first being Receptor activator of nuclear factor kappa-B ligand (RANKL) and second being macrophage-colony stimulating factor (M-CSF).

Osteoclasts collect at the site of bone resorption and fuse together to form the multinucleated mature osteoclasts (Fig II & IV). The mature osteoclasts digest mineralized bone using either lysosomal enzymes (collagenase or low pH activated cathepsins, tartrate-resistant acid phosphatase) collagenase or low pH activated cathepsins. After one cycle of resorption osteoclasts undergo apoptosis and cellular marker is tartrate-resistant acid phosphatase (TRAP) staining. Osteocytes constitute about >90-95% of all the bone cells, whereas osteoblasts make up 4-6% and osteoclasts constitute about 1-2%.

**Bone remodeling:**
The three cells which were described above work together to remodel the bone. Bone remodeling is a natural and life-long process whereby old & damaged bone is replaced by new bone. Osteocytes acts as a mechanosensors, during mechanical loading or microfractures, osteocytes detect the changes in the fluid pressure in the tiny channels called canaliculi and release nitric oxide, prostaglandins, glutamates or VEGF and recruits osteoclasts to resorb the damaged part of bone with the help of collagenases. Osteoblasts enter the tunnel behind them and line its walls forming new bone as described in the Fig III. In adults, the entire skeleton is replaced every 7 years with remodeling occurring at ~ 10-14% per year.

**Bone cell development:**

The abrogated and unbalanced bone turnover can result in low bone mass and low bone mineral density that leads to osteopenia and subsequent osteoporosis (bone disease). Thus, an increase in osteoclasts relative to the need for bone resorption, and decrease or shortage of osteoblasts relative to the need for cavity repair, are critical pathogenic factors which can also lead to osteoporosis [2]. Osteoporosis is characterized by decreased bone mass and declined bone architecture which results in increased bone fragility and bone fracture risk. Interestingly, although OCs and OBs arise from different progenitor cells, OC maturation is regulated by pre-OBs. Pre-OBs secrete RANKL, which positively regulates OC maturation and also secretes OPG which acts as a decoy receptor for RANKL and negatively regulates OC maturation. Osteoblasts on the other hand, have the same ancestor as adipocytes. Studies show that early activation of TGFβ
signaling promotes differentiation towards OB whereas the expression & activation of PPARγ2 drives the cells towards adipocytes. PPARγ not only positively regulates adipocytes but also osteocytes (Fig IV).

**Peroxisome proliferator-activated receptor gamma:**

PPARγ belongs to the DNA-binding nuclear hormone receptor family, and has been shown to control bone mass, glucose metabolism and energy.
expenditure [6-9]. In Humans there are about 2 major isoforms of $PPAR_{\gamma}$ generated through alternative splicing & alternative promoter usage. $PPAR_{\gamma}1$, expressed in many tissues like fat, muscle, bone, liver, brain, macrophages and $PPAR_{\gamma}2$ (has 30 additional residues on the N-terminus) and is restricted to preadipocytes and adipocytes.

Structure of $PPAR_{\gamma}$ nuclear receptor includes DNA binding domain (DBD) and ligand binding domain (LBD) whereas cell or tissue or ligand specific activation of genes is regulated by the activation function regions present at the C-terminal of the LBD. The AF-1 domain on the N-terminus of the $PPAR_{\gamma}$ controls the ligand independent activation function of the N terminus. Furthermore, gene expression profiling determines that the AF1 regions of the different PPAR family members are the main determinants of isotype-selective gene expression. Phosphorylation of a mitogen-activated protein kinase site in the A/B region of $PPAR_{\gamma}$ inhibits both ligand-independent and ligand-dependent transactivation functions. The AF-2 domain or the helix-12 domain of the LBD which is molecular switch is considered to be the key player for recruiting the coactivators needed for the transcription of target genes. Under normal conditions, most of the $PPAR_{\gamma}$ is in complex with $RXR$ and co-repressor
complex and is bound to DNA within the nucleus. Gene transcription is silenced. In the presence of a ligand, the $PPAR\gamma$ undergoes a conformational change, releases the co-repressor complex, and recruits the co-activator complex and activates transcription of target genes (Fig V). Along with TZDs which acts as synthetic ligands for $PPAR\gamma$; natural ligands such as prostaglandin PGJ2, linolenic, eicosapentaenoic, docohexanoic, and arachidonic acids also exist [10].

**Insulin sensitization by thiazolidinediones:**

Thiazolidinediones (or glitazones) represent a class of oral anti-diabetic agents which improve insulin sensitivity in diabetic patients [11]. Thiazolidinediones (TZDs) improve insulin sensitivity in muscle, liver, and adipocytic tissues. TZD’s lower blood glucose levels by inhibiting hepatic gluconeogenesis. They reduce insulin levels and decrease the levels of circulating triglycerides and FFAs [12, 13]. Commonly used TZDs are rosiglitazone (Rosi) and pioglitazone (Pio). TZDs acts via Peroxisome Proliferator–activated receptor-$\gamma$ ($PPAR\gamma$), and increases the levels of insulin sensitizing hormones in adipocytes [14, 15] like adiponectin, leptin and inhibit the insulin resistance hormones like resistin. TZDs improve insulin sensitivity in muscle, liver, and adipocytic tissues and inhibit gluconeogenesis from the liver. Lower blood sugar levels by 15% and insulin levels by 20%. TZDs also decrease levels of triglycerides and FFAs. TZDs are considered to be one of the most efficient insulin sensitizers, however prolonged administration of these drugs leads to development of detrimental side effects including
cardiovascular risk factors, edema, fluid retention, and weight gain, which have a clinical significance [12, 16, 17].

**TZD-induced bone loss:**

Apart from these, TZDs also cause severe bone loss both in animals and humans, and increase fracture risk in humans by unbalancing bone remodeling [18]. Previously we observed that older postmenopausal TZD users experienced an annual bone loss rate of 1% when as compared to non-TZD users [14]. TZD’s affect mesenchymal stem cell (MSC) lineage allocation toward osteoblast and decrease bone formation, and enhance osteoclasts development and increase bone resorption [18, 19]. Besides PPARγ2 role in glucose metabolism, it controls osteoblasts and adipocyte

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**Anti-diabetic drugs TZDs, high affinity ligands for PPARγ, affect bone cell differentiation and cause bone loss**

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• TZDs cause bone loss in animals and humans by unbalancing bone remodeling, and increases fracture risk in humans by 2 folds.

• *Increased risk of bone fractures* has been observed in *women* who received *rosiglitazone*.

(Lecka-Czernik, Curr Opin Invest)

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development from common marrow mesenchymal progenitor cells. In a \( PPAR\gamma 2 \) controlled cellular model of marrow MSCs differentiation, U-33/\( \gamma 2 \) cells, Rosi administration results in the formation of adipocytes and simultaneous suppression of osteoblast phenotype [20, 21] (Fig VI). In humans, administration of TZD leads to severe bone loss and is associated with decreased circulating bone formation markers especially in older women [21, 22].

Bone-forming osteoblasts and adipocytes in the marrow cavity originate from the bone marrow mesenchymal stem cells (MSCs). Lineage determination of MSCs depends on the tissue specific signaling factors present in the extracellular milieu. Osteogenic signaling, such as \( TGF\beta/BMP \) and \( Wnt \), induce expression of osteoblast- specific transcription factors \( Runx2, Dlx5, \) and \( Osterix \), and stimulates osteoblast differentiation [19]. Alternatively, activation of the adipocyte- specific transcription factor \( PPAR\gamma 2 \) drives MSCs terminally towards an adipocyte lineage. Hematopoietic precursor cells also reside in the marrow space and give rise to the bone resorbing osteoclasts. Activation of \( PPAR\gamma 2 \) with TZDs drives adipocyte differentiation by upregulating expression of adipocyte markers such as \( aP2/FABP4 \) and by suppressing osteoblast specific-transcription factors \( Runx2, Dlx5, \) and \( Osterix \). Rosiglitazone suppresses the osteoblast phenotype mainly by downregulating the expression of different members of \( TGF\beta/BMP \) and \( Wnt \) Signaling pathway [21] (Fig 8A).

In order to understand the molecular mechanisms underlying the TZD induced bone loss, extensive studies have been done in our laboratory. For this study, we have generated \textit{in vitro} cell-models of MSC differentiation under the control of \( PPAR\gamma 2 \). These cell lines were mainly generated in order to understand whether the Rosi effect is
by \textit{PPARγ2} or \textit{PPARγ1} as Rosi can activate both the \textit{PPARs}. Two cell lines were generated from spontaneously immortalized pre-osteoblastic UAMS-33 cells. These cells were stably transfected with either an empty vector (U-33/c) or a \textit{PPARγ2} expression plasmid under the EF-1α promoter (U-33/γ2). The pro-adipocytic and anti-osteoblastic effects were only seen with activated \textit{PPARγ2} whereas \textit{PPARγ1} only promoted adiponectin expression. In this system, adipogenesis was seen only in the U-33/γ2 (28.6 cells) cell lines under the influence of Rosi. As expected, Rosi induced \textit{PPARγ2} is able to induce and affect the differentiation of pre-osteoblastic (pre-OB) cells to adipocytes, as evidenced by large fat depots stained by Oil Red O (\textbf{Fig 1A}). Interestingly, these cells completely lost their potential to differentiate into osteoblasts. At this stage osteoblastic stimulators, such as ascorbic acid and beta-glycerophosphate, are no longer able to initiate mineralization and bone development [20]. Therefore, it is suggested that Rosi treatment results in adipogenesis and complete suppression of osteoblastogenesis or bone formation. Northern blot analysis of bone specific and fat specific gene markers confirmed these results [20].

Activation of \textit{PPARγ2} in this system resulted in a marked downregulation of \textit{procollagen} and \textit{osteopontin} whereas adipogenic markers increase to aid in the differentiation of MSC to fat cells [20]. The adverse bone effect of long term treatment with \textit{PPARγ2} agonist and anti-diabetic drugs TZDs has been well documented in humans and rodents. The diversion of MSCs to an adipocyte lineage at the expense of an osteoblastic phenotype may partially explain the dramatic loss in bone mass, especially in older diabetic women. Bone remodeling in these individuals is at an imbalance due to inadequate bone formation from the MSCs.
Microarray analysis of $PPAR\gamma 2$-controlled gene expression in the marrow MSCs in the presence of Rosiglitazone:

Rosiglitazone exerts two major physiological changes in the bone microenvironment; increases marrow adiposity and decreases bone mineral density. In order to examine the changes in the gene expression of different genes which are involved in the transition of MSCs to adipocytes, and also to determine all the genes and signaling pathways involved in regulating these cellular modifications, we have conducted gene expression studies using microarrays containing 39,000 murine transcripts (Mouse 430 v. 20, Affymetrix Gene Chip, Affymetrix). The experiments 2x3x2 factorial design corresponds to the usage of 2 cell lines (U-33/c and U-33/$\gamma 2$), 3 time points of Rosiglitazone treatment (2, 24, 72hrs) and 2 independently performed experiments. Each time point represents a separate stage of Rosiglitazone treated U-33/$\gamma 2$ cell conversion from osteoblast phenotype to adipocyte like phenotype and includes the induction at 2hrs, intermediate transitions in phenotype progression at 24hrs and, finally a terminal differentiated adipocytic phenotype at 72hrs where osteoblastic phenotype is completely suppressed. There was a consistent increase in the expression of genes which are involved in lipid and energy metabolism and are induced early, demonstrating the pro-adipocytic activities of $PPAR\gamma 2$ and its ability to control the energy metabolism [21].

Positive regulators of osteoblastic differentiation and development like $Wisp1$, $Dmp1$, and $BMP4$, were downregulated whereas the genes which are known to downregulate or inhibit the osteoblastic development are upregulated, like $Tob1$ and $Tle3$. 
*PPARγ2*, besides regulating the genes involved in fat and bone cell development, it also regulating the expression of genes controlling cell death, cancer, inflammation, immune system, nervous, and cardiovascular system development [21]. Rosiglitazone activated *PPARγ2* suppressed the expression of many genes which are linked to the signaling pathways which are involved in osteoblast differentiation, and in extracellular matrix formation and also in mineralization. Effected genes are different types of collagen, fibronectin, alkaline phosphatase, osteocalcin, and bone sialoprotein. Gene expression of members of *TGFβ/BMP* and *Wnt* signaling pathways, are altered early and are completely suppressed by the end, showing that in cells which are converted into adipocytes these pathway activities are completely abolished. The results, as depicted in Fig 1B, reveal *TGFβ/BMP* signaling proteins, *BMP4, Smad 2/3*, and its suppressor *Tob1* to be among the early responders to Rosi and are the regulators responded at 24 hrs time point. Finally, *IGF-1, FGF*, and PTH signaling pathways were the latest responders.

**Early responders of Rosiglitazone treatment:**

Clinical evidence indicated TZD use leads to bone loss. Microarray data suggests that when *PPARγ2* is activated, it suppresses the expression of osteoblastic-specific genes (Fig 1B). Our lab identified at least three well-established osteoblast-specific signaling pathways affected by *PPARγ2*. The activities and the expression of the components of the *TGFβ/BMP, Wnt* and *IGF-1* pathways were affected by Rosiglitazone activated *PPARγ2*. All three pathways are involved, in regulating MSC differentiation to some extent. *BMP’s* and *Wnt* family of morphogenic members which are extracellular signals are
involved in the induction of the osteoblast phenotype and are essential for the commitment and differentiation of the osteoblast lineage [23]. BMP’s promote osteoblastic differentiation both \textit{in vivo} and \textit{in vitro} and ectopic bone formation \textit{in vivo} [24] [25]. Wnt signaling also plays an important role in osteoblastogenesis, operating through the \textit{GSK3/β-catenin} and non-canonical pathways [26]. Gene expression of the essential osteoblast specific transcription factors, such as \textit{Dlx5}, \textit{Runx2}, and \textit{osterix}, were downregulated only after 24 hrs treatment with Rosi. This implies that the anti- osteoblastic effect of activated \textit{PPARγ2} is mediated through the early responders, and not through the osteoblast specific transcriptional regulators.

Among the early responders in the members of the \textit{TGFβ/BMP} pathway, expression of \textit{BMP4}, involved in bone formation and \textit{Tob1}, suppressor of \textit{TGFβ/BMP} signaling pathway activity were affected. Distinguishing whether \textit{PPARγ2} activated \textit{Tob1} affects the pro-adipocytic activity or the anti-osteoblastic activity of \textit{PPARγ2}, would aid in understanding of \textit{PPARγ2} regulation of bone remodeling and may benefit future innovations in fracture repair. It also helps in understanding whether \textit{Tob1} has any direct effects on the osteoblastic phenotype and/or on the adipogenesis. Identifying whether \textit{Tob1} is the candidate gene involved directly or mediating the TZD induced anti-diabetic properties is important and in this study we examined whether \textit{Tob1} mediates the TZD induced bone loss.

\textbf{The \textit{TGFβ/BMP} signaling pathway regulating bone regeneration:}

The \textit{TGFβ} and \textit{BMP} signaling pathways belong to the transforming growth factor beta (\textit{TGFβ}) superfamily, a large family of growth and differentiation factors that induce
bone formation and fracture repair. Decrease in the expression of these proteins is associated with a variety of bone diseases. TGFβs are the key osteotropic polypeptides involved in both bone formation and bone resorption [27]. These are effective stimulators of bone by inducing proliferation and differentiation of MSCs into osteoblasts [3, 28]. Regulation of osteoblast differentiation by TGF-β is biphasic. TGF-β, a cytokine present in the bone matrix stimulates early osteoblasts development and differentiation by promoting the recruitment and proliferation of the osteoblast precursors and by increasing the matrix proteins. Conversely it inhibits the late osteoblast differentiation and mineralization by inhibiting the expression of phenotype-specific genes, such as osteocalcin and alkaline phosphatase (ALP) [29-31]. TGF-β has been shown to inhibit the adipocyte differentiation by inactivating the C/EBP transcription factors probably by physically interacting with Smad3 protein [32].

In contrast, the osteogenic proteins, BMPs are a group of growth factors and cytokines belonging to the subfamily within TGF-β superfamily and have ability to induce ectopic bone formation [33, 34]. These are important regulators of osteoblast proliferation and differentiation, and genetic disorders in the genes belonging to the BMP signaling pathway are associated with many skeletal disorders such as fibrodysplasia ossificans progressive (Fig 1C), brachydactyly type B [24, 35]. BMP’s are essential for osteoblasts to become mature, which is characterized by the ability to form collagen-based extracellular matrix and mineral deposits induces bone formation. Many clinical studies done in animal’s shows that BMPs are very successful in restoring large segments of bone defects [36]. BMP2/4 cytokines positively regulate expression of osteoblast-specific genes, such as Runx2/Cbfa1 (Runt-related transcription factor 2), Dlx5 (Distal-
less Homeobox 5), A1coll (Alpha1Collagen1) and ALP. BMP’s induces expression of the BMP antagonist noggin, a natural antagonist by negative feedback loop, and leads to suppression of the osteoblast phenotype and lack of mineralization in vitro and in vivo [37] [38]. Cytokines BMP2/4 are shown to stimulate adipocyte differentiation either by committing the precursor cell to adipocyte lineage or increasing adipocyte differentiation [39].

BMPs interact with specific receptors on the cell membranes known as bone morphogenetic protein receptors (BMPRs) and TGFβ interacts with TGFβRs. These are serine/threonine kinase receptors, that is type I and type II receptors, which on ligand binding form a heteromeric complex [40]. Small mothers against decapentaplegic (SMADs) are the signal transducers that modulate the activity of the TGFβ and BMP ligands. When the ligands binds to the receptors, type II receptors phosphorylate the type I receptor which in turn phosphorylates the c-terminal of the receptor regulated R-SMADS (Smad 1, 5, 8 for BMP’s or SMAD 2, 3 in response to TGF-β, nodal, and activins). These phosphorylated R-SMADs associates with Co-SMADS (SMAD-4), and form a complex and translocates into the nucleus, where together with other transcription factors binds to the transcription promoters and initiates transcription of various osteoblastic genes like RUNX2 and Dlx5 [41, 42] (Fig 2A). Signaling through Smad 1 and Smad 5 are very important for BMP-induced osteoblast differentiation and overexpression of these Smad proteins in the MSCs are enough to drive the differentiation of cells towards the osteoblast lineage [43].

The Smad Signaling pathway also increases the expression of two inhibitory Smads (I-Smads), Smad6, and Smad7, which negatively regulate the TGFβ/BMP
signaling pathway whose expression is under the positive control of activated R-Smads [44-46]. These I-Smads interact with the active type-1 receptors and prevent the phosphorylation of R-Smad or interact with phosphorylated Smads and prevent the complex formation, they can form also a complex with histone deacetylases and act as transcriptional corepressor in the nucleus or induce the degradation of the receptors by recruiting the Smurfs, the E3 ubiquitin ligases [47] (Fig 2A). Besides, Smad-dependent signaling, BMPs also activate Smad independent signaling pathway, such as p38 mitogen activated protein (MAP) kinase and phosphatidylinositol 3-kinase (PI3K), which also play an important role in BMP induced osteoblast differentiation [48] [49]. Its been shown that there is a crosstalk between BMP signaling and other signaling pathways like Wnt signaling which controls the BMP signaling by inducing the phosphorylation of Smad1 through extracellular signal-regulated kinase (ERK), p38 and JNK and MAP kinases [50, 51]. Canonical Wnt signaling is important for the BMP-2 induced bone formation in vivo and early induction of osteogenic genes like Runx2, Dlx5, and Msx2 in primary cultures [52]. This crosstalk is crucial for the normal skeletal development and for the bone homeostasis. Extensive research work done on BMPs reveals that they can regulate osteogenesis and adipogenesis in vitro [53]. BMP4 was one of the early responders to rosi treatment and thus it is important to investigate whether the anti-osteoblastic activity of rosi is mediated through BMP4 and in this study we investigated that aspect.

**Evidence for TOB1 suppression of TGFβ/BMP signaling pathway:**
Transducer of ERBB2 (Tob1) belongs to the TOB/BTG1 (B-cell translocation gene) family of anti-proliferative proteins. This family is also known as APRO and includes six members; Tob1, Tob2, BTG1, BTG2/PC3/TIS21, ANA/BTG3, and PC3B which have similar amino-terminal region essential for their anti-proliferative property. Tob1 which can inhibit and regulate cell growth [54] [55] is a nuclear protein that interacts with ErbB-2, a receptor tyrosine kinase [56], and overexpression suppresses the cell growth in tissue culture [57]. This growth suppression was abrogated in the presence of activated ErbB2 kinase. This is due to the association of a protein kinase called p90rsk1 which phosphorylates Tob on serine and threonine residues and regulates its function [58] downstream to MAPK. This anti-proliferative activity of Tob1 is regulated by phosphorylation at 3 serine/threonine residues which are modified by Erk MAP kinases.

Tob1 blocks cell proliferation by suppressing Cyclin D1 and thus restricting the cell cycle progression [57, 59] from G1/S transition. Tob1 can also hamper the cell growth suppression by recruiting and associating with Caf1 (CCR4 associated factor 1), a component of the CCR4-NOT large histone deadenylase transcriptional complex belonging to Ribonuclease D family [60]. Through Caf1 Tob1 binds to deadenylase complex and gathers poly (A) binding proteins (PABP) and enhances the deadenylation of the poly (A) tail of mRNA [61], thus promoting the degradation of mRNA’s and controlling the expression of various genes [62] by controlling the RNA stability. Tob1 which is an anti-proliferative protein acts as a tumor suppressor protein. Tob1 can act as a radiosensitizer and its levels are downregulated in lung and breast cancers [63, 64] and higher tumor occurrence rate was observed in Tob null mice. This tumor suppressor and
anti-proliferative abilities of \textit{Tob1} are abolished when it is phosphorylated at the amino terminal, which is seen in most of the cancers [64].

Recent studies done in targeted deletion of \textit{Tob1} in mice demonstrated a high bone mass, indicates that \textit{Tob1} negatively regulates osteogenesis. Overexpression of Tob1 suppresses BMP induced Smad mediated transcriptional activation of osteoblastic genes resulting in decreased osteoblasts [65]. \textit{Tob1} suppresses the osteoblast phenotype by downregulating \textit{BMP/Smad} signaling pathway [65] and lack of \textit{Tob1} in ovariectomised mice, increases the osteoblastic activity [66]. \textit{Tob1} inhibits the \textit{TGF/BMP} pathway by interacting with the \textit{BMP Type II receptor} and inhibiting \textit{R-SMADs} from being phosphorylated, or by binding to the \textit{Co-SMADS} and prohibiting the \textit{R-SMADs} from forming a complex with it, or by enhancing the inhibitory \textit{SMADS} (I- \textit{SMAD 6, 7}) [67, 68] (Fig 4). \textit{Tob1} can also inhibit the \textit{BMP} pathway by strongly interacting with the \textit{Smad6} and by co-localizing along with the complex to the plasma membrane and thereby increasing the interaction between the \textit{Smad6} and \textit{BMP type I receptor} [47]. By inhibiting the \textit{BMP} pathway \textit{Tob1} negatively regulates the bone formation or osteogenesis [65]. Recent studies done on \textit{Tob1}, a multifunctional protein which is ubiquitously expressed, revealed that it plays an essential role in hippocampus dependent learning and memory and increased acquisition of motor skills [69]. Apart from its role in suppressing osteoblast phenotype, it also suppresses the T-cell proliferation and activation by associating with Smad 2 and 4 proteins in the quiescent cells [70].
Selective \( \text{PPAR}_\gamma 2 \) Modulators (SPPARMS):

In marrow MSCs, activation of \( \text{PPAR}_\gamma 2 \) with (TZD) Rosiglitazone induces adipocyte and suppresses osteoblastic differentiation [19, 21]. Previously our lab has shown that the beneficial anti-diabetic and pro-adipocytic (pro-AD) activities of \( \text{PPAR}_\gamma 2 \) can be separated from this unwanted anti-osteoblastic (anti-OB) activity, by using selective \( \text{PPAR}_\gamma \) agonists with different chemical structures [71, 72]. These kinds of molecules are referred to as Selective \( \text{PPAR}_\gamma \) Modulators (SPPARMs). These modulators binds to \( \text{PPAR}_\gamma \) in a very distinct manner to the ligand-binding pocket of the \( \text{PPAR}_\gamma \) receptor, leading to the displacement of repressors and recruitment of different cofactors to the receptor, ultimately causing a selective expression of a subset of genes [73]. One such SPPARM is Telmisartan (Tel). Tel is a unique angiotensin II receptor blocker (ARB) which is a partial \( \text{PPAR}_\gamma \) agonist activates the receptor, only 30% of the maximum level which is achieved by the full agonists like TZD’s [74]. Tel is currently used as a long-duration antihypertensive agent, and in the treatment of Chronic Heart Failure (CHF), and diabetic nephropathy [75] [76] [77].

Tel binds to AT1R and prevents the binding of angiotensin II to the receptor, thus acting like an anti-hypertensive agent (Fig 9). Tel has a high affinity for the AT1R which belongs to G-protein coupled receptor superfamily (GPCRs) [78] with \( K_i \) value of 3.7nM and dissociates very slowly [79] and this high affinity is due to its unique “delta lock” structure where the high lipophilic distal benzimidazole portion of Tel occupies the entire hydrophobic pocket of AT1R [80]. In contrast, the AT1R-selective antagonist Losartan (Los) is less potent, with \( K_i \) value of 23.7nM [81]. Due to this high binding
affinity to AT1R and greater half-life period of Tel, it is considered to be the most potent antihypertensive drug with longer duration of action than Los [82]. Tel cardiovascular protective effects are mediated through $PPAR_\gamma$ where eNOS expression and phosphorylation is increased and bioavailability of NO is enhanced by decreasing the ROS production and thus decreases the vasoconstriction [83-85]. Tel was the first ARB which was approved to treat cardiovascular events in cardiovascular high risk patients mainly due to its high tolerability [86].

Metabolic syndrome is characterized by the insulin resistance, and collection of various cardiovascular risk factors including hypertension, visceral obesity, glucose intolerance, dyslipidemia and pro-inflammatory state in the same individual [87, 88]. Clinical studies reveal that about 15% to 18% of hypertensive patients are glucose intolerant, insulin resistant, and contributing to the cardiovascular risk factors [89, 90]. In the diabetic population, occurrence of hypertension is about 1.5 to 3 times higher than in the non-diabetic individuals (age-matched) [91]. There are evidences supporting the link between Renin Angiotensin Aldosterone system (RAAS) and metabolic syndrome and studies shows a positive correlation between diabetes and hypertension in patients [92]. Angiotensinogen which is a precursor molecule of angiotensin II (Ang II) is primarily expressed in liver, but it is also seen in adipose tissue, and its expression is shown to be increased in obese conditions revealing its role in hypertension and visceral adiposity [93, 94].

Endothelial dysfunction and increase in Angiotensin II is also considered to be a link between insulin resistance and hypertension [89]. Many clinical studies revealed that ARB’s tend to reduce the incidence of new-onset diabetes [89, 95, 96]. Blockade or
decrease in the Ang II which is a vasoconstrictor, is shown to reduce plasma glucose levels and increase the expression of GLUT4 in soleus muscle. A possible mechanism is acting through the Opioid µ-receptors in streptozotocin (STZ)-induced diabetic rats [97-99]. Other mice studies shows that lack of Angiotensin-converting enzyme (ACE) resulted in increase in whole body energy expenditure, fatty acid hydrolysis, beta oxidation and a decrease in visceral adiposity [100].

Tel is the only structurally unique angiotensin II receptor antagonist, which can function as a selective PPARγ partial agonist, activating receptor (even at low concentration as 1 to 5 µM) to 1/3rd of the maximum level achieved by full agonists (Rosiglitazone and Pioglitazone) [74]. The binding affinity of Rosi (112nM) to PPARγ is about four times stronger than binding affinity of Tel (463nM) to PPARγ [101]. Tel is a Selective PPARγ modulator which can activate PPARγ and act as an anti-diabetic agent acting independently with its ability to block the Angiotensin II Type IA Receptor and influence the expression of PPAR target genes involved in carbohydrate and lipid metabolism [74, 102]. By using GST-Pull down assays, it’s been shown that Tel induces a different PPARγ conformation compared to TZD-full PPARγ agonist. Rosi mediated activation of PPARγ is defined as ‘mouse trap’ model of receptor activation, where AF2 helix of the Ligand binding domain (LBD) closes on the ligand-binding site in response to Rosi and establishes a transcriptionally active form of the receptor and recruits important coactivators like SRC-1/NCoA-1, TIF2/GRIP-1/NCoA-2, DRIP205. Whereas, Tel does not bind to the AF-2 helix of the PPARγ LBD and so the activation functional domain is not completely exposed to recruit and interact with the important Co-activators. Moreover, conformational change induced upon Tel is such that there is a less
efficiency to release corepressor NcoR, or slight recruitment of coactivator TIF-2 to the LBD of \(PPAR_\gamma\) in GST-Pull down assays, characterizing Tel as agonists inducing selective cofactor docking and resulting in a different gene expression profile to that of Rosi [103]. These NCoR or (SMRT) or (TRAC-1) recruits histone deacetylases to DNA promoter regions. Due to this property, Tel activate only a subset of the functions induced by Rosi, it decreases the fat mass, but still improves the diabetic conditions.

Tel can exert its action either through AT1R or through \(PPAR_\gamma\). Literature provides vast supporting evidence, that Tel activities via \(PPAR_\gamma\) are mainly anti-diabetic improving insulin resistance, lipid profile and inhibiting atherosclerosis progression by increasing serum adiponectin levels. Tel exerts its insulin sensitizing properties by increasing the expression of \(PPAR_\gamma\) target molecules like aP2, adiponectin, and also upregulates \(GLUT4\) expression and glucose uptake in preadipocytes [104-106]. Tel also enhances the upregulation of phosphorylated insulin receptor, insulin receptor substrate-1 (IRS-1) and Akt in response to insulin, suggesting its insulin sensitizing properties [107]. Tel decreases steatohepatitis progression by suppressing macrophage infiltration into the liver due to its Anti-inflammatory properties [108]. Tel exerts its anti-atherosclerotic effects through \(PPAR_\gamma\), by downregulating the MCP-1, TNF-\(\alpha\) expression, and suppresses the oxidized low-density lipoprotein (LDL) induced macrophage proliferation in the aorta [109]. Tel protects pancreatic islet function even with high fat diet by downregulating the inflammation, decreasing the oxidative stress, ER stress, and apoptosis in pancreas by blocking the RAAS pathway [110]. Tel through \(PPAR_\gamma\) improves glucose and lipid metabolism and also gives protection against diet-induced weight gain and visceral obesity [111, 112]. It’s been shown that Tel decreases the food
intake, and thus abrogates the weight gain in obese diabetic mice mainly by upregulating the α-MSH signaling pathway in hypothalamus, and acting through the melanocortin pathway. Through $PPAR_\gamma$ dependent pathways, Tel increases caloric expenditure and fatty acid oxidation in skeletal muscles mainly by increasing the expression of mitochondrial encoded genes that are important for mitochondrial energy metabolism [113]. There is increasing substantial evidence that $PPAR_\gamma$ agonists also exert anti-inflammatory, anti-oxidative and anti-proliferative effects on vasculature thus decreasing the atherosclerosis [114].

Many clinical studies and trials done in patients support the fact that Tel indeed, has some insulin sensitizing effects in patients taking Tel daily [115-119] which is absent in patients taking Losartan (Los) or Valsartan (Val) suggesting the possible mechanism through the $PPAR_\gamma$ [120] [102] [121]. Tel also improves the insulin sensitivity and lowers the fasting plasma insulin levels and HOMA-IR in non-diabetic hypertensive patients [122]. The usual antihypertensive dose of Tel can improve insulin resistance in diabetic patients, by increasing adiponectin levels [123]. A 12- month Double-blind placebo controlled trial provided evidence that Tel treatment to the patients with hypertension and Type 2 Diabetes lowered their plasma total cholesterol (TC), LDL-C, triglycerides (TG) and highly sensitive (hs-CRP) when compared to patients who were treated with Eprosartan (Epr) or placebo [91]. Clinical evidence also shows that Tel was well tolerated by patients who were hypertensive and had metabolic syndrome. There has been no sign of edema or weight gain in the patients who were taking Tel, which were seen with the patients who were administered with TZD’s [118, 124-126].
Many studies were also done to investigate the role of RAAS on Bone mineral density (BMD) and in bone remodeling. With aging the prevalence and incidence of hypertension and osteoporosis increases and in about 50% of the women population, hypertension was also associated with an increase in fracture risk in women [127]. One of the studies done revealed that there was an inverse relationship between the blood pressure and bone mass which was measured by DEXA scan. 24hr urine calcium secretion was high in hypertensive female population compared to normotensive ones [128]. This inverse relation between hypertension and bone mineral content was observed not only in females but also in males [129]. There is adequate evidence, that bone expresses Renin, Angiotensin converting enzyme (ACE) and Angiotensin II Receptors [130]. Both osteoblasts and osteoclast express the AT1 and AT2 Receptors and Ang II is shown to activate osteoclasts by inducing the expression of RANKL and IL-6 in osteoblasts thus exacerbating osteoporosis [131]. Ang II also decreases the alkaline phosphatase activity and osteocalcin expression in \textit{in vitro} experiments [132, 133]. It’s been shown that anti-hypertensive treatment either by using β-blockers or ACE inhibitors or ARBs decreases fracture risk and improves the BMD [134, 135]. These evidences suggest that Ang II is involved in bone metabolism. Other studies done on AT2R indicated that Blockade of this receptor with antagonist (PD123319) increased the bone mass, mainly by increasing the bone formation and decreasing the bone resorption [130]. Reports show that Los did not have any significant affects on bone mass [130], but till now there are no studies have been done to investigate the effects of Tel on bone phenotype. Therefore it is very important to investigate the effects of Tel (AT1R blocker)
on bone mass, as Tel is demonstrated to be called as a “Metabosartan”, with dual properties to treat both metabolic and cardiovascular diseases.
Chapter 2: Thiazolidinediones, the Anti-Diabetic $PPAR_\gamma$ Agonists, Suppress Osteoblast Differentiation and the Activity of $TGF\beta/BMP$ Signaling Pathways

Introduction

Osteoporosis, both age-related (type II) and post-menopausal (type I) occurs commonly in animals and humans. Bone homeostasis is maintained throughout life by a process called bone remodeling. It is a process where old and damaged bone is continuously replaced with new bone. With aging there is a decrease in rate of bone formation and osteoblast number and is inversely correlated with an increase in the fat content and adipocytes number in bone marrow [19]. This inverse relationship between the osteoblast and adipocyte differentiation and their common progenitor origin led to the formulation of hypothesis that these two phenotypes are inseparable [136] [137]. This shared precursor hypothesis suggests that an increase in adipocyte differentiation occurs at an expense of osteoblast differentiation, and vice versa. But in some certain situations, adipocytic and osteoblastic differentiations occur independently, indicating these there might be a separate pool of progenitor cells responding to different stimuli in adult marrow or there might be separate regulatory mechanisms for osteoblast and adipocytic differentiation.
Osteoblasts are the bone forming cells and adipocytes are the fat cells which originate form the same bone marrow MSCs. Lineage determination of MSCs depends on the tissue specific signaling factors present in the extracellular milieu. Osteogenic signaling, such as $TGF\beta$/BMP and $Wnt$, induce expression of osteoblast- specific transcription factors $Runx2$, $Dlx5$, and $Osterix$, and stimulates osteoblast differentiation [19]. Alternatively, activation of the adipocyte- specific transcription factor $PPAR\gamma2$ drives MSCs terminally towards an adipocyte lineage. Hematopoietic precursor cells also reside in the marrow space and give rise to the bone resorbing osteoclasts. $PPAR\gamma2$ is a nuclear receptor and is essential for the lipid and glucose metabolism [11]. Natural ligands for $PPAR\gamma$ include polyunsaturated fatty acids and metabolites of prostaglandins $J_2$, and synthetic ligands include the anti-diabetic thiazolidinedoines (TZDs). Through $PPAR\gamma$ TZDs drives adipocyte differentiation by upregulating expression of adipocyte markers such as $aP2/FABP4$ and by suppressing osteoblast specific-transcription factors $Runx2$, $Dlx5$, and $Osterix$.

To understand the molecular mechanisms underlying the TZD induced bone loss, extensive studies done in our laboratory reveal that $PPAR\gamma2$ downregulates the expression of $TGF\beta$/BMP signaling genes thereby antagonizing this well known system of bone formation. For this U33/γ2 cells were used, which is an in vitro cell-model of MSC differentiation under the control of $PPAR\gamma2$. These cell lines were mainly generated in order to understand whether the Rosi effect mediated through $PPAR\gamma2$ or $PPAR\gamma1$ as Rosi can activate both the $PPAR$s. Two cell lines were generated from spontaneously immortalized pre-osteoblastic UAMS-33 cells. These cells were stably transfected with either an empty vector (U-33/c) or a $PPAR\gamma2$ expression plasmid under the EF-1α.
promoter (U-33/γ2). The pro-adipocytic and anti-osteoblastic effects were only seen with activated PPARγ2 whereas PPARγ1 only promoted adiponectin expression [20, 21].

Results suggested that Rosi treatment results in adipogenesis and complete suppression of osteoblastogenesis or bone formation. Activation of PPARγ2 in this system resulted in a marked downregulation of procollagen and osteopontin whereas adipogenic markers increase to aid in the differentiation of MSC to fat cells [20]. The adverse bone effect of long term treatment with PPARγ2 agonist and anti-diabetic drugs TZDs has been well documented in humans and rodents. The diversion of MSCs to an adipocyte lineage at the expense of an osteoblastic phenotype may partially explain the dramatic loss in bone mass, especially in older diabetic women. Bone remodeling in these individuals is at an imbalance due to inadequate bone formation from the MSCs.

Microarray analysis also confirmed that there was a consistent increase in the expression of genes which are involved in lipid and energy metabolism and are induced early, demonstrating the pro-adipocytic activities of PPARγ2 and its ability to control the energy metabolism [21]. Positive regulators of osteoblastic differentiation and development like Wisp1, Dmp1, and BMP4, were downregulated whereas the genes which are known to downregulate or inhibit the osteoblastic development are upregulated, like Tob1 and Tel3. PPARγ2, besides regulating the genes involved in fat and bone cell development, it also regulating the expression of genes controlling cell death, cancer, inflammation, immune system, nervous, and cardiovascular system development [21].

Rosiglitazone activated PPARγ2 suppressed the expression of many genes which are linked to the signaling pathways which are involved in osteoblast differentiation, and
in extracellular matrix formation and also in mineralization. Effected genes are different types of collagen, fibronectin, alkaline phosphatase, osteocalcin, and bone sialoprotein. Gene expression of members of TGFβ/BMP and Wnt signaling pathways, are altered early and are completely suppressed by the end, showing that in cells which are converted into adipocytes these pathway activities are completely abolished. The results reveal that TGFβ/BMP signaling proteins, BMP4, Smad 2/3, and its suppressor Tob1 to be among the early responders to Rosi and are the regulators responded at 24 hrs time point. Finally, IGF-1, FGF, and PTH signaling pathways were the latest responders.

Clinical evidence indicated TZD use leads to bone loss. Microarray data suggests that when PPARγ2 is activated, it suppresses the expression of osteoblastic-specific genes. Our lab identified at least three well-established osteoblast-specific signaling pathways affected by PPARγ2. The activities and the expression of the components of the TGFβ/BMP, Wnt and IGF-1 pathways were affected by Rosiglitazone activated PPARγ2. All three pathways are involved, in regulating MSC differentiation to some extent. BMP’s and Wnt family of morphogenic members which are extracellular signals are involved in the induction of the osteoblast phenotype and are essential for the commitment and differentiation of the osteoblast lineage [23]. BMP’s promote osteoblastic differentiation both in vivo and in vitro and ectopic bone formation in vivo [24] [25]. Wnt signaling also plays an important role in osteoblastogenesis, operating through the GSK3/β-catenin and non-canonical pathways [26]. Gene expression of the essential osteoblast specific transcription factors, such as Dlx5, Runx2, and osterix, were downregulated only after 24 hrs treatment with Rosi. This implies that the anti-
osteoblastic effect of activated \( PPAR_2 \) is mediated through the early responders, and not through the osteoblast specific transcriptional regulators.

Among the early responders in the members of the \( TGF_2/BMP \) pathway, expression of \( BMP_4 \), involved in bone formation and \( Tob1 \), suppressor of \( TGF_2/BMP \) signaling pathway activity were affected. Distinguishing whether \( PPAR_2 \) activated \( Tob1 \) affects the pro-adipocytic activity or the anti-osteoblastic activity of \( PPAR_2 \), would aid in understanding of \( PPAR_2 \) regulation of bone remodeling and may benefit future innovations in fracture repair. It also helps in understanding whether \( Tob1 \) has any direct effects on the osteoblastic phenotype and/or on the adipogenesis. Identifying whether \( Tob1 \) is the candidate gene involved directly or mediating the TZD induced anti-diabetic properties is important and in this study we examined whether \( Tob1 \) mediates the TZD induced bone loss.

The \( TGF_2 \) and \( BMP \) signaling pathways belong to the transforming growth factor beta (\( TGF_2 \)) superfamily, a large family of growth and differentiation factors that induce bone formation and fracture repair. Decrease in the expression of these proteins is associated with a variety of bone diseases. \( TGF_2 \)s are the key osteotropic polypeptides involved in both bone formation and bone resorption [27]. These are effective stimulators of bone by inducing proliferation and differentiation of mesenchymal stem cells (MSCs) into osteoblasts [3, 28]. Regulation of osteoblast differentiation by \( TGF-\beta \) is biphasic. \( TGF-\beta \), a cytokine present in the bone matrix stimulates early osteoblasts development and differentiation by promoting the recruitment and proliferation of the osteoblast precursors and by increasing the matrix proteins. Conversely it inhibits the late osteoblast
differentiation and mineralization by inhibiting the expression of phenotype-specific genes, such as osteocalcin and alkaline phosphatase (ALP) [29-31].

In contrast, the osteogenic proteins, BMPs are a group of growth factors and cytokines belonging to the subfamily within TGF-β superfamily and have ability to induce ectopic bone formation [33, 34]. These are important regulators of osteoblast proliferation and differentiation. BMP’s are essential for osteoblasts to become mature, which is characterized by the ability to form collagen-based extracellular matrix and mineral deposits induces bone formation. Many clinical studies done in animal’s shows that BMPs are very successful in restoring large segments of bone defects [36]. BMP2/4 cytokines positively regulate expression of osteoblast-specific genes, such as Runx2/Chfa1 (Runt-related transcription factor 2), Dlx5 (Distal-less Homeobox 5), Al1coll (Alpha1Collagen1) and ALP. BMP’s induces expression of the BMP antagonist noggin, a natural antagonist by negative feedback loop, and leads to suppression of the osteoblast phenotype and lack of mineralization in vitro and in vivo [37] [38]. Cytokines BMP2/4 are shown to stimulate adipocyte differentiation either by committing the precursor cell to adipocyte lineage or increasing adipocyte differentiation [39].

BMPs interact with specific receptors on the cell membranes known as Bone morphogenetic protein receptors (BMPRs) and TGFβ interacts with TGFβRs. These are serine/threonine kinase receptors, that is type I and type II receptors, which on ligand binding form a heteromeric complex [40]. Small mothers against decapentaplegic (SMADs) are the signal transducers that modulate the activity of the TGFβ and BMP ligands. When the ligands binds to the receptors, type II receptors phosphorylates the type I receptor which in turn phosphorylates the c-terminal of the receptor regulated R-
SMADS (Smad 1, 5, 8 for BMP’s or SMAD 2, 3 in response to TGF-β, nodal, and activins). These phosphorylated R-SMADs associates with Co-SMADS (SMAD-4), and form a complex and translocates into the nucleus, where together with other transcription factors binds to the transcription promoters and initiates transcription of various osteoblastic genes like RUNX2 and Dlx5 [41, 42]. Signaling through Smad 1 and Smad 5 are very important for BMP-induced osteoblast differentiation and overexpression of these Smad proteins in the MSCs are enough to drive the differentiation of cells towards the osteoblast lineage [43].

The Smad Signaling pathway also increases the expression of two inhibitory Smads (I-Smads), Smad6, and Smad7, which negatively regulates the TGFβ/BMP signaling pathway whose expression is under the positive control of activated R-Smads [44-46]. These I-Smads interact with the active type-1 receptors and prevent the phosphorylation of R-Smad or interact with phosphorylated Smads and prevent the complex formation, they can form also a complex with histone deacetylases and act as transcriptional corepressor in the nucleus or induce the degradation of the receptors by recruiting the Smurfs, the E3 ubiquitin ligases [47]. Besides, Smad-dependent signaling, BMPs also activate Smad independent signaling pathway, such as p38 mitogen activated protein (MAP) kinase and Phosphatidylinositol 3-kinase (PI3K), which also play an important role in BMP induced osteoblast differentiation [48] [49]. Its been shown that there is a crosstalk between BMP signaling and other signaling pathways like Wnt signaling which controls the BMP signaling by inducing the phosphorylation of Smad1 through extracellular signal-regulated kinase (ERK), p38 and JNK and MAP kinases [50, 51]. Canonical Wnt signaling is important for the BMP-2 induced bone formation in vivo
and early induction of osteogenic genes like Runx2, Dlx5, and Msx2 in primary cultures [52]. This crosstalk is crucial for the normal skeletal development and for the bone homeostasis. Extensive research work done on BMPs reveals that they can regulate osteogenesis and adipogenesis *in vitro* [53]. *BMP4* was one of the early responders to Rosiglitazone treatment and thus it is important to investigate whether the anti-osteoblastic activity of Rosiglitazone is mediated through *BMP4* and in this study we investigated that aspect.

Transducer of *ERBB2* (*Tob1*) belongs to the *TOB/BTG1* (B-cell translocation gene) family of anti-proliferative proteins. This family also known as APRO includes six members; Tob1, Tob2, BTG1, BTG2/PC3/TIS21, ANA/BTG3, and PC3B which have similar amino-terminal region essential for their anti-proliferative property an. *Tob1* which can inhibit and regulate cell growth [54] [55] is a nuclear protein that interacts with *ErbB*-2, a receptor tyrosine kinase [56], and overexpression suppresses the cell growth in tissue culture [57]. This growth suppression was abrogated in the presence of activated ErbB2 kinase. This is due to the association of a protein kinase called p90rsk1 which phosphorylates *Tob* on serine and threonine residues and regulates its function [58] downstream to MAPK. This anti-proliferative activity of *Tob1* is regulated by phosphorylation at 3 serine/threonine residues which are modified by Erk MAP kinases.

*Tob1* blocks cell proliferation by suppressing Cyclin D1 and thus restricting the cell cycle progression [57, 59] from G1/S transition. *Tob1* can also hamper the cell growth suppression by recruiting and associating with *Caf1* (CCR4 associated factor 1), a component of the CCR4-NOT large histone deadenylase transcriptional complex belonging to Ribonuclease D family [60]. Through Caf1 Tob1 binds to deadenylase
complex and gathers poly (A) binding proteins (PABP) and enhances the deadenylation of the poly (A) tail of mRNA [61], thus promoting the degradation of mRNA’s and controlling the expression of various genes [62] by controlling the RNA stability. *Tob1* which is an anti-proliferative protein acts as a tumor suppressor protein. *Tob1* can act as a radiosensitizer and its levels are downregulated in lung and breast cancers [63, 64] and higher tumor occurrence rate was observed in *Tob* null mice. This tumor suppressor and anti-proliferative abilities of *Tob1* are abolished when it is phosphorylated at the amino terminal, which is seen in most of the cancers [64].

Recent studies done in targeted deletion of *Tob1* in mice demonstrated a high bone mass, indicates that *Tob1* negatively regulates osteogenesis. Overexpression of *Tob1* suppresses BMP induced Smad mediated transcriptional activation of osteoblastic genes resulting in decreased osteoblasts [65]. *Tob1* suppresses the osteoblast phenotype by downregulating *BMP/Smad* signaling pathway [65] and lack of *Tob1* in ovariectomised mice, increases the osteoblastic activity [66]. *Tob1* inhibits the *TGF/BMP* pathway by interacting with the *BMP Type II receptor* and inhibiting *R-SMADs* from being phosphorylated, or by binding to the *Co-SMADS* and prohibiting the *R-SMADs* from forming a complex with it, or by enhancing the inhibitory *SMADS (I-SMAD 6, 7)* [67, 68]. By inhibiting the *BMP* pathway *Tob1* negatively regulates the bone formation or osteogenesis [65]. Apart from its role in suppressing osteoblast phenotype, it also suppresses the T-cell proliferation and activation by associating with Smad 2 and 4 proteins in the quiescent cells [70]. As *Tob1* was one of the early responders to Rosi treatment and its expression was increased by approximately two folds after 2hrs of Rosi
post treatment, it is crucial to investigate whether Tob1 can completely mediate the anti-osteoblastic activity caused by Rosi, and in this study we investigate this.

**Study Objective:**

The observation that Rosiglitazone an insulin sensitizer causes severe bone loss increases bone fractures, and microarray data revealing the suppression of TGFβ/BMP pathway associated genes, and BMP4 and Tob1 (suppressor of BMP pathway) to be the early responders to Rosiglitazone treatment provided the momentum to investigate whether these early responders can completely mimic the anti-osteoblasticity caused by Rosiglitazone activated PPARγ2. To this date, we began by fully characterizing the molecular signaling pathways affected in TZD induced bone loss which are crucial for osteogenesis like TGFβ/BMP pathway. To further elucidate the role of these early responders in mediating the anti-osteoblastic activities of PPARγ2, we will investigate the effects of Tob1 and BMP4 on osteoblast- and adipocytic-specific phenotypes by regulating their gene expression using overexpression and gene silencing techniques. Other aim of this study is to evaluate whether, available glitazones of different anti-hyperglycemic potencies differ in their anti-osteoblastic and pro-adipocytic activities, and to determine their effect on pro-osteoblastic TGFβ/BMP signaling pathways. Understanding the mechanism underlying PPARγ2 mediated bone loss (*via Tob1 or BMP4*) allows in successfully eliminating the negative effects of the PPARγ2 in bone while retaining the insulin sensitizing functions of PPARγ2 on diabetic patients and design better anti-diabetic drugs.
Results

In this section we present evidence that Rosiglitazone treatment inhibits bone formation by strongly suppressing $TGF/BMP$ signaling pathway both on RNA and protein levels. The action of Rosiglitazone is mediated through the activation of a critical adipocyte specific transcription factor $PPAR\gamma$. As described in the introduction (microarray analysis data), Rosiglitazone inhibited bone formation by strongly downregulating the expression of $TGF\beta/BMP$ signaling genes (Fig 1B & 8B) thereby antagonizing this well known system of bone formation. In this study we confirmed these results of effects of $PPAR\gamma$ on the expression of different members of $TGF\beta/BMP$ pathway in our well known system of \textit{in vitro} model of cell differentiation. Among the early responders in the members of the $TGF\beta/BMP$ pathway, expression of BMP4, involved in bone formation and Tob1, suppressor of $TGF\beta/BMP$ signaling pathway activity were affected significantly. Distinguishing whether $PPAR\gamma_2$ activated Tob1 affects the pro-adipocytic activity or the anti-osteoblastic activity of $PPAR\gamma_2$ would aid in understanding of $PPAR\gamma_2$ regulation of bone remodeling and may benefit future innovations in fracture repair.

\textit{Glitazones through $PPAR\gamma_2$ suppresses $TGF\beta/BMP$ signaling genes:}

As explained from the Microarray data and as shown in Fig 1B, Rosiglitazone decreased the expression of $TGF\beta/BMP$ signaling pathway and completely suppressed the integrity of the pathway, we investigated effects of different TZDs on this pathway
both *in vitro* and *ex vivo*. For this we treated the U33/γ2 cells with either Rosiglitazone (1µM) or Pioglitazone (6µM) or Troglitazone (10µM) as described in methods section and their effects on Alkaline phosphatase activity (ALP) and fat accumulation was measured and results indicate that all the three TZDs had a similar effects in suppressing the ALP activity and in increasing the adipogenesis which is measured as stained oil red O cells Fig 2B. The effects of tested glitazones on adipocytic specific and osteoblastic specific gene expression in primary bone marrow cells were similar to that observed in U33/γ2 cells, indicating that not only *in vitro*, that *in vivo* also the anti-osteoblastic activity of TZDs are mediated through PPARγ2 Fig 2C. From the results it’s very clear that all the three TZDs have a similar effect on the gene expression increasing the adipocytic genes and decreasing the osteoblastic genes in the order of Rosiglitazone > Pioglitazone > Troglitazone corresponding to their binding affinity towards PPARγ2 Fig 2A. Tested glitazones had similar suppressive effects on TGFβ/BMP signaling members. Rosiglitazone inhibited TGFβ/BMP signaling genes in a similar fashion both in primary bone marrow cells and in U33/γ2 cells represented as yellow shaded lines in Fig 2D, which is consistent to what we observed in Microarray analysis data.

Rosiglitazone not only hampered the gene suppression but only inhibited the protein expression of the members of the pathway. To investigate this functional assay of TGFβ1 or BMP4 was performed as described in methods section. Results shown in Fig 3B clearly demonstrates that Rosiglitazone decreased the protein expression of Smad1 and treatment with BMP4 also did not rescue the protein, whereas BMP4 treatment alone increased Smad 1 expression (Fig 3B.1). Similar results were observed in case of TGFβ1 functional assay, where Smad 3 protein expression was detected, data revealed that
Rosiglitazone decreased Smad3 protein levels even in the presence of TGFβ1. But in case of gene expression, TGFβ1 could rescue the PAI1 and Smad7 expressions which are direct target genes of TGFβ signaling whereas in case of BMP4 gene expression of Dlx5 was not rescued from Rosiglitazone’s effect (Fig 3B. 2). Above results indicate that Glitazones had a similar affect on the expression of TGFβ/BMP signaling components. All of them specifically decreased expression of TGFβ2, TGFβ3, TGFβRII, Smad3, Smad4 and Smad7, as well as BMP2, BMP4, Smad1 and Smad6. Glitazones significantly decreased activities of TGFβ and BMP4 cytokines suggesting that an inhibitory effect of PPARγ2 on osteoblast phenotype is, at least in part, due to suppressive effects on TGFβ/BMP signaling pathways.

**Tob1 inhibited both osteoblastic and adipocytic phenotype:**

Microarray data analysis reveals that Tob1 is an early responder to the Rosiglitazone treatment, where its expression was increased by 2fold. We also show that Rosiglitazone treatment not only increases the gene expression but also increases its protein levels by more than 2fold (Fig 7B). In order to examine the role of Tob1 in PPARγ2 mediating pro-adipocytic and anti-osteoblastic activities, Tob1 mRNA was overexpressed using TOB1 expression plasmid under the CMV promoter in cells and alterations in gene expression of OB and AD-specific markers were analyzed. Overexpression of Tob1 by 5.6 folds decreased the gene expression of both AD and OB markers and members of TGF-β/BMP signaling pathway. Osteoblastic specific genes like Runx2, Dlx5, Osteocalcin, and A1Col1 decreased by 50%, 70%, 50%, and 40%
respectively and adipocytic specific genes like \(aP2\) decreased by 50%. \(\text{Tob1}\) overexpression resulted in downregulation of genes involved in \(TGF-\beta/BMP\) pathway, like \(TGF\beta3, TGF\beta R, BMP4, Smad1, Smad2\) were downregulated by 0.3, 0.6, 0.4, 0.4, and 0.4 folds respectively (Fig 5A). Anti-proliferative properties of \(\text{Tob1}\) were tested, and results showed that \(\text{Tob1}\) overexpression downregulated the expression of Cyclin D1 which was one of the important regulators of the Cyclin kinases playing a role in transition of G1/S phase in cell cycle. Similar results were observed when MTS proliferation assay was done in a stable cell line overexpressing \(\text{Tob1}\) (using the same \(\text{Tob1}\) expression vector and U33/γ2 cells) (Fig 7A). In order to identify the direct effects of \(\text{Tob1}\) in Rosi activated \(PPAR\gamma2\) induced pro-adipocytic anti-osteoblastic activities, we silenced cellular \(\text{Tob1}\) using specific siRNA and then analyzed alterations in gene expression of OB and AD specific gene markers. Downregulation of \(\text{Tob1}\) transcript by 70%, paralleled with a slightly increased basal transcript levels for adipocytic–specific genes like \(aP2\) and \(Ccrn4l\) both by 1.8 folds and osteoblast-specific genes like \(Dlx5, OC, BMPRIA\) and \(TGF\beta3\) by 1.6, 1.3, 1.2, and 1.4 folds respectively (Fig 5B). Taken together, these results demonstrates that suppressive effect of \(\text{Tob1}\) on OB specific genes is partial as changes in \(\text{Tob1}\) levels did not significantly alter the expression of all the osteoblastic markers, and both OB and AD phenotypes were equally affected with changes in \(\text{Tob1}\) gene expression. Therefore, we came to a conclusion that \(\text{Tob1}\) is not the ‘master’ regulator directly controlled by \(PPAR\gamma2\) and is not mimicking all of its anti-osteoblastic activities in this case. \(\text{Tob1}\) may play a pivotal role in maintaining the “stemness” of the MSC. In otherwords, \(\text{Tob1}\) may be playing an important role in preventing the MSC, in progressing towards any of the lineages. Thus we came to a conclusion that
Tob1 is not the best candidate or not the pivotal candidate that completely mediates the anti-osteoblastic activity caused by activated PPARγ2.

**Cellular silencing of BMP4 using specific siRNAs did not completely downregulate osteoblastic specific gene expression:**

One of the other early responder to Rosi was BMP4 and its expression was decreased by 1.8 folds in 2hrs post treatment with Rosi in the Microarray data analysis. To investigate whether BMP4 can completely mimic the anti-osteoblasticity of the activated PPARγ, we silenced cellular BMP4 using specific siRNA and then analyzed the alterations in gene expression of both OB and AD specific gene markers. Downregulation of BMP4 transcript by 65%, paralleled with a significant decrease in the expression of Runx2, Dlx5, Osteocalcin, and Smad6 by 40%, 45%, 60%, and 30% respectively (Fig 6).

In summary, BMP4 does not completely mediate the anti-osteoblastic activity caused by activated PPARγ2. This is also consistent with what we observed previously in BMP4 functional assay, where BMP4 treatment couldn’t rescue the Dlx5 expression in the presence of Rosiglitazone indicating that BMP4 is not the pivotal player in mediating the anti-osteoblastic activities of PPARγ2. Furthermore, whether BMP4 overexpression can rescue the OB phenotype or can reverse the negative effects caused by Rosiglitazone activated PPARγ2 has to be investigated.

As there was no single candidate gene which completely mediates the Anti-osteoblastic activity of activated PPARγ2, there is a need to identify the PPARγ2 ligands
which can selectively activate the beneficial Anti-diabetic and not the anti-osteoblastic activities.
Discussion

In this study, we have presented evidence that, TZD treatment affects the status of the marrow mesenchymal stem cells with respect to their central differentiation potential. TZD treatment also alters the ability of the mMSC to induce and produce the signaling molecules which are vital for the formation and maintenance of the bone homeostasis. TZDs were the drugs which were developed to treat insulin resistant in type 2 diabetic patients [138]. TZD treatment improves insulin sensitivity resulting in better glycemic control. On the other hand, there were many side effects of clinical importance, such as weight gain, edema and cardiovascular problems [6, 15]. In addition; there is also emerging concerns about the deleterious effects of TZDs on bone [10, 44, 45]. These TZD’s are the synthetic ligands of PPARγ and we have previously showed that with aging both the PPARγ expression and its natural ligands were increased which might be a driving force for pro-adipocytic and anti-osteoblastic changes in the differentiation potential of the mMSC which finally contributing to the age-related bone loss.

With aging the mMSC which are committed to the adipocyte lineage increases where precursors for osteoblast lineage decrease [3, 8]. These results are similar to the ones which were observed with Rosiglitazone treatment where adipogenesis was increased in the marrow with post treatment. There was a significant bone loss due to suppression of osteoblast differentiation and increase in adipocytic progenitors expressing PPARγ [6]. Microarray analysis shows that TGFβ/BMP signaling pathway is negatively regulated and is suppressed by Rosiglitazone treatment; similarly in aging we observe that activity of TGFβ/BMP signaling pathway is decreased [3]. This may lead to
decrease in new osteoblasts and increase in the formation of new adipocytes. This may be due to the changes in expression of TGFβ cytokines and decrease in the receptors of the pathway, indicating the importance of the pathway in development and maintenance of the bone.

Treatment of the cells with different TZDs demonstrated similar effects of anti-osteoblastic and pro-adipocytic activities indicating that all the glitazones simultaneously inhibited the osteoblastic differentiation and stimulated adipocyte development in a rank order, rosiglitazone > pioglitazone > troglitazone, corresponding their binding affinity for PPARγ2. The effects of tested glitazones on osteoblast- and adipocyte-specific gene expression in bone marrow was similar to that in U-33/γ2 cells, suggesting that in vivo their anti-osteoblastic effects are mediated through PPARγ2 transcription factor which we see in C57BL/6J mice administered with Rosiglitazone. Glitazones similarly affected the expression of TGFβ/BMP signaling components both in vitro and ex vivo indicating that TZDs are acting at the initial steps of mMSC commitment to the different lineages. Glitazones significantly decreased activities of TGFβ1 and BMP2/4 cytokines suggesting that an inhibitory effect of PPARγ2 on osteoblast phenotype is, at least in part, due to suppressive effects on TGFβ/BMP signaling pathways.

The well established in vitro cell model which we used in these studies i.e., U-33/γ2 cells serve as a valuable tool for assessing the osteoblastic and adipocytic activities of different PPARγ ligands. In these cells osteoblast and adipocyte differentiation are explicitly under the control of PPARγ2 which is constitutively expressed [71]. Microarray results indicate a sequence of events that led the osteoblastic cells to the adipocytic lineage and early responders to rosiglitazone treatment gives us
some information about the initial triggering events necessary for that transition. We also
saw that adipocytic genes were upregulated at early treatment and continued to increase
throughout the 72hrs. Therefore there was a need to investigate whether these early
responders may play a crucial role in mediating the pro-adipocytic and anti-osteoblastic
activities. Interestingly changes in \( Tob1 \) levels did not significantly alter the expression of
all osteoblastic markers and both osteoblast and adipocyte phenotypes are equally
affected with \( Tob1 \). This may suggest that \( Tob1 \) may have a negative effect on both the
osteoblastic and adipocytic phenotypes. \( Tob1 \) may play a pivotal role in maintaining the
stem-ness of the MSC. In otherwords, \( Tob1 \) may be playing an important role in
preventing the MSC, in progressing towards any of the lineages. This aspect of Tob1 on
stem cells is needed to investigate further. Results may indicate that \( Tob1 \) is not the best
candidate or not the only one that completely mediates the anti-osteoblastic activity
caused by activated \( PPAR\gamma2 \). Similarly changes in \( BMP4 \) gene resulted decrease in
expression of osteoblastic markers but did not completely suppress the expression of
pathway genes indicating that there must some compensatory mechanism through which
the pathway is rescued like mediating through \( BMP2 \) which is structurally very similar to
\( BMP4 \). In microarray analysis we observed complete abrogation of expression of other
members eliminating the possibility of any compensatory mechanisms. This has to be
further investigated.

In summary, TZDs appear to be the full agonists of \( PPAR\gamma \) which completely
suppresses the osteoblast phenotype and induces adipocytic phenotype both \textit{in vivo} and in
bone MSCs. Our results show that TZD’s causes bone loss mainly by inhibiting and
suppressing the integrity of the \( TGF\beta/BMP \) signaling pathway. This interesting finding
provides necessary information that selective $PPAR_\gamma$ modulators can be used to separate necessary anti-diabetic and unwanted anti-osteoblastic activities and which does not have any negative effects on on $TGF\beta/BMP$ pathway or which may protect the integrity of the pathway from TZD’s. This property of selective $in\ vivo$ activity of novel $PPAR_\gamma$ modulators may be of clinical relevance. As, patients with metabolic syndrome, loose more bone than non-diabetic patients. These patients are at very high risk of falls and fractures, [17, 139], and treatment with TZD (Rosi) may increase the risk of bone loss. So to such patients, in order to improve their bone mass, we should use a drug which does not have any adverse effects on bone but still possess the beneficial anti-diabetic effects.
Materials and Methods

Cell Culture, treatment regime, and cell phenotype assays:

Rosiglitazone maleate (BRL 49653), Pioglitazone and Troglitazone were obtained from Tularik, Inc. (South Francisco, CA). Murine marrow-derived U-33 cells stably transfected with a $PPAR_\gamma$2 expression construct, referred to as U-33/$\gamma$2 cells, and cells transfected with an empty vector control, referred as U-33/c cells, as previously described [20] were used. Cell were maintained in αMEM (GIBCO) supplemented with 10% FBS (Hyclone), 0.5 mg/ml of G418, 100 U/ml penicillin, 100µg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO$_2$.

To measure the effect of the ligands on alkaline phosphatase activity which is an early marker of the osteoblast phenotype, cells were plated in 96-well plates, in triplicates and after cultures achieved ~70% confluence (or after 24 hrs), growth medium was supplemented with either the tested ligands at different concentrations or with DMSO (as vehicle control) and cells were treated for 3 days. After treatment, alkaline phosphatase activity was measured using p-nitrophenol as a Substrate (Phosphatase Substrate, Sigma), and data was normalized to the number of cells which was measured with Cell Titer 96 Non-Radioactive Cell Proliferation assay kit (Promega, Madison, WI). One microunit ($\mu$U) of activity was defined as the amount of enzyme capable of hydrolyzing 1 pmol of p-nitrophenol substrate per minute at room temperature.

To measure adipocyte formation, cells were cultured in 48-well plates and treated with tested ligands at different concentrations for 3 days, as described above and after 72
hrs of treatment, cells were fixed with 10% phosphate-buffered formalin, and stained for 30 min with 0.15% Oil Red O (Sigma, St. Louis, MO) in a 55:45 (vol/vol) mixture of isopropanol and water for lipid detection [71]. Cells were rinsed with water and counterstained with 0.5% methyl green (Fisher Scientific, Fair Lawn, NJ) in 0.1M sodium acetate (pH 4.0). Quantification of the adipocyte cells were performed by in each replicate wells, by counting the approximately 100 cells in 5 different randomly selected fields and percentage of cells that had lipid droplets positively stained for Oil Red O was calculated.

Plasmids, expression constructs and siRNA:

Tob1 expression construct was created in pSport6 vector and purchased from Invitrogen. This Tob1 was subcloned into pcDNA3.1 (-) along with 2 linker molecules. Tob1 specific siRNAs and BMP4 specific siRNA’s were purchased from Santa Cruz and consists of a mixture of four different 20-25 nucleotides oligonucleotides. U-33/γ2 cells were seeded in 6 well plates as 100,000 cells per well and after 24hrs cells were transfected with a pool of 75 picomoles (pmoles) of siRNAs/100,000 cells using Lipofectamine 2000 (Invitrogen). Seventy two hours after transfection, total RNA was extracted and used for gene expression analysis by real time PCR, as described previously [140].

RNA isolation and gene expression analysis:
For RNA isolation Primary Bone Marrow cells (PBMC) was isolated by aspiration from the femora of 6 months old male C57BL/6J, as previously described [20] using a University of Toledo-Medical Centre IACUC approved protocol. Marrow was isolated and cultures were established by plating cells at a density \(2 \times 10^5\) cells/cm\(^2\) on 100-mm plates. After 10 days of growth, RNA was isolated using RNeasy (Qiagen, Valencia, CA, USA) and was subjected to DNase I digestion [3]. To measure the effect of the ligands on gene expression, a U-33/\(\gamma\)2 and U-33/c were grown in basal media until cultures obtained 80% confluency and they were treated with tested compounds for 3 days and then RNA was isolated. Total RNA from cell lines were isolated using RNeasy kit (QIAGEN Inc., Valencia, CA). RNA isolation from tissues and intact femur of experimental animals was performed using TRIzol reagent (Invitrogen, Carlsbad, CA) as described previously [6]. 1\(\mu\)g of total RNA was treated with DNase I (Invitrogen, Carlsbad, CA) and made into cDNA using the iScript cDNA synthesis kit (Biorad, Hercules, CA). Gene expression analysis was performed using real-Time PCR (Step One plus, Applied Biosystem, Foster City, CA), as previously described [8]. Relative change in the expression was measured by the comparative \(C_T\) method after each sample was normalized to the quantity of 18S RNA. Real time PCR analysis was performed using the following Primer sets:

- **18S**
  - F: TTCGAACGTCTGCCCTATCAA
  - R: ATGGTAGGCACCGGCAGACTA

- **Runx2**
  - F: GGGCACAAGTTCTATCTGGAAAA
  - R: CGGTGTCACTGCGCTGAA

- **Dlx5**
  - F: TGACAGGAGTTGTGACAGAAGGT
  - R: CCGGAACGGAGCTTGGA

- **A1Col1**
  - F: ACTGTCCCAACCCCAAAAAG
  - R: CGTATTCTTCCGGGCAGAAA
Osteocalcin     : F: CGGCCCTGAGTCTGACAAA
                  : R: GCCGGAGTCTGTTCACTACCTT
Ccnnb1            : F: TGCGGGAACAGGGTGCTA
                  : R: TGCGCCGTTGCTGTTGTC
Phex                : F: GAAAGACATTGGTCCCTCGG
                  : R: TGGCAATGGTTTTTCTTCTTCTC
Dmp1              : F: TGTCATTCTCCTTGTGTTCTTTTG
                  : R: AGAGCTTTTCAGATTGATTTGTTAT
BMP4                : F: TCAAGGGAGTGGAGATTGGG
                  : R: GCCATCATGGGAAAAAGTG
Smad 1            : F: TCCGTCTCTTGGCAAACTATCGA
                  : R: TTTGCCAGTGCTCTCATGCTG
Smad 2            : F: CCCTTCAGTGCGATGCTCA
                  : R: GAATACTACGACCGAGGAGCTGT
Smad 3            : F: CACGCAGAAGTCCACACC
                  : R: GCCAGTAGAATAACGTTAGGG
Smad 4            : F: ACAGAGAACATTGGATGGACGA
                  : R: ACCGGCATAGATCATGAGG
BMPR1A            : F: TGCAATGACACTCCAATCTGCA
                  : R: ACAGAAAGCACCACCTTTATGGACA
BMPR2            : F: TCCACCTTGGGTACATCTCCA
                  : R: CCCCAGTACTCCATTGTG
TGFb1            : F: TACAGCAAGTCTCTTGCCCT
                  : R: GCAGCAGTGGTACGCC
TGFb2            : F: CAACACCATAAATCCCGAAGC
                  : R: GGTCAGTGGTCCAGATCTCT
TGFb3            : F: GCAACTAGCTATCTCAGGTCCCTT
                  : R: CCAGGGAATAATACGAGAGAACC
TGFbR1            : F: AGCAGTGACTGCCATGCC
                  : R: CAGGCTAAACGTCTCAACTGCA
TGFbRII          : F: TCTGGAGACGTTTTGCCAC
                  : R: GCAGCAGTCCAGATGTA
Smad7            : F: GCCGGGCTTCCCTCTGCT
                  : R: CCAGGCAAGGATGGTACC
Smad6            : F: TGGCTGAGATCCTACTCAACA
                  : R: GGAGCGCTGCGGCACAG
Tob1            : F: AATGAAAGATAGTGCCGCTAGCA
                  : R: GGAGGCTGTTACATTTACAGTCA
CyclinD1        : F: CCAGAGGGCGATGAGAAACAA
                  : R: GGCAAGAGGGGCCACAAA
aP2            : F: GCG TGG AAT TCG ATG AAA TCA
                  : R: CCC GCC ATC TAG GGT TAT GA
Adiponectin      : F: GGCCGTCTCTTCCATACCTACG
                  : R: TGGAGGAGCAGAGGACG
Protein isolation and Western Blot analysis

Proteins were isolated from U-33/γ2 cells after cells were washed with PBS and scrapped into Lysis Buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% NP-40, 50 mM NaF, Triton X 100, 50mM Sodium Orthovanadate, PMSF and Protease and Phosphatase inhibitors) and spun at 17000xg for 10min to extract proteins. Protein concentration was measured using BCA Protein Assay kit (Thermo Scientific) and separated on SDS-PAGE. Proteins were probed with 1:200 dilution of anti-Tob1 (Santa Cruz) or 1:200 dilution of anti-Smad3 or 1:200 dilution of anti-Smad1 (Santa Cruz), 1:5000 dilution of anti-b-actin (Sigma), and developed with near-infrared (NIR) fluorescence detection system using LI-COR secondary antibodies at a dilution of 1:10,000.

Immunoprecipitation

U-33/γ2 cells were lysed using buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% NP-40, 50 mM NaF, and protease inhibitors and centrifuged at 7,000xg for 5 min. The supernatant containing the cellular proteins was tested for protein concentration using BCA Protein Assay kit and 200ug of the lysate was incubated with either Tob1 antibody (Santa Cruz) or with Smad 3 antibody (Santa Cruz) bound Protein G magnetic beads (Invitrogen) at 4 degrees for 1 h. The immunoprecipitated complex was then washed four times and the proteins in the complex were eluted using eluting buffer.
(provided by the kit), SDS sample buffer, and heat. The proteins were then run on an SDS-PAGE acrylamide gel for detection of proteins in the complex.

Statistical Analysis:

Statistically significant differences between the groups were detected using one-way ANOVA followed by post-hoc analysis by Dunnetts and Tukey within the SPSS Statistics 17.0 Software (SPSS, Inc., Chicago, IL). All data shown represent means plus standard errors of the means (SEMs) and in all cases, P< 0.05 was considered significant.
Chapter 2: An anti-hypertensive drug Telmisartan selectively activates \( PPARY \) anti-diabetic and pro-adipocytic but not anti-osteoblastic activities \textit{in vitro} and \textit{in vivo}.

**Introduction & Literature Review**

Obesity and diabetes are major public health concerns and are widely increasing in sedentary lifestyle all over the world. Diabetes is the seventh leading cause of death in the United States, affecting an estimated 25.8 million children and adults [1]. The most common form is insulin–independent, or Type 2 Diabetes which is characterized by insulin resistance and glucose intolerance and associated with occurrence of hyperinsulinemia and hyperglycemia.

Age-related bone loss otherwise known as Type II osteoporosis occurs both in animals and humans, whereas post-menopausal bone loss or type I osteoporosis, affects individuals regardless of their glucocorticoid status [2] [3]. Two types of cells are involved in the bone remodeling. Osteoblasts, which originate from mesenchymal stem cells (MSCs), and osteoclasts originate from hematopoietic stem cells. In marrow MSCs, activation of \( PPARY2 \) with (TZD) Rosiglitazone induces adipocyte and suppresses osteoblastic differentiation [19, 21]. Previously our lab has shown that the beneficial anti-diabetic and pro-adipocytic (pro-AD) activities of \( PPARY2 \) can be separated from this unwanted anti-osteoblastic (anti-OB) activity, by using selective \( PPARY \) agonists with
different chemical structures [71, 72]. These kinds of molecules are referred to as Selective $PPAR_\gamma$ Modulators (SPPARMs). These modulators binds to $PPAR_\gamma$ in a very distinct manner to the ligand-binding pocket of the $PPAR_\gamma$ receptor, leading to the displacement of repressors and recruitment of different cofactors to the receptor, ultimately causing a selective expression of a subset of genes [73]. One such SPPARM is Telmisartan (Tel). Tel is a unique angiotensin II receptor blocker (ARB) which is a partial $PPAR_\gamma$ agonist activates the receptor, only 30% of the maximum level which is achieved by the full agonists like TZD’s [74]. Tel is currently used as a long-duration antihypertensive agent, and in the treatment of Chronic Heart Failure (CHF), and diabetic nephropathy [75] [76] [77].

Tel binds to AT1R and prevents the binding of angiotensin II to the receptor, thus acting like an anti-hypertensive agent (Fig 9). Tel has a high affinity for the AT1R which belongs to G-protein coupled receptor superfamily (GPCRs) [78] with $K_i$ value of 3.7nM and dissociates very slowly [79]. In contrast, the AT1R-selective antagonist Losartan (Los) is less potent, with $K_i$ value of 23.7nM [81]. Due to this high binding affinity to AT1R and greater half-life period of Tel, it is considered to be the most potent antihypertensive drug with longer duration of action than Los [82]. Tel was the first ARB which was approved to treat cardiovascular events in cardiovascular high risk patients mainly due to its high tolerability [86].

Metabolic syndrome is characterized by the insulin resistance, and collection of various cardiovascular risk factors including hypertension, visceral obesity, glucose intolerance, dyslipidemia and pro-inflammatory state in the same individual [87, 88]. Clinical studies reveal that about 15% to 18% of hypertensive patients are glucose
intolerant, insulin resistant, and contributing to the cardiovascular risk factors [89, 90]. There are evidences supporting the link between Renin Angiotensin Aldosterone system (RAAS) and metabolic syndrome and studies shows a positive correlation between diabetes and hypertension in patients [92]. Angiotensinogen which is a precursor molecule of angiotensin II (Ang II) is primarily expressed in liver, but it is also seen in adipose tissue, and its expression is shown to be increased in obese conditions revealing its role in hypertension and visceral adiposity [93, 94].

Tel is the only structurally unique angiotensin II receptor antagonist, which can function as a selective $PPAR\gamma$ partial agonist, activating receptor (even at low concentration as 1 to 5 µM) to $1/3^{rd}$ of the maximum level achieved by full agonists (Rosiglitazone and Pioglitazone) [74]. The binding affinity of Rosi (112nM) to $PPAR\gamma$ is about four times stronger than binding affinity of Tel (463nM) to $PPAR\gamma$ [101]. Tel is a Selective $PPAR\gamma$ modulator which can activate $PPAR\gamma$ and act as an anti-diabetic agent acting independently with its ability to block the Angiotensin II Type IA Receptor and influence the expression of $PPAR$ target genes involved in carbohydrate and lipid metabolism [74, 102]. By using GST-Pull down assays, it’s been shown that Tel induces a different $PPAR\gamma$ conformation compared to TZD-full $PPAR\gamma$ agonist. Rosi mediated activation of $PPAR\gamma$ is defined as ‘mouse trap’ model of receptor activation, where AF2 helix of the Ligand binding domain (LBD) closes on the ligand-binding site in response to Rosi and establishes a transcriptionally active form of the receptor and recruits important coactivators like SRC-1/NCoA-1, TIF2/GRIP-1/NCoA-2, DRIP205. Whereas, Tel does not bind to the AF-2 helix of the $PPAR\gamma$ LBD and so the activation functional domain is not completely exposed to recruit and interact with the important Co-
activators. Moreover, conformational change induced upon Tel is such that there is a less efficiency to release corepressor NcoR, or slight recruitment of coactivator TIF-2 to the LBD of \textit{PPAR}_\gamma \textit{in GST-Pull down assays, characterizing Tel as agonists inducing selective cofactor docking and resulting in a different gene expression profile to that of Rosi [103]. These NCoR or (SMRT) or (TRAC-1) recruits histone deacetylases to DNA promoter regions. Due to this property, Tel activate only a subset of the functions induced by Rosi, it decreases the fat mass, but still improves the diabetic conditions.}

Tel can exert its action either through AT1R or through \textit{PPAR}_\gamma. Literature provides vast supporting evidence, that Tel activities \textit{via} \textit{PPAR}_\gamma are mainly anti-diabetic improving insulin resistance, lipid profile and inhibiting atherosclerosis progression by increasing serum adiponectin levels. Tel exerts its insulin sensitizing properties by increasing the expression of \textit{PPAR}_\gamma target molecules like aP2, adiponectin, and also upregulates \textit{GLUT4} expression and glucose uptake in preadipocytes [104-106]. Tel also enhances the upregulation of phosphorylated insulin receptor, insulin receptor substrate-1 (IRS-1) and Akt in response to insulin, suggesting its insulin sensitizing properties [107]. Tel decreases steatohepatitis progression by suppressing macrophage infiltration into the liver due to its Anti-inflammatory properties [108]. Tel exerts its anti-atherosclerotic effects through \textit{PPAR}_\gamma, by downregulating the MCP-1, TNF-\alpha expression, and suppresses the oxidized low-density lipoprotein (LDL) induced macrophage proliferation in the aorta [109]. Tel through \textit{PPAR}_\gamma improves glucose and lipid metabolism and also gives protection against diet-induced weight gain and visceral obesity [111, 112]. It’s been shown that Tel decreases the food intake, and thus abrogates the weight gain in obese diabetic mice mainly by upregulating the \alpha-MSH signaling pathway in
hypothalamus, and acting through the melanocortin pathway. Through $PPAR_\gamma$ dependent pathways, Tel increases caloric expenditure and fatty acid oxidation in skeletal muscles mainly by increasing the expression of mitochondrial encoded genes that are important for mitochondrial energy metabolism [113]. There is increasing substantial evidence that $PPAR_\gamma$ agonists also exert anti-inflammatory, anti-oxidative and anti-proliferative effects on vasculature thus decreasing the atherosclerosis [114].

Many clinical studies and trials done in patients support the fact that Tel indeed, has some insulin sensitizing effects in patients taking Tel daily [115-119] which is absent in patients taking Losartan (Los) or Valsartan (Val) suggesting the possible mechanism through the $PPAR_\gamma$ [120] [102] [121]. Tel also improves the insulin sensitivity and lowers the fasting plasma insulin levels and HOMA-IR in non-diabetic hypertensive patients [122]. The usual antihypertensive dose of Tel can improve insulin resistance in diabetic patients, by increasing adiponectin levels [123]. A 12-month Double-blind placebo controlled trial provided evidence that Tel treatment to the patients with hypertension and Type 2 Diabetes lowered their plasma total cholesterol (TC), LDL-C, triglycerides (TG) and highly sensitive (hs-CRP) when compared to patients who were treated with Eprosartan (Epr) or placebo [91]. Clinical evidence also shows that Tel was well tolerated by patients who were hypertensive and had metabolic syndrome. There has been no sign of edema or weight gain in the patients who were taking Tel, which were seen with the patients who were administered with TZD’s [118, 124-126].

Many studies were also done to investigate the role of RAAS on Bone mineral density (BMD) and in bone remodeling. With aging the prevalence and incidence of hypertension and osteoporosis increases and in about 50% of the women population,
hypertension was also associated with an increase in fracture risk in women [127]. There is adequate evidence, that bone expresses Renin, Angiotensin converting enzyme (ACE) and Angiotensin II Receptors [130]. Both osteoblasts and osteoclast express the AT1 and AT2 Receptors and Ang II is shown to activate osteoclasts by inducing the expression of \textit{RANKL} and \textit{IL-6} in osteoblasts thus exacerbating osteoporosis [131]. It’s been shown that anti-hypertensive treatment either by using ß-blockers or ACE inhibitors or ARBs decreases fracture risk and improves the BMD [134, 135]. These evidences suggest that Ang II is involved in bone metabolism. Other studies done on AT2R indicated that Blockade of this receptor with antagonist (PD123319) increased the bone mass, mainly by increasing the bone formation and decreasing the bone resorption [130]. Reports show that Los did not have any significant affects on bone mass [130], but till now there are no studies have been done to investigate the effects of Tel on bone phenotype. Therefore it is very important to investigate the effects of Tel (AT1R blocker) on bone mass, as Tel is demonstrated to be called as a “Metabosartan”, with dual properties to treat both metabolic and cardiovascular diseases.

\textbf{Study Objective:}

The observation that TZD’s an insulin sensitizer causes severe bone loss, increases bone fractures, and Ang II causes bone loss in hypertensive patients and the blockade of RAAS increases bone mass, and Tel an ARB being a partial and selective \textit{PPARγ} agonist with anti-diabetic properties provided the momentum to investigate the underlying mechanisms and the effects of Tel alone or in combination with TZDs on
bone mass. Main objective of this study is to test whether Tel or combination with Rosiglitazone, with similar anti-hyperglycemic potencies differ in their anti-osteoblastic and pro-adipocytic activities, and to determine their effect on pro-osteoblastic $TGF\beta/BMP$ signaling pathways. To this date, we began by fully characterizing the molecular signaling pathways affected in TZD induced bone loss. To further elucidate the mechanisms underlying the effects of SPPARMs in the diabetic bone phenotype, we will investigate the effects of Tel on osteoblasts, osteoclasts and on adipogenesis in the presence or absence of TZDs.

**Significance:**

TZDs are high affinity ligands for PPAR$\gamma$ and are the only anti-diabetic insulin sensitizing drugs on the market. Recent studies done on different TZDs like Rosiglitazone, Pioglitazone, and troglitazone showed that prolonged use of these drugs cause a detrimental effect on the bone skeleton, especially in the regions of hip, wrist and lower back, especially in diabetic post-menopausal women. With menopause, women undergo a dramatic loss in estrogen levels. Estrogen is anabolic for bone and therefore a decrease in estrogen levels decrease bone formation, leading to osteopenia or low bone mineral density. The evidence for TZD mediated bone loss is well established and it justifies the necessity for a study in the mechanism of prevention of TZD mediated bone loss. The effectiveness of anti-diabetic therapy using TZDs in humans is unparalleled and recent studies point to the beneficial effect of TZDs in improving diabetic conditions [141]. Hence, the study of bone loss preventive mechanisms of TZD therapy is vital.
Since Tel has partial anti-diabetic properties, the studies outlined below will determine whether Tel in combination with TZD-therapy aids in the prevention of TZD mediated bone loss. Identifying the downstream mechanisms of Tel activated $PPAR_\gamma^2$ may aid in eliminating the negative TZD mediated effects of $PPAR_\gamma^2$ in bone while retaining the insulin sensitizing functions of $PPAR_\gamma^2$ in diabetic patients.
**Results**

In this section we present evidence of the effects of Rosi and Tel treatments on bone phenotype. As described in the introduction (Microarray analysis data), Rosi inhibited bone formation by strongly suppressing the TGFβ/BMP signaling pathway and downregulating the expression of different members of this pathway. Distinguishing whether Rosi or Tel activated PPARγ2 affects the proadipocytic activity or the anti-osteoblastic activity of PPARγ2 differently would aid in understanding of PPARγ2 regulation of bone remodeling and may benefit future innovations in fracture repair. In the previous study we saw that there was no single candidate gene which completely mediates the anti-osteoblastic activity of activated PPARγ2, there is a need to identify the PPARγ2 ligands which can selectively activate the beneficial Anti-diabetic and not the anti-osteoblastic activities. One such SPPARMs is the Tel which has the capacity to activate anti-diabetic and proadipocytic (weak) activities, however no clinical evidence or scientific research has been done to investigate the effects of Tel on OB phenotype. Infact, our lab is the first one to investigate the effects of Tel on Bone mass and the mechanisms involved in that activity.

**Effects of Telmisartan on adipocyte and osteoblast differentiation:**

Previously we have shown the effects of PPARγ agonists on the OB and AD phenotypes in a well established cellular model. This model is a bipotential mesenchymal
progenitor cell line (U-33/γ2) stably transfected with a \( PPAR_\gamma 2 \) expression construct or empty vector (U-33/c cells) [20, 71]. For these tested agonists U-33/c cells serve as negative control for \( PPAR_\gamma 2 \)-dependant effects. U-33/γ2 cells are derived from a clonal murine pre-osteoblastic cell line which are bipotential and have capacity to terminally differentiate into both osteoblast and adipocytes based on the osteogenic or adipogenic media. These cells have capacity to mineralize the extracellular matrix in the presence of osteoblastic stimuli (Ascorbic acid and β-glycerophosphate). In contrast, addition of \( PPAR_\gamma \) ligand, such as Rosi, induces fat accumulation and adipocyte differentiation, and simultaneously suppresses the osteoblast phenotype in these cells [6] (Fig 1A).

It is well established and characterized that \( PPAR_\gamma \) activation by TZDs or by other ligands has a negative affect on cell growth i.e. \( PPAR_\gamma \) plays an active role in process of departure of the cell cycle [142]. \( PPAR_\gamma \) ligands were also proven to inhibit growth especially in breast and prostrate cancer cells where they also induce the cell apoptosis [143, 144]. In Fig 10A anti-proliferative properties of Rosi and Tel were compared using the MTS assay. Effects of both compounds on the activity of enzyme that reduce MTS to formazan dyes were measured using a plate reader as described in materials and methods section. Results indicate that both drugs have a common significant inhibitory effect on the proliferation of cells when compared to control. These data along with others shows that Tel induces growth arrest in U-33/γ2 cells and prostate cancer cells [145] and other ARB’s did not had any effect on cell proliferation (Fig 10C). These results were consistent with the gene expression data provided in Fig 18, showing the effects of \( PPAR_\gamma \) ligands on expression of Cyclin D1, which is a cell-cycle marker.

To dissect and to clearly understand the \( PPAR_\gamma \) mediated effects of Tel (as it is a partial
agonist), we treated U-33/γ2 cells with another ARB called Los which is a non PPARγ agonist and also we treated U33/control cells (which doesn’t express PPARγ2) with these drugs and tested their anti-proliferative effects. Results shown in Fig 10 B &C were supporting the idea, that anti-proliferative properties of Tel are mediated through PPARγ2 and not through the RAAS, as inhibition on the proliferation was not seen in U-33/control cells or with use of Los. We also tested whether this decrease in cell number in U-33/γ2 cells is due to effect on cell proliferation or decrease in viable cells and we confirm that this decrease in cell number is not due to increase in cell death (data not shown). In summary we report that Tel has an inhibitory effect on cell growth in vitro experiments, suggesting that the anti-proliferative effect of Tel is not a result of direct toxicity or apoptotic induction but of an direct effect on cell division i.e. Tel did not induce a reduction of cell viability and early apoptosis of U-33/γ2 cells and prostate cells [145].

In order to see the effects and compare the pro-adipocytic activities of Tel and Rosi in U-33/γ2 cells, we performed experiments and looked at the effects on the number of the adipocytes counted by the number of oil-red O positive cells. As expected Rosi treatment showed a significant increase in oil red O positive cells (i.e.) adipocytes with lipid droplets (Fig 11 A &B). Though (Tel was a partial and a selective agonist of PPARγ) there was a positive effect of Tel on adipocyte number, we dint see any drastic increase in adipocyte number with different doses of Tel as compared to Rosi. To support this, we also measured the lipid accumulation in the U-33/γ2 cells measured by the intensity of the fluorescent lipophilic stain known as Nile red (as described in the Materials and methods). Results shown in Fig 11D, clearly shows the intensity of lipid accumulation in these cells with Rosi treatment is much higher than with the Tel
treatment. Then to investigate the pro-adipocytic effect of these drugs, we treated the U-33/γ2 cells for 72hrs and then effects on adipocyte-specific genes like aP2 and adiponectin were analyzed using Real-Time RT-PCR. As expected Rosi increased the expression of aP2 and adiponectin dramatically and Tel alone and combined treatment also had a positive effect on the expression of AD-specific genes (Fig 11C). In the same cells, effects of Tel and Rosi on Brown adipose tissue specific gene markers like UCP1, Dio1, FoxC2, PRDM16 and Adrenergic Receptor Beta 3 (ARB3) were analyzed (Fig 12). For our surprise we found that Tel was increasing the genes involved in thermogenesis UCP1 and Dio1 by 10 folds, whereas we did not observe any significant change with Rosi treatment in vitro, though we saw an increase in UCP1 expression in whole bone RNA samples. But with combinational treatment, we saw 4 to 5 folds increase in expression of the BAT genes (Fig 12). There was an increase in FoxC2, ADBR3 expression with Tel alone, and combinational treatment but there was significant increase in ADBR3 expression with Rosi treatment which was not seen in FoxC2 expression. These data reveals that proadipocytic activity of Tel is comparatively weak and is less extensive when compared with Rosi. Results also indicate that Tel increases the BAT genes especially the genes involved in thermogenesis more extensively than Rosi. Further analysis is needed to confirm these results and see effects on activity of BAT. Also Tel administered animals, showed an improved glucose and fat homeostasis in liver tissues. Gluconeogenesis genes like PEPCK and Glucose-6-Phosphatase (Glc6ase), and Fox01 and FAS expression were increased in Rosi and Tel and combinational treated liver tissues at fasting condition (data not shown).
Anti-osteoblastic properties of Tel and Rosi were compared by using an osteoblast-specific assay called alkaline phosphatase activity (ALP) assay. This ALP activity is an early marker of osteoblastic differentiation [146]. As shown in Fig 13, Rosi effectively inhibited the ALP activity both in the cells and in primary bone marrow cells. In contrast, Tel (12 µM to 50 µM) did not affect ALP activity at all and when treated with Rosi (1 µM), it even reversed the negative effect of Rosi on ALP activity (Fig 13 A & C). We investigated the effects of other ARB, Losartan (a non PPARγ2 agonist and an AT1R blocker) on ALP activity, Results indicated that Losartan alone has no effect on ALP and Losartan did not had any potential to reverse the negative effects of Rosi and rescue the ALP which we saw with Tel treatment, indicating that these effects of Tel are mediated through PPARγ2 and not through RAAS system (Fig 13 B & D). Next, we compared the results of primary ex vivo murine bone marrow cultures to Rosi or, Tel or combinational treatment. Murine primary bone marrow mesenchymal progenitor cells were harvested as described above in materials and methods and treated with either Rosi (1 µM) or Tel (50 µM), or combination of both. Similar results were observed in ex vivo treatment of Primary bone marrow cells with Rosi and Tel (Fig 13E) indicating that Tel has no effect on osteoblastic phenotype (ALP) and is very effective in protecting the ALP activity and reversing the anti-osteoblastic effects of Rosi in U-33/γ2 cells. We can say that in vitro Tel did not affect osteoblastic phenotype (ALP) and indeed it protected the OB phenotype from negative effects of Rosi which was evident from the combinational treatment.
**Effects of Telmisartan on osteoclast differentiation:**

Analysis of the expression of the genes which are essential for the osteoclast differentiation and maturation showed that Rosi decreased the expression of osteoprotegerin (*OPG*), and *RANKL*, whereas Tel did not had any significant effect on *OPG* or *RANKL* expression, whereas in combinational treatment did not had any significant effect on expression of *OPG* or *RANKL* (Fig 14). Results indicate that Tel had no significant effects on osteoclast differentiation and in fact it inhibited Rosi’s pro-osteoclastic effects in combined treatment in cells. Products of these genes play an important role in formation of a mature multinucleated osteoclasts [147]. To further investigate the effects of Tel and Rosi on osteoclastogenesis, we compared the ability of these drugs in recruiting the osteoclast precursors from the pool of hematopoietic cells and their differentiation potential towards the mature osteoclasts in the presence of Vit D3 and co-culturing with supporting mesenchymal cells (which can produce cytokines like *RANKL*) such as U-33/γ2 cells as described in methods section. There were no significant changes in the number of TRAP$^+$ cells in either of the pretreatments, whereas in combination treatment there is a trend towards decrease in the number of TRAP$^+$ cells (Fig 14). This effect on osteoclastogenesis has to be further studied. Similar effects were observed in the serum of the mice which were administered with different drugs. Decreased trend in osteoclast bone specific markers were observed in combined treatment (Table 2).
Comparison of the anti-hyperglycemic effects of Telmisartan and Rosiglitazone in agouti mice:

Our lab has previously demonstrated that Rosi administration, at an anti-hyperglycemic dose (20mg/kg/day), to diabetic or non-diabetic C57BL/6 mice for 7 weeks induced significant bone loss[6]. Therefore, in order to determine the effect of Tel on bone, the effective dose of Tel that elicits a similar anti-hyperglycemic effect as Rosi, was used i.e. 3mg/kg/day [148]. To show the anti-hyperglycemic effects, we used Co-morbid obese yellow agouti A^v^y mice which has human type-2 diabetes features [17]. The diabetic phenotype of these mice, which results from a mutation in the agouti gene locus, includes hyperglycemia, hyperinsulinemia, and insulin resistance [149]. Previous studies in which C57BL/6 mice and agouti mice (unpublished data) mice were used, demonstrated that a dose of 20mg/kg/day of Rosi is effective in lowering plasma glucose levels [6]. Therefore, the anti-hyperglycemic effect of Rosi in A^v^y males at two doses (10 and 20 mg/kg/day) was compared to the two doses of Tel (1.5 and 3mg/kg/day).

Fasting plasma glucose levels measured after 4 days administration of different doses of Rosi and Tel compounds demonstrated that the dose of 3mg/kg.day of Tel had a similar anti-hyperglycemic effect as the 20mg/kg/day dose of Rosi (Fig 15). But in case of 10mg/kg/day dose of Rosi, though the dose had an anti-hyperglycemic effect it was not sufficient to bring down the serum glucose levels as efficiently as the other higher dose of Rosi which is evident from the Figure 15. (Though the values were not significant, but there was a trend especially at 120min time point.). From the same figure it was clear that lower dose of Tel i.e. 1.5mg/kg/day was insufficient in reducing
the serum glucose levels when compared to Rosi (20mg/kg/day) or Tel (3mg/kg/day). Area under curve (AUC) clearly shows this effect. So for further experiments we used Rosi and Tel at 20mg/kg/day and Tel 3mg/kg/day doses respectively.

**Effects of Telmisartan on the skeleton of Agouti A^vy mice:**

Telmisartan (3mg/kg/day) or Rosiglitazone (20mg/kg/day) or combination of both the drugs were administered to 4-7 months old male agouti A^vy mice, for 4 weeks, using an experimental protocol identical to that described for Rosi [6] and for Tel [108]. In 4 wks of treatment mice consumed about 15.6 mg of Rosi/day/kg weight of mice and 16.6 mg of Rosi /day/kg weight of mice by Rosi alone administered mice and Tel+Rosi mice respectively. About 14 mg of Tel/day/kg weight of mice and 11 mg of Tel/day/kg weight of mice in Tel alone and Tel+Rosi administered mice respectively. Figure 16 shows the effects of Tel administration on body weight, the weights of white adipose tissue (WAT), brown adipose tissue (BAT), and changes in random glucose levels. Four weeks of administration of Tel alone or Rosi alone or combination of both drugs did not affect food intake or water consumption or body weight in black mice a/a (data not shown). In case of yellow agouti A^vy mice, none of the groups showed any significant changes in food intake or water consumption, but in case of Rosi treatment alone or combination of drugs significantly increased body weight (BW) and BAT (interscapular fat) weight when compared to Tel alone. Similar effects were previously shown in C57BL/6J mice treated with Rosi (20mg/kg/day for 7 weeks) [6]. Random glucose levels in these mice after 2 weeks of treatment also shows that Tel alone or Rosi alone were very efficient in
decreasing the Glucose levels when compared to mice fed with normal chow diet. These results suggest that Tel (3mg/kg/day) is as effective as Rosi (20mg/kg/day) in lowering random blood glucose levels in these co-morbid A\textsuperscript{vy} mice and effective in fat metabolism.

As we previously showed in U33/\gamma2 cells, in \textit{intro} assays, Tel did not exhibit any effects on ALP; similarly, treatment of mice with Tel did not show any significant effects on trabecular bone content in vertebrae when compared to control group mice (Table 1). There was a decrease in trabecular bone volume (BV/TV) in L4 Vertebrae by 50\% (P ≤ 0.0005), connective density by 75\% (P ≤ 0.0007), number of trabeculae (Tb.N) by 30\% (P ≤ 0.0006) and trabecular thickness (Tb.Th) by 15\% (P ≤ 0.03). Consistently, the trabeculae separation (Tb.Sp) was significantly increased by 33\% (P ≤ 0.0005) in case of Rosi treated mice when compared to control or Tel treated mice (Fig 17 A & B). We could see the same effects in trabecular bone region in proximal tibia and distal femur (data not shown) when compared to control group mice (Table 1). Structure model index (SMI) data indicates that trabecular bone architecture of mice treated with Tel was more platelike, whereas, more rodlike bone microarchitecture was found in mice treated with Rosi. Together, these data suggests that microCT analysis of vertebrae and femur did not show any changes in the bone microarchitecture of animals treated with Tel (at this dose and duration), whereas all the parameters of bone were significantly affected by Rosi treatment. No changes were observed in serum levels of bone resorption markers, such as TRAP5b enzyme activity and CTX, a marker of collagen degradation. The activity of bone–specific alkaline phosphatase in serum of mice administered Rosi or Tel or combination of both were not different from control mice (Table 2). Further dynamic bone histomorphometric analysis is needed to study the effects of Tel on new bone
formation. Further histological assessment of the bone has to be done after it is
decalcified and embedded in paraffin sections and bone specimens should be stained
using H&E and later examined for osteoblast, osteoclast, and adipocytic markers.

From these results, it is clear that Tel did not have any effect on bone and even it has a potential role in protecting bone from negative effects of Rosi. To support this, we further studied the architecture of the bone, by using BioDent indentation instrument, where the structural design and construction of the bone along with biomedical properties of the bone is investigated. BioDent, Osteoprobe II (Figure 18A) is a bone diagnostic instrument developed to measure bone material properties of the bone as described in the paper [150, 151]. Results from the analysis shows that Rosi increases the Indentation distance increase (IDI) and Creep Indentation Distance (CID), whereas Tel did not increase IDI and CID (Fig 18B) which shows that Rosi treatment has a negative impact on the bone structure and thus decreases the capacity of bone in resisting the continuing fracture events caused by the indentation tip of the Biodent instrument. This also supports the statement that IDI increases for a bone which has a greater tendency of fractures or the bone with degraded biomedical and mechanical properties [150].

**Mechanisms involved in Telmisartan effects on osteoblast phenotype:**

In order to investigate the mechanisms involved in Tel effects on osteoblasts, we examined the expression of OB-specific genes and transcription factors in the U-33/γ2 cells using Real-time RT-PCR (Fig. 19). Treatment with Rosi had a suppressive or negative effect on the expression of the genes as expected, after 72 h of treatment. On the
other hand Treatment with TEL alone had either no effect or had a positive effect on the OB-specific transcription factors like Runx2 and Dlx5, where their gene expression was unchanged and indeed in the combinational treatment Tel protected or rescued the expression of Runx2 from negative effects of Rosi. In the same way expression of β-catenin, a main transducer of Wnt pathway was unaffected with Tel alone and in combination with Rosi. As shown previously [21] in the Figure 1B from the microarray analysis, Rosi activated PPARγ2 causes the bone loss mainly by decreasing the bone formation by affecting the TGFβ/BMP signaling pathway and decreasing the gene expression of the members of the pathway. Therefore we analyzed gene expression of different members of TGFβ/BMP signaling pathway BMP4, TGFβ3, TGFβR2, Smad6, and Smad7 and data shows that Tel has no effect on expression of these genes and Rosi decreased the expression of these genes, Whereas, in combinational treatment Tel rescued or protected the expression of those genes from the negative effects of Rosi (Fig 20).
**Discussion**

In this study, we demonstrated for the first time, that telmisartan alone does not effect bone mass and infact protects OB phenotype from negative effects of TZD’s by protecting the integrity of TGF/BMPβ signaling pathway via \( PPAR_\gamma \)-dependent pathway *in vitro*.

TZDs were the drugs which were developed to treat insulin resistant in type 2 diabetic patients [138]. TZD treatment improves insulin sensitivity resulting in better glycemic control. On the other hand, there were many side effects of clinical importance, such as weight gain, edema and cardiovascular problems [6, 15]. In addition; there is also emerging concerns about the deleterious effects of TZDs on bone [10, 44, 45]. Therefore, identification and development of improved \( PPAR_\gamma \) ligands which retain their insulin-sensitizing activities, but doesn’t cause any adverse effects i.e. having more specific mode of activation is necessary. A promising selective \( PPAR_\gamma \) modulator is Tel which activates only a subset of genes and functions which are induced by full cognate ligands [29]. Tel binds to \( PPAR_\gamma \) in such away that entire LBD is not involved thereby causing a distinct conformational receptor change occurs after binding when compared to typical \( PPAR_\gamma \) ligands like TZDs.

The well established *in vitro* cell model which we used in these studies i.e. U33/\( \gamma \)2 cells serve as a valuable tool for assessing the osteoblastic and adipocytic activities of different \( PPAR_\gamma \) modulators. In these cells osteoblast and adipocyte differentiation are explicitly under the control of \( PPAR_\gamma 2 \) which is constitutively expressed [71]. Using this in vitro cell model we have clearly showed that Tel did not
have any significant effect on osteoblast phenotype, as determined by the alkaline phosphatase activity and osteoblast-specific gene expression and in combinational treatment Tel rescued the osteoblast phenotype from the negative effects of Rosi. This protective effect of Tel on ALP was mediated through PPARγ and not through RAAS system, as ALP activity was decreased and was not protected from Rosi when cells were treated with combination of Losartan and Rosi. Losartan is an ARB which can efficiently bind and modify functions of AT1R but not PPARγ (Fig 13 B & D). Tel had a selective protective effect from Rosi on the OB-specific genes, as BMP4 expression was unaffected which has control on Runx2 expression which in turn controls ALP expression. Similar protective effects were seen in murine primary bone marrow mesenchymal cells treated with combination of Tel and Rosi Figure 13E, demonstrating that primary bone marrow progenitors respond to both PPARγ ligands in the similar fashion as U-33/γ2 cells does which reinforces the statement that U-33/γ2 cells are valuable tool which can be used to determine the selective effects of PPARγ modulators on both the OB and AD phenotypes. Similarly effects on cell proliferation were shown to be PPARγ dependent (Fig 10), as the cell proliferation which was determined by the optical density (OD) readings was unchanged with Losartan treatment (non-PPARγ agonist) or in U-33/Control cells. Rosi decreased the expression of OPG and RANKL and Tel did not effect the expression of these genes indicating that it does not affect the MSCs or the osteoblastic phenotype and in combination treatment completely blocked the Rosi’s inductive effect on osteoclastogenesis.

*In vitro*, Tel showed a weak pro-AD activity when compared to Rosi (Fig 11). This was evident in U-33/γ2 cells, from number of adipocytes and lipid accumulation in
Nile red assay and also from the gene expression data. These results strongly support the notion that Tel activates \(PPAR_{\gamma}\) in a different fashion than Rosi by acting as a partial agonist and activating only a subset of genes. Similar results were observed in whole bone RNA samples of mice administered with Tel and Rosi (Data not shown). The adipocytes which we saw with Tel treatment may be of brown adipose tissue phenotype, as in U-33/\(\gamma\)2 cells, as Tel significantly activates the brown adipose genes like \(UCP1\), \(Dio2\) and \(FoxC2\) either when treated alone or in combination with Rosi. This shows that brown adipose phenotype or thermogenesis is upregulated with Tel, though we did not see any increase in these thermogenic genes in RNA samples of whole bone from mice fed with Tel alone, but there was a significant increase in whole bone samples from mice administered both the drugs (Data not shown). This difference may be due to the fact that RNA is made from the whole bone and there is a pool of cells and not from the bone marrow. This increase in BAT phenotype may be contributing to the decrease in weight gain with Tel administration both in patients and mice along with increase in \(\beta\)-oxidation in skeletal muscles by increasing the mitochondrial genes [112, 113, 152, 153] which were not seen with Rosi administration and weight gain is the significant problem faced by the patients taking TZD’s.

The absence or lack of anti-osteoblastic activity of Tel \textit{in vitro} also correlated with observed effects on bone \textit{in vivo}. \textit{In vivo} studies on agouti mice shows that Tel at a dose of 3mg/kg/day, which lowered blood glucose levels as effectively as Rosi dose of 20mg/kg/day, did not affect the skeleton and infact, even protected the bone from negative effects of Rosi which supports that Tel has no effect on bone in both diabetic mice and ovariectomised rats [154]. Tel did not affect BMD, bone microarchitecture (Fig
17A), bone-specific gene expression (data not shown). MicroCt images clearly shows the trabecular loss in Mice fed with Rosi and preservation of trabecular bone in combinational treatment, though the analysis of bone parameters in Table 1 shows a decrease in trabecular number, and increase in trabecular spacing. But unchanged connective density and decreased SMI suggests a potential protective of Tel and raises the possibility that higher doses and longer treatments times may even reduce those slight negative skeletal effects and can completely protect the bone.

Other studies did also provide enough evidence to the fact that Tel reduces the food intake and decreases the weight gain in the animal models along with decreasing abdominal circumference in human subjects. But in Agouti model we did not see any significant changes in food intake or water consumption, but weight changes were significantly less in Tel fed mice compared to Rosi, but this decrease was not seen in combinational group. This may be due to the fact that Tel dose given to the mice and duration of treatment (4 wks) was not enough to overcome the Rosi’s weight gain effect.

Analysis of fat and glucose metabolism genes in liver samples from mice fed with Tel or Rosi or both shows similar kind of effects on Gluconeogenesis and FAS genes, indicating that both the drugs have similar abilities to control glucose and fat metabolism.

These weak pro-adipocytic and lack of suppressive effects on osteoblast differentiation activities of Tel in vivo is conveyed via PPARγ dependent mechanisms. Another interesting feature about Tel activity is its ability to stimulate adipocytes and lack of a suppressive effect on osteoblast differentiation. Even though there are many evidences showing that PPARγ drives the cells towards the adipocytic phenotype simultaneously suppressing the osteoblastic phenotype [20], and there are numerous...
examples to show the complex relationship between the phenotypes and role of $PPAR_\gamma$ involvement [155], Tel was able to stimulate adipocytic activity without effecting osteoblast phenotype. Previously we demonstrated in Primary bone marrow cells and in U-33/γ2 cells, that ligands with different chemical structure can selectively activate and regulate $PPAR_\gamma$ adipocytic and osteoblastic activities [17, 71]. This lack of suppressive effect of Tel should be considered and further research has to be done.

The studies presented here provide a clear cut separation of the anti-diabetic (pro-adipocytic) and anti-osteoblastic activities of Tel, which were performed in an animal model which was previously used to demonstrate how Rosi was controlling both the activities simultaneously. Our study is the first one to show that Tel does not affect osteoblast phenotype and in fact protects from Rosi induced bone loss both in diabetic mice and spontaneously hypertensive ovariectomized rats [156]. This work also provides a possible mechanism involved in the improvement of bone phenotype from Rosi induced bone loss. We show that Tel is protecting the bone from negative effects of Rosi by protecting the $TGF_\beta/BMP$ signaling pathway. As we saw from Figure 20 that Tel did not change the expression of different members of the signaling pathway but protected them from Rosi’s negative effects. Therefore we can conclude that Tel abrogates the Rosi induced bone loss by protecting the integrity of the $TGF_\beta/BMP$ pathway. To further demonstrate and understand these effects we used Los which is non-$PPAR_\gamma$ agonist but can act as an Antihypertensive agent with ARB properties, but could not protect the OB phenotype from Rosi’s negative effect. This is consistent with a previous report showing that Los did not show any effect on bone mass [130]. Thus, we hypothesize that the major contribution of Tel towards the protection against the negative effects caused by Rosi
might be mediated through $PPAR_\gamma$ and there may be competition between the ligands for the nuclear receptor and not through the RAAS blockade, as described above. However, further investigations are needed in this respect.

In summary, Tel appears to be a partial and selective $PPAR_\gamma$ ligand which separates anti-diabetic and proadipocytic activities from the anti-osteoblastic activity both \textit{in vivo} and in bone MSCs. This interesting finding provides necessary information that selective $PPAR_\gamma$ modulators can be used to separate necessary anti-diabetic and unwanted anti-osteoblastic activities and this property of selective \textit{in vivo} activity of novel $PPAR_\gamma$ modulators may be of clinical relevance. As, patients with metabolic syndrome, loose more bone than non-diabetic patients and are at very high risk of falls and fractures, [17, 139] treatment with TZD (Rosi) alone may increase the risk of bone loss. But such patients who are hypertensive it may be beneficial to use combination of both TZD and Tel. Thus, Tel represents a paradigm of multifunctional drug, which can be used alone or in combination with Rosi, for simultaneous treatment of hypertension and diabetes without any adverse effects on bone. Further detailed clinical studies are needed to provide sufficient information regarding the potential side effects in patients who receive Tel or the combined therapy.
Materials and Methods

Cell Culture, treatment regime, and cell phenotype assays:

Rosiglitazone maleate (BRL 49653) was obtained from Pharmacy (University of Toledo, Medical Centre, Toledo, OH), Telmisartan was obtained from Sigma-Aldrich, St. Louis, MO. Murine marrow-derived U-33 cells stably transfected with a PPARγ2 expression construct, referred to as U-33/γ2 cells, and cells transfected with an empty vector control, referred as U-33/c cells, as previously described [20] were used. Cell were maintained in αMEM (Gibco) supplemented with 10% FBS (Hyclone), 0.5mg/ml of G418, 100 U/ml penicillin, 100µg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO2.

To measure the effect of the ligands on alkaline phosphatase activity which is an early marker of the osteoblast phenotype, cells were plated in 96-well plates, in triplicates and after cultures achieved ~70% confluence (or after 24 hrs), growth medium was supplemented with either the tested ligands at different concentrations or with DMSO (as vehicle control) and cells were treated for 3 days. After treatment, alkaline phosphatase activity was measured using p-nitrophenol as a Substrate (Phosphatase Substrate, Sigma), and data was normalized to the number of cells which was measured with Cell Titer 96 Non-Radioactive Cell Proliferation assay kit (Promega, Madison, WI). One microunit (µU) of activity was defined as the amount of enzyme capable of hydrolyzing 1 pmol of p-nitrophenol substrate per minute at room temperature.
To measure adipocyte formation, cells were cultured in 48-well plates and treated with tested ligands at different concentrations for 3 days, as described above and after 72 hrs of treatment, cells were fixed with 10% phosphate-buffered formalin, and stained for 30 min with 0.15% Oil Red O (Sigma, St.Louis, MO) in a 55:45 (vol/vol) mixture of isopropanol and water for lipid detection [71]. Cells were rinsed with water and counterstained with 0.5% methyl green (Fisher Scientific, Fair Lawn, NJ) in 0.1M sodium acetate (pH 4.0). Quantification of the adipocyte cells were performed by in each replicate wells, by counting the approximately 100 cells in 5 different randomly selected fields and percentage of cells that had lipid droplets positively stained for Oil Red O was calculated.

To quantitate the lipid accumulation in the cells, cells were cultured and treated similarly as described above. Nile Red stock solutions in DMSO were prepared and stored and protected from light as described somewhere else [157]. After 72 hrs, Nile red (Sigma Chemical Co., San Louis, MO, USA) stock solution (10 mg/ml in DMSO) was added to 1 ml of sample, resulting in a final Nile red concentration of 0.05 mg/ml, and then incubated for 30 min at room temperature in the dark and then visualized and pictures were captured in blue light using QCapture Pro software in QImaging microscope (Leica DMIRB Microsystems).

**RNA isolation and gene expression analysis:**

U-33/γ2 and U-33/c were grown in basal media until cultures obtained 80% confluency and they were treated with tested compounds for 3 days and then RNA was
isolated. Total RNA from cell lines were isolated using RNeasy kit (QIAGEN Inc., Valencia, CA). RNA isolation from tissues and intact femur of experimental animals was performed using TRlZol reagent (Invitrogen, Carlsbad, CA) as described previously [6]. 1µg of total RNA was treated with DNaseI (Invitrogen, Carlsbad, CA) and made into cDNA using the iScript cDNA synthesis kit (Biorad, Hercules, CA). Gene expression analysis was performed using real-Time PCR (StepOneplus, Applied Biosystem, Foster City, CA), as previously described [8]. Relative change in the expression was measured by the comparative C_T method after each sample was normalized to the quantity of 18S RNA. Real time PCR analysis was performed using the following Primer sets:

18S                  : F: TTCGAACGTCTGCCCCTATCAA
                      : R: ATGGTGGCAGCGGCGACTA
Runx2                : F: GGGCAACGTTCTATCTGGAAAA
                      : R: CGGTGTCGCTCGCTGGAA
Dlx5                 : F: TGACAGGAGTGTGACACAGAAGCT
                      : R: CCGGAACCGGAGCTTGGGA
A1Col1               : F: ACTGTCCCAAACCCCAAAAG
                      : R: CGTATTCTCCGGGACAGAAA
Osteocalcin           : F: CGGCCCTGAGTCTGACAAA
                      : R: GCCGGAGTCTGCTACACCTT
Ccnb1                 : F: TGCAGGAACAGGCTGCTA
                      : R: TGGCAGGTCGCTTTCCTACCT
Phex                  : F: GAAAGACATTGCGCTCGG
                      : R: TGGCAATGCTGTCCTTCCT
Dmp1                  : F: TGTCATTCTCCTGTTCTTCCTT
                      : R: AGAGCTTTACATCTAGTTGTTAT
BMP4                  : F: TCAAGGAGTGGAGATTGG
                      : R: GCCATCATGGCTAAGTGG
Smad 1                : F: TCCGTCTCTGCAACTATCGA
                      : R: TTTCTGGTCTGCTCTC
Smad 2                : F: CCCTTCAGTGCGATGCTCA
                      : R: GAATACAGACGGAGGACGT
Smad 3                : F: CACGCAAGACGTGAACACC
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*Primary bone marrow cultures, treatment, and differentiation assays:*
Primary Bone Marrow cells (PBMC) was isolated by aspiration from the femora of 4 months old male black a/a mice, as previously described [20] using a University of Toledo-Medical Centre IACUC approved protocol. Marrow was isolated and cultures were established by plating cells at a density of $2.5 \times 10^6$ cells/well on 6-well plates for differentiation assays or at $5 \times 10^6$ cells per 60 mm$^2$ plates for RNA isolation. Cells were plated in triplicates and grown in the presence of 15% FBS (Hyclone, Waltham, MA) in αMEM medium (Invitrogen, Carlsbad, CA). One half of the medium was changed every 6 days. To measure the effect of the ligands on alkaline phosphatase activity, a marker of the osteoblast phenotype, Potential of MSC to differentiate towards osteoblasts was assessed by seeding PBMC on 96 well plates at a density of $1 \times 10^5$ cells/well. Cultures were grown for 6 days and half of the medium was replaced with new 15% FBS, on tenth day total media was replaced by 15% FBS supplemented either with DMSO, or 50µM Tel or 1µM Rosi or a combination of 50µM Tel and 1µM Rosi for 6 days. Cell proliferation was assessed by MTS assay and ALP activity was measured and normalized with proliferation assay after 6 days of treatment as described previously [17]. Each experiment was repeated three times.

Osteoclastogenesis:

PBMC were harvested as previously described as previously described [20], and seeded at the density of $2.5 \times 10^5$/cm$^2$ in the presence of 15% FBS (Hyclone, Waltham, MA) in αMEM medium (Invitrogen, Carlsbad, CA). In the co-culture
experiments, non-adherent bone marrow cells were harvested after 24 hrs which are a source of osteoclast progenitors and were plated at the density $2 \times 10^5$ cells/cm$^2$ over the U-33/$\gamma$2 cells, which represent marrow cells of mesenchymal lineage which naturally produces RANKL and M-CSF and able to support osteoclastogenesis [8]. U-33/$\gamma$2 cells were plated at the density $1 \times 10^4$ cells/cm$^2$ on 48-well plate 3 days before non-adherent PBMC were added (at $2 \times 10^5$ /cm$^2$). These U-33/$\gamma$2 cells were treated either with DMSO, or 50µM Tel or 1µM Rosi or a combination of 50µM Tel and 1µM Rosi for 3 days. Cultures were grown in the media supplemented with $10^{-8}$ M 1, 25-dihydroxy vitamin D3 for 8 days with a change of one-half of the medium after 4 days of culture. Osteoclasts were identified as previously described by staining for tartrate–resistant acid phosphatase (TRAP5b) [20].

*Western blotting analysis:*

Proteins were isolated from U-33/$\gamma$2 cells or from the liver tissue samples from the mice treated with either of the drugs as described above. For isolating proteins from cells, cells were washed with PBS and scrapped into Lysis Buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% NP-40, 50 mM NaF, Triton X 100, 50mM Sodium Orthovanadate, PMSF and Protease and Phosphatase inhibitors) and spun at 17000xg for 10min to extract proteins. For isolating proteins from Liver tissues liver was cut and lysis buffer was added and by using the Tissue Lyser (Qiagen) and magnetic beads, sample was homogenized at 3500rpm for 10 min and later was spun at 17000xg for 10min in a centrifuge and supernatant was collected. Protein concentration was measured using BCA
Protein Assay kit (Thermo Scientific) and separated on SDS-PAGE. Proteins were probed with AKT, pAKT, b-catenin (BD Transduction Laboratories) and developed with near-infrared (NIR) fluorescence detection system using LI-COR secondary antibodies at a dilution of 1:10,000

Animal maintenance

Yellow agouti A\textsuperscript{vy} diabetic mice which are Co-morbid obese (4 to 5 months old male) and black a/a mice which were age matched with the yellow mice. Animals were housed in groups of 4-8 per cage with a constant temperature on a 12-hour light-dark cycle. All animal treatments and care protocols were approved by the Institutional Animal Care and Utilization Committee. Mice were fed a standard chow (control) or a Rosiglitazone mixed diet (Rosi) and normal water or Telmisartan dissolved water (TEL) for 4 weeks.

To establish a dose of telmisartan with the same anti-hyperglycemic effect as the previously reported rosiglitazone dose [6] [108], A\textsuperscript{vy} mice (4-8 animals per group) were fed water supplemented with either telmisartan (1.5 mg/kg body weight /day or 3 mg/kg body weight /day ), or fed a diet supplemented with rosiglitazone (10 mg/kg/day or 20 mg/kg/day), or fed with non-supplemented water and diet (control) for 4 days. Animals were fed once in every 3 days for 4 wk, and food and water intake per cage and body weights of individual animals were monitored. At the end of the experiment the average intake of rosiglitazone and telmisartan per gram body weight per cage was calculated. All
animals were fed for 4 weeks with food and water monitored once in every 2 days and the body weights of individual animals were monitored weekly.

_Blood collection and plasma glucose measurements_

Plasma glucose levels were determined for each animal at the beginning and throughout the experiment. Animals were fasted for 18h and serum samples were prepared from blood collected by cardiac puncture immediately after euthanasia. Bone-specific alkaline phosphatase was measured colorimetrically using alkaline phosphatase substrate and Alkaline Buffer solution (Sigma-Aldrich, St. Louis, MO) in the presence of L-Phenylalanine to exclude intestinal enzymatic activity. N-terminal propeptide of type I procollagen (P1NP), C-terminal telopeptide of type I collagen (CTX), and tartrate-resistant acid phosphatase form 5b (TRAP5b) were measured using diagnostic kits provided by Immunodiagnostic Systems Inc (IDS, Scottsdale, AZ). Plasma Glucose levels were measured through out of the experiment at each week for four weeks along with beginning of the experiment for each animal fasted 4 hrs before blood collection from tail vein. Glucose levels in serum were determined using AlphaTRAK Blood Glucose Monitoring system.

_Micro-Computed Tomography (MicroCT) analysis:_

After 4 wks of feeding mice were sacrificed, the vertebrae, left femur and the right tibia of each animal was dissected and fixed in 10% Phosphate buffered formalin,
pH 7.4, at 4°C. MicroCT analysis was performed using a SCANCO µCT 35 (SCANCO Medical AG, Bassersdorf, Switzerland) equipped with a 10-mm focal spot microfocus x-ray tube. MicroCT scans were performed at the following instrument settings: E=70 KVp, I=110µA, increment 7µm, threshold value =300 [8]. Two hundred slices of the proximal and 33 slices of the midshaft tibia were used for trabecular and cortical bone analysis respectively. Trabecular bone in vertebrae was measured in the vertebral body after manual exclusion of the superior and inferior end plates, which consisted of approximately 10% of vertebral height on each side. Carefully contouring of the regions yielded volumetric information of bone volume (BV), total volume (TV), and calculated ratio of BV/TV, as well as trabecular thickness (Tb.Th.), Trabecular number (Tb.N), and trabecular spacing (Tb.Sp.). Plate or rod-like characteristics of the bones were measured and estimated by using Structure Model index (SMI). For an ideal plate or rod structure, the SMI values are 0 and 3, respectively. For a mixed structure, the value is between 0 and 3, depending on the ratio of plates and rods ratio. The Connectivity density (Conn.D.) is calculated [158].

The Osteoprobe II: Indentation instrument:

The Osteoprobe II™ bone diagnostic instrument (BDI) is the improved new control system, which is used to perform in vivo diagnostic measurements. Osteoprobe II, containing a novel test probe and reference probe, used to do indentation measurements on bone that is covered with skin and soft tissue as described previously [150]. Tibia from different treatment groups were sent to Biodent, Reference Point Indenter (Active
Life Scientific, Inc. California, USA). Reference Point Indentation (RPI) was on the bones, in order to directly measure the bone tissue strength. The outer reference probe present protects the inner probe from surrounding soft tissues, at the same time; the test probe initiates a reference point at the site of indentation for measuring displacement (Fig 18A). Along with other parameters Indentation distance increase (IDI), and Creep indentation distance (CID) were measured. IDI is a measure of continuing damage that results from repeated loading. IDI with repeating cycling to a fixed force at a point on the bone differentiates the bone that is more easily fractured from bone that is less easily fractured [151].

Statistical Analysis:

Statistically significant differences between the groups were detected using one-way ANOVA followed by post-hoc analysis by Dunnetts and Tukey within the SPSS Statistics 17.0 Software (SPSS, Inc., Chicago, IL). All data shown represent means plus standard errors of the means (SEMs) and in all cases, P< 0.05 was considered significant.
Figure Legends:

Figure 1: Characterization of marrow-derived cell lines and results from Microarray data analysis of TGFβ/BMP pathway:

(A). U-33/γ2 cells were cultured with ASC+BGP to stimulate osteoblastogenesis or with Rosiglitazone to stimulate adipogenesis. Cells were stained with Oil Red O to visualize fat droplets (red) or with von Kossa reagent to stain mineral and counterstained with methyl green. (B). Schematic representation of effects of Rosiglitazone activated PPARγ2 on expression of gene members of TGFβ/BMP pathway from Microarray data: (C).

Fibrodysplasia Ossificans Progressiva (FOP): Rare genetic disorder (1 in 1.5 million) characterized by heterotopic ossification of soft tissues (joints, muscle, cartilage). Due to improper expression of BMP4 by inflammatory cells (lymphocytes) and disregulation in BMP signaling in cells of mesenchymal lineage and there is no established medical treatment.

Figure 2: Comparison of glitazones effects, on osteoblastic and adipocytic phenotypes in U-33/γ2 cells:

(A). Characteristics of tested glitazones. (B). U-33/γ2 cells were treated with glitazones in different concentrations for 3 days, than cultures were either stained with Oil Red O to visualize fat droplets or enzymatic assay for alkaline phosphatase was performed. (C). Glitazones effect on the expression of phenotype-specific gene markers measured by real
time RT-PCR. * Numbers reflect a fold change of an analyzed gene mRNA expression in treated versus non-treated cells. (D). U-33/γ2 cells or primary bone marrow cultures established from 6 mo old C57BL/6 mice were treated with either vehicle or tested glitazones for 3 days. The mRNA expression for genes that are components of either TGFβ or BMP signaling pathways was analyzed using quantitative real time PCR. Bars represent average values (+/- SD) from two experiments. Yellow, transparent boxes indicate genes, which expression is similarly affected by rosiglitazone in both U-33/γ2 cells and bone marrow.

Figure 3: Schematic representation of TGFβ/BMP signaling pathway and effects of Rosiglitazone on this pathway:

(A). Schematic representation of TGFβ/BMP signaling pathways. (B). U-33/γ2 cells were treated in duplicate cultures with either vehicle or 1 mM Rosiglitazone. After for 3 days of treatment, media in one vehicle and one Rosiglitazone-treated plate were supplemented with either 10 ng/ml TGFβ1 or 50 ng/ml BMP4 for 2 hrs followed by either protein or RNA isolation. (1). The Smad1 protein expression was detected using Western blot analysis. Smad3 protein was first immunoprecipitated from the protein lysate and than visualized using Smad3-specific polyclonal antibodies by Western blot. (2). The mRNA expression for genes that are directly regulated by either TGFβ1 or BMP4 signaling pathway was analyzed using quantitative real time PCR. C. U-33/γ2 cells or primary bone marrow cultures established from 6 mo old C57BL/6 mice were treated with either vehicle or tested glitazones for 3 days. The mRNA expression for
genes that are components of either TGFβ or BMP signaling pathways was analyzed using quantitative real time PCR. Bars represent average values (+/- SD) from two experiments. Yellow, transparent boxes indicate genes, which expression is similarly affected by Rosiglitazone in both U-33/γ2 cells and bone marrow.

**Figure 4:** Pictorial representation of Tob1 suppressing BMP signaling

**Figure 5:** Effects of changes in expression of Tob1 on Pro-osteoblastic markers and members of TGFβ/BMP signaling pathway:

(A). U-33/γ2 cells were transiently transfected with 2µg of pcTOB 3.1(-) for 24 hrs. And effects of Tob1 overexpression on different osteoblast (Runx2, Dlx5, A1Col1, Osteocalcin and β catenin), adipocyte (aP2) and gene expression of different members of TGFβ/BMP signaling pathway were analyzed using real time PCR were represented. Values are expressed as means ± SD of 3 separate experiments. *p≤ 0.05 vs control. (B). U-33/γ2 cells were transiently transfected with 75 picomoles of Tob1 siRNA for 48 hrs. And effects of Tob1 overexpression on different osteoblast (Runx2, Dlx5, A1Col1, Osteocalcin and β-catenin), adipocyte (aP2, Ccrn4l) and gene expression of different members of TGFβ/BMP signaling pathway were using real time PCR were represented. Values are expressed as means ± SD of 3 separate experiments. *p≤ 0.05, **p≤ 0.01 vs control.

**Figure 6:** Effects of Cellular Silencing of BMP4 on Pro-osteoblastic markers and members of TGFβ/BMP signaling pathway:
U-33/γ2 cells were transiently transfected with 75 picomoles of *BMP4* siRNA for 48 hrs. And effects of *Tob1* overexpression on different osteoblast (*Runx2, Dlx5, AlCol1, OC* and *β-cat*), *adipocyte* (*aP2, Ccrn4l*) and gene expression of different members of *TGFβ/BMP* signaling pathway were using real time PCR were represented. Values are expressed as means ± SD of 3 separate experiments. *p≤ 0.05, **p≤ 0.01 vs control.*

*Figure 7: Effects of Tob1 expression on Cell proliferation and effects of Rosiglitazone on Tob1 protein as detected by Tob1 Antibody after immunoprecipitation:*

(A). Anti-proliferative activity of *Tob1 in 3.1 cells (U33 cells stably transfected with Tob1)* using the MTS- proliferation assay. ** *p ≤ 0.002. (B). Tob1 protein was first immunoprecipitated from the protein lysate and than visualized using Tob1-specific polyclonal antibodies by Western blot.

*Figure 8: Mechanism of TZD-induced Bone loss:*

(A). Schematic representation of showing how Anti-diabetic drugs TZDs, high affinity ligands for PPARγ, affects bone cell differentiation and cause bone loss. (B). Schematic representation of mechanism of TZD-induced bone loss.
Figure 9: Schematic representation of Renin Angiotensin aldosterone system (RAAS) and Telmisartan Blocks the AT1R, and decreases the hypertension.

Figure 10: Effects of Rosiglitazone and different Sartans on Cell proliferation

(A). U-33/γ2 cells were treated with either Vehicle (DMSO) or Rosiglitazone (1µM) or Telmisartan (12µM, 25µM, and 50µM) or (B). U-33/Control cells were treated with either Vehicle (DMSO) or Rosiglitazone (1µM) or Telmisartan with different concentrations (12µM, 25µM, and 50µM) or (C). U-33/γ2 cells were treated with either Vehicle (DMSO) or Rosiglitazone (1µM) or Losartan (12µM, 25µM, and 50µM) for 72 hrs and their effects on cell proliferation were measured MTS assay. p-value vs Vehicle * ≤ 0.001.

Figure 11: Telmisartan has weak proadipocytic activity than Rosiglitazone

(A). U-33/γ2 cells were treated with either Vehicle (DMSO) or Rosiglitazone (1µM) or Telmisartan (12µM, 25µM, and 50µM) or in combination of Rosiglitazone (1µM) and Telmisartan (50µM) for 3 days, then cultures were stained with Oil Red O to visualize fat droplets and Oil Red O positive cells were counted. (B). U-33/γ2 cells were treated either Vehicle (DMSO) or Rosiglitazone (1µM) or Telmisartan (50µM) or in combination of Rosiglitazone (1µM) and Telmisartan (50µM) as described in Fig11A and pictures were captured. (C). As described above, after 3 days of treatment. gene expression of adipocytic markers (aP2 and adiponectin) was analyzed using real time PCR. Values are
expressed as means ± SD of 3 separate experiments. *p≤ 0.05, **p≤ 0.01 vs control, and p-value vs Rosi # p≤ 0.05, ## p≤ 0.01 ≤ 0.001. (D). U-33/γ2 cells were treated with either vehicle (DMSO) or rosiglitazone (1µM) or telmisartan (12µM, 25µM, and 50µM) or in combination of rosiglitazone (1µM) and telmisartan (50µM) for 3 days, then nile red was added to stain lipid molecules in the adipocytes fluorescent nile red was visualized and pictures were captured.

Figure 12: Telmisartan induces Brown adipose tissue specific genes

U-33/γ2 cells were treated with either Vehicle (DMSO) or Rosiglitazone (1µM) or Telmisartan (50µM) or in combination of Rosiglitazone (1µM) and Telmisartan (50µM) for 3 days, and then expression of BAT specific genes (UCP1, Dio2, FoxC2, PRDM16, and ADBR3) was analyzed using real time PCR. Values are expressed as means ± SD of 3 separate experiments. *p≤ 0.05, **p≤ 0.01 vs control, and p-value vs Rosi # p≤ 0.05.

Figure 13: Comparison of different sartans on OB-phenotype in invitro cultures

(A). U-33/γ2 cells were treated with either Vehicle (DMSO) or Rosiglitazone (1µM) or Telmisartan (12µM, 25µM, and 50µM) for 3 days and alkaline phosphatase activity in these cells was measured. (B). U-33/γ2 cells were treated with either Vehicle (DMSO) or Rosiglitazone (1µM) or Losartan (12µM, 25µM, and 50µM) for 3 days and alkaline phosphatase activity in these cells was measured. (C). U-33/γ2 cells were treated with either Vehicle (DMSO) or Rosiglitazone (1µM) or a combination of Rosiglitazone (1µM)
and Telmisartan (50µM) for 3 days and alkaline phosphatase activity in these cells was measured. (D). U-33/γ2 cells were treated with either Vehicle (DMSO) or Rosiglitazone (1µM) or a combination of Rosiglitazone (1µM) and Losartan (50µM) for 3 days and alkaline phosphatase activity in these cells was measured. (E) Primary bone marrow cultures were established from 8 mo old C57BL/6 mice and after 10 days of culturing cells were treated with either Vehicle (DMSO) or Rosiglitazone (1µM) or Telmisartan (50µM) or a combination of Rosiglitazone (1µM) and Telmisartan (50µM) for 3 days. The alkaline phosphatase activity was measured. Bars represent average values (+/- SD) from triplicates. *p≤ 0.05 and ** p≤ 0.005 vs control, #p≤ 0.05.

**Figure 14:** *In vitro* analysis of Rosiglitazone and Telmisartan effects on osteoclast differentiation and gene expression

(A). U-33/γ2 cells were treated with either Vehicle (DMSO) or Rosiglitazone (1µM) or Telmisartan (50µM) or in combination of Rosiglitazone (1µM) and Telmisartan (50µM) for 3 days, then expression of Osteoclast regulating genes (*OPG*, and *RANKL*) was analyzed using real time PCR. Values are expressed as means ± SD of 3 separate experiments. *p≤ 0.05, **p≤ 0.01 vs control, and p-value vs Rosi # p≤ 0.05. (B). Non-adherent marrow cells were derived from C57BL/6J animals and cultured as described in materials and methods. The experiment was repeated 3 times. Calculated number of TRAP⁺ cells was represented.
Figure 15: Telmisartan effectively lowers glucose similarly as Rosiglitazone does in Agouti mice

Comparing the Anti-hyperglycemic effects, of different doses of Rosiglitazone and Telmisartan in obese yellow AVY mice. Mice were fed for 4 days with either non-supplemented chow (Grey circle), or chow supplemented with Rosi (20mg/kg/day; open square) or with Rosi (10mg/kg/day; closed square) or Tel supplemented in water (3mg/kg/day; open triangle) or Tel (1.5 mg/kg/day; closed triangle). Blood was collected from animals fasted for 4hrs and serum glucose levels determined as described in materials and methods. lines and points shows the serum glucose levels after 4 days of treatment, whereas vertical lines indicate standard deviation in each treatment group. *p≤ 0.05 and ** p≤ 0.005 vs control.

Figure 16: Comparison of anti-diabetic PPARγ ligands (Rosi and Tel) effects, on different metabolic parameters.

Mice were fed for 4 weeks with either non-supplemented chow (dark grey bar, or chow supplemented with Rosi (20mg/kg/day; Red bar) or Telmisartan supplemented in water (3mg/kg/day; Green bar) or both Rosi and TEL (Blue bar). Mice were weighed and random glucose was measured throughout the 4wks treatment and after they were sacrificed organs were isolated and weighed as described in materials and methods. (A) Body weight percentage, (B) BAT tissue weight, (C) percentage of WAT tissue, (D)
Random glucose.*p≤ 0.05 and ** p≤ 0.005 vs control. *p≤ 0.05 , ** p≤ 0.005 vs control , #p≤ 0.05 . ##p≤ 0.001 Vs Rosiglitazone and $ p≤ 0.05 vs Telmisartan.

Figure 17: MicroCT analysis of bone microarchitecture and its images:

(A). MicroCT images of Vertebrae: Compared to Control, Rosi administration resulted in Trabecular bone loss of Avy/a mice, but not of TEL or TEL+Rosi administered animals, Even TEL+Rosi demonstrated a protection in bone loss caused by Rosi. Animal model; 5 to 6 month, old males. (B). MicroCT analysis of bone microarchitecture. Graphs represent the bone parameters of L4 vertebrae and proximal tibia in all 4 groups of mice. Vertical Bars are expressed as means ± SD of n= 4 to 9 animals per group. BV/TV is ratio of bone volume to total volume, Tb.N is trabecular number, Tb.th trabecular thickness, Tb.Sp trabecular spacing, ConnD is connective density and SMI represents Structure Model Index. *p≤ 0.05, ** p≤ 0.005 vs control and # p≤ 0.05.

Figure 18: The Osteoprobe II™ bone diagnostic instrument and measurement of different parameters of bone strength:

(A). Represents The Osteoprobe II™ bone diagnostic instrument consisting of a measurement head mounted on a stand, an electronics box, and a laptop computer from Biodent Laboratories. in vivo to measure the material properties of bone, the reference probe/test probe assembly is inserted through the skin down to the bone. The reference probe serves as a reference for measuring the distance that the test probe indented into the
bone. (B) The Indentation Distance Increase (IDI) and Creep Increase Distance for Control and Telmisartan Tibia bone is smaller than that for Rosiglitazone treated bone. This difference is statistically significant at the level of *p≤ 0.05 vs control and # p≤ 0.05 for these samples.

Figure 19: Comparison of Rosiglitazone and Telmisartan effects on OB phenotypic–specific gene expression in vitro cultures.

U33/γ2 cells were treated with either Vehicle (DMSO) or Rosi (1µM) or Tel (50µM) or a combination of Rosi and Tel. Total RNA was isolated and analyzed using Quantitative real time RT-PCR. Bars represent a relative mRNA expression of tested gene normalized to the levels of 18S rRNA. Vertical lines indicate standard deviation (SD) calculated from three independent PCR analyzes. Effects of treatments on OB specific genes Cola1 represents α1 (I) collagen, OC- Osteocalcin, Ctnnb1- β-catenin, Ccnd1- Cyclin D1 were analyzed.*p≤ 0.05 and ** p≤ 0.005 vs control. # p≤ 0.05 and ## p≤ 0.005 vs Rosi.

Figure 20: Comparison of Rosiglitazone and Telmisartan effects on TGFβ/BMP signaling pathway in vitro cultures.

U33/γ2 cells were treated with either Vehicle (DMSO) or Rosi (1µM) or Tel (50µM) or a combination of Rosi and Tel. Total RNA was isolated and analyzed using Quantitative real time RT-PCR. Bars represent a relative mRNA expression of tested gene normalized to the levels of 18S rRNA. Vertical lines indicate standard deviation (SD) calculated from
three independent PCR analyzes. Effects of different treatments on expression of TGFβ/BMP signaling pathway members like BMP4, TGFβ3, Smad1, Smad3, Smad6, and Smad7 were analyzed.*p ≤ 0.05, and ** p ≤ 0.005 vs. control. # p ≤ 0.05, and ## p ≤ 0.005 vs. Rosi.
### Table 1: Trabecular Parameters of L4 vertebrae and proximal Tibia

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<td>0.3±0.05</td>
<td>78.64±35.06</td>
<td>3.11±0.15</td>
</tr>
<tr>
<td>Rosi</td>
<td>0.10±0.03*</td>
<td>0.03±0.01**</td>
<td>2.44±0.38**</td>
<td>0.03±0.003</td>
<td>0.42±0.06**</td>
<td>20.11±15.18**</td>
<td>3.46±0.19**</td>
</tr>
<tr>
<td>TEL</td>
<td>0.18±0.04</td>
<td>0.06±0.01</td>
<td>3.55±0.55</td>
<td>0.04±0.004</td>
<td>0.29±0.05</td>
<td>71.92±26.9</td>
<td>3.09±0.21</td>
</tr>
<tr>
<td>TEL+Rosi</td>
<td>0.15±0.02#</td>
<td>0.05±0.01</td>
<td>2.73±0.17*</td>
<td>0.04±0.001#</td>
<td>0.37±0.02*</td>
<td>54.1±5.76##</td>
<td>2.97±0.07##</td>
</tr>
<tr>
<td><strong>Tibia</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.24±0.05</td>
<td>0.08±0.02</td>
<td>3.2±0.25</td>
<td>0.03±0.003</td>
<td>0.3±0.03</td>
<td>424.1±105</td>
<td>2.3±0.41</td>
</tr>
<tr>
<td>Rosi</td>
<td>0.16±0.05*</td>
<td>0.05±0.01**</td>
<td>2.61±0.3**</td>
<td>0.03±0.003</td>
<td>0.4±0.04**</td>
<td>232.7±85**</td>
<td>2.8±0.28*</td>
</tr>
<tr>
<td>TEL</td>
<td>0.20±0.11</td>
<td>0.07±0.03</td>
<td>2.8±0.44</td>
<td>0.03±0.005</td>
<td>0.4±0.06</td>
<td>380.1±202.8</td>
<td>2.53±0.5</td>
</tr>
<tr>
<td>TEL+Rosi</td>
<td>0.23±0.05#</td>
<td>0.07±0.005#</td>
<td>2.61±0.15**</td>
<td>0.04±0.002</td>
<td>0.4±0.03**</td>
<td>385.7±123.7#</td>
<td>2.15±0.1##</td>
</tr>
</tbody>
</table>

MicroCT analysis of bone microarchitecture: Table represents the bone parameters of L4 vertebrae and proximal tibia in all 4 groups of mice. Values are expressed as means ± SD of n= 4 to 9 animals per group. BV represents Bone Volume, BV/TV is ratio of bone volume to total volume, Tb.N is trabecular number, Tb.th trabecular thickness, Tb.Sp trabecular spacing, ConnD is connective density and SMI represents Structure Model Index. *p≤ 0.05, ** p≤ 0.005 vs control and # p≤ 0.05 ## p≤ 0.05 vs Rosi
Table 2: Serum Bone turnover markers

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Rosi</th>
<th>TEL</th>
<th>TEL+Rosi</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRAP5b(U/L)</td>
<td>2.22±0.18</td>
<td>2.85±0.5</td>
<td>1.67±0.21</td>
<td>1.35±0.24*#</td>
</tr>
<tr>
<td>ALP activity</td>
<td>0.35±0.16</td>
<td>0.17±0.13</td>
<td>0.34±0.27</td>
<td>0.36±0.11</td>
</tr>
</tbody>
</table>

Bone turnover markers in serum samples: Values are expressed as means ± SD of n= 4 to 9 animals per group. TRAP5b indicates tartrate-resistant acid phosphatase form 5b; ALP activity; Alkaline phosphatase activity which is an early osteoblast marker. *p≤ 0.05, ** p≤ 0.005 vs control and # p≤ 0.05 ## p≤ 0.005 vs Rosi
Figures:

Figure 1: Characterization of marrow-derived cell lines and results from microarray data analysis of TGFβ/BMP pathway:

A. Terminal differentiation in U-33γ2 cells

B. Microarray data representation of changes in gene expression of TGFβ/BMP pathway members

<table>
<thead>
<tr>
<th>Genes</th>
<th>Microarray (fold change)</th>
<th>Increase or Decrease</th>
<th>Function in OB phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tob1</td>
<td>1.8 folds</td>
<td>Increase at 2 hrs</td>
<td>Suppressor</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>1.8 folds</td>
<td>Increase at 24 hrs</td>
<td>Inducer</td>
</tr>
<tr>
<td>TGF-β3</td>
<td>1.6 folds</td>
<td>Decrease at 24 hrs</td>
<td>Inducer</td>
</tr>
<tr>
<td>TGF-βR2</td>
<td>1.5 folds</td>
<td>Decrease at 24 hrs</td>
<td>Inducer</td>
</tr>
<tr>
<td>SMAD1/3</td>
<td>2 folds</td>
<td>Decrease at 24 hrs</td>
<td>Inducer</td>
</tr>
<tr>
<td>SMAD4</td>
<td>1.7 folds</td>
<td>Decrease at 24 hrs</td>
<td>Inducer</td>
</tr>
<tr>
<td>SMAD7</td>
<td>1.7 folds</td>
<td>Decrease at 24 hrs</td>
<td>Suppressor</td>
</tr>
<tr>
<td>BMPR2</td>
<td>1.7 folds</td>
<td>Decrease at 72 hrs</td>
<td>Inducer</td>
</tr>
<tr>
<td>BMP4</td>
<td>1.7 folds</td>
<td>Decrease at 2 hrs</td>
<td>Inducer</td>
</tr>
</tbody>
</table>
C. Fibrodysplasia Ossificans Progressiva (FOP)
Figure 2: Comparison of glitazones effects, on osteoblastic and adipocytic phenotypes in U-33/γ2 cells:

A.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Drug</th>
<th>PPAR-γ binding</th>
<th>Rank order of anti-hyperglycemic potency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rosiglitazone</td>
<td>Avandia (GlaxoSmithKline, Inc)</td>
<td>0.04 µM</td>
<td>1</td>
</tr>
<tr>
<td>Pioglitazone</td>
<td>Actos (Takeda Pharmaceuticals, Inc.)</td>
<td>0.5 µM</td>
<td>2</td>
</tr>
<tr>
<td>Troglitazone</td>
<td>Rezulin (withdrawn from the market)</td>
<td>0.8 µM</td>
<td>3</td>
</tr>
</tbody>
</table>

B.

C.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cell type</th>
<th>PPAR-γ2</th>
<th>aP2</th>
<th>Dlx5</th>
<th>Runx2</th>
<th>OC</th>
<th>Coll</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rosiglitazone</td>
<td>U-33/γ2</td>
<td>4.0 *</td>
<td>2,558.0</td>
<td>0.18</td>
<td>0.23</td>
<td>0.01</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td>Bone</td>
<td>74.8</td>
<td>94.4</td>
<td>0.27</td>
<td>0.14</td>
<td>0.13</td>
<td>0.18</td>
</tr>
<tr>
<td>Pioglitazone</td>
<td>U-33/γ2</td>
<td>2.4</td>
<td>1,857.0</td>
<td>0.15</td>
<td>0.21</td>
<td>0.01</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>Bone</td>
<td>367.8</td>
<td>84.0</td>
<td>0.40</td>
<td>0.39</td>
<td>0.16</td>
<td>0.14</td>
</tr>
<tr>
<td>Troglitazone</td>
<td>U-33/γ2</td>
<td>2.9</td>
<td>2,234.0</td>
<td>0.14</td>
<td>0.19</td>
<td>0.01</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>Bone</td>
<td>160.8</td>
<td>108.0</td>
<td>0.39</td>
<td>0.32</td>
<td>0.07</td>
<td>0.17</td>
</tr>
</tbody>
</table>
Figure 2D: The effect of tested glitazones on the expression of components of TGFβ/BMP signaling

Bar charts showing the fold expression in treated cells vs. vehicle for TGF-β pathway and BMP pathway components. The graphs display the expression levels of TGF-β cytokines, receptors, and mediators (SMADs) across different treatments with different glitazones at various concentrations.
Figure 3: Schematic representation of TGFβ/BMP signaling pathway and effects of rosiglitazone on this pathway:

A. TGF-β1/BMP4 signaling pathway

B. Rosiglitazone effects on Smad1 and Smad3 protein expression (1), and TGF-β1 and BMP4 pathway activities (2)
Figure 4: Pictorial representation of *Tob1* suppressing *BMP* signaling
Figure 5: Effects of changes in expression of Tob1 on pro-osteoblastic markers and members of TGFβ/BMP signaling pathway:

A: Effects of overexpression of Tob1 on expression of Ob specific genes and on members of TGFβ/BMP signaling

B: Effects of Tob1 gene silencing on expression of Ob specific genes and on members of TGFβ/BMP signaling
Figure 6: Effects of BMP4 gene silencing on expression of OB specific genes and on members of TGFβ/BMP signaling
Figure 7: Effects of Tob1 expression on cell proliferation and effects of rosiglitazone on Tob1 protein as detected by Tob1 antibody after immunoprecipitation:

A. Tob1 overexpression on Cell proliferation

B. Effects of Rosi treatment on Tob1 Protein detected by Tob1 ab after Co-IP
Figure 8: Mechanism of TZD-induced bone loss: (A). Schematic representation of showing how anti-diabetic drugs TZDs, high affinity ligands for PPARγ, affects bone cell differentiation and cause bone loss. (B). Schematic representation of mechanism of TZD-induced bone loss.
Figure 9: Schematic representation of renin angiotensin aldosterone system (RAAS) and telmisartan blocks the AT1R, and decreases the hypertension.
Figure 10: Effects of rosiglitazone and different sartans on cell proliferation
Figure 11: Telmisartan has weak pro-adipocytic activity compared to rosiglitazone:
Figure 12: Telmisartan induces brown adipose tissue specific genes
Figure 13: Comparison of different sartans on OB-phenotype in invitro cultures

A. ALP Activity (uUnits/Min) for Vehicle 1R 12T 25T 50T

B. ALP Activity (uUnits/min) for Vehicle 1R 12L 25L 50L 100L

C. ALP Activity in PBMC cells for Vehicle 1R 50T 50T+1R

D. ALP Activity (uUnits/Min) for Vehicle 1R 50T+R

E. ALP Activity in PBMC cells for Vehicle 1R 50T 50T+1R
Figure 14: In vitro analysis of rosiglitazone and telmisartan effects on osteoclast differentiation and gene expression:
Figure 15: TEL effectively lowers glucose similar to rosiglitazone in agouti mice:
Figure 16: Comparison of anti-diabetic PPARγ ligands (rosiglitazone & telmisartan), effects on different metabolic parameters:

A. Percentage Change in Weights within the groups

B. BAT Weight (gms)

C. WAT in Percentage

D. Random Glucose
Figure 17: MicroCT analysis of bone microarchitecture and its images:

### A.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Rosi</th>
<th>TEL</th>
<th>T+R</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ratio of BV/TV</strong></td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td><strong>Trabecular Number</strong></td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td><strong>Trabecular Spacing</strong></td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td><strong>Trabecular Thickness</strong></td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td><strong>Connective Density</strong></td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
</tbody>
</table>

### B.

![Graphs showing BV/TV, Trabecular Number, Trabecular Spacing, Trabecular Thickness, and Connective Density]
Figure 18: The Osteoprobe II™ bone diagnostic instrument and measurement of different parameters of bone strength:

A.

B.

![Graphs showing indentation distance increase and creep indentation distance for Control, Rosi, and TEL groups with statistical significance markers (*) and (#).]
Figure 19: Comparison of rosiglitazone and telmisartan effects on OB phenotypic-specific gene expression in vitro cultures.
Figure 20: Comparison of rosiglitazone and telmisartan effects on TGFβ/BMP signaling pathway in vitro cultures.
References:


analysis of the dual roles of BMPs in regulating adipogenic and osteogenic differentiation of mesenchymal progenitor cells. Stem Cells Dev l2009;18: 545-59.


receptor antagonist with selective PPARgamma-modulating activity. Hypertension 12004;43: 993-1002.


[119] Yamana A, Arita M, Furuta M, Shimajiri Y, Sanke T. The angiotensin II receptor blocker telmisartan improves insulin resistance and has beneficial effects in hypertensive


