Original Research Article

Angiotensin receptor I stimulates OSTEOPROGENITOR PROLIFERATION through tgfβ - mediated signaling†

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Running head: AT1R signaling in osteoblast differentiation

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Abstract

Clinical studies of large human populations and pharmacological interventions in rodent models have recently suggested that anti-hypertensive drugs that target angiotensin II (Ang II) activity may also improve loss of bone mineral density. Here we identified in a genetic screen the Ang II type I receptor (AT1R) as a potential determinant of osteogenic differentiation and, implicitly, bone formation. Silencing of AT1R expression by RNA interference severely impaired the maturation of a multipotent mesenchymal cell line (W20-17) along the osteoblastic lineage. The same effect was also observed after the addition of the AT1R antagonist losartan but not the AT2R inhibitor PD123,319. Additional cell culture assays traced the time of greatest losartan action to the early stages of W20-17 differentiation, namely during cell proliferation. Indeed, addition of Ang II increased proliferation of differentiating W20-17 and primary mesenchymal stem cells and this stimulation was reversed by losartan treatment. Cells treated with losartan also displayed an appreciable decrease of activated (phosphorylated)-Smad2/3 proteins. Moreover, Ang II treatment elevated endogenous transforming growth factor β (TGFβ) expression considerably and in an AT1R-dependent manner. Finally, exogenous TGFβ was able to restore high proliferative activity to W20-17 cells that were treated with both Ang II and losartan. Collectively, these results suggest a novel mechanism of Ang II action in bone metabolism that is mediated by TGFβ and targets proliferation of osteoblast progenitors.

Introduction

Genetic and environmental factors are implicated in the onset and progression of hypertension and osteopenia/osteoporosis, two major age-related diseases that are an increasing healthcare burden in industrialized countries. Different degrees of low bone mineral density (BMD) characterize disease progression from the largely asymptomatic osteopenia to the increased risk for fractures in osteoporosis (NIH Consensus Development Panel on Osteoporosis Prevention, 2001). Low BMD reflects the imbalance between bone formation by osteoblasts and bone resorption by osteoclasts during physiological bone remodeling. This homeostatic process involves a large number of systemic signals that are produced by different organ systems, such as parathyroid hormone, blood calcium and leptin, as well as local signals that are secreted by cells or released from the extracellular matrix (ECM), such as chemokines and transforming growth factor β (TGFβ) signaling molecules (Karsenty, 2006; Kitahara et al., 2003; Ono et al., 2007). Equally complex is the clinical presentation of hypertension in which dysregulation of the renin-angiotensin system (RAS) contributes greatly to disease progression. RAS normally regulates water fluid balance and blood pressure by promoting renin-induced production of angiotensin II (Ang II) both systemically and locally in several tissues, including bone (Daniels et al., 2007; Izu et al., 2009). Indeed, a number of clinical studies have suggested a potential association between hypertension and BMD and between the latter and stroke in selected cohorts of patients (Dennis et al., 2002; Kanis et al., 2001; von der Recke et al., 1999). Furthermore, Lynn et al. have recently reported higher than normal BMD in elderly individuals who had been treated with angiotensin-converting enzyme (ACE) inhibitors (Lynn et al., 2006). These observations have, therefore, raised the intriguing possibility that anti-hypertensive drugs may also reduce bone loss. Unfortunately, progress in this direction has been hampered by our limited understanding of RAS activity in the skeleton and by controversial findings of RAS action in rodent models of bone loss.

Ang II signals through receptors 1 and 2 (AT1R and AT2R, respectively) to activate G protein and non-G protein-related transduction pathways with a myriad of highly contextual cellular outcomes. For example, whereas AT1R antagonism prevents experimentally induced abdominal aortic aneurysm, AT2R blockade accelerates it (Lucarini et al., 2009). In spite of the aforementioned clinical evidence, the role of Ang II signaling in bone metabolism remains controversial with reports of both positive and negative results (Broulik et al., 2001; Gu et al., 2012; Izu et al., 2009; Shimizu et al., 2009). On the one hand, the angiotensin receptor blocker (ARB) olmesartan has been shown to attenuate bone loss in ovariectomized hypertensive rats and to inhibit Ang II stimulation of receptor activator of nuclear factor κ-B ligand (RANKL) production in cultured osteoblasts (Shimizu et al., 2008). Moreover, the systemic administration of the ARB blocker telmisartan has been proven effective in improving bone callus formation and fracture healing in mice (Zhao et al., 2014). On the other hand, no changes in baseline bone density have been noted in rodents systemically treated with the ARB losartan, and pharmacological or genetic inactivation of AT2R was found to increase bone mass in mice (Broulik et al., 2001). Additionally, earlier cell culture experiments have indicated that Ang II treatment inhibits the maturation of primary calvaria osteoblasts, which represent a highly heterogenous population of cells at different stages of differentiation (Hagiwara et al., 1998). More recently, it has been shown that Ang II administration induces mitochondrial alterations and apoptosis in the same cellular system (Li et al., 2014).

Blockade of AT1R activity has also been shown to lower promiscuous TGFβ signaling in experimental renal and cardiac fibrosis, and in mouse models of and in patients afflicted with Marfan syndrome (MFS), (Brooke et al., 2008; Cohn et al., 2007; Habashi et al., 2006). MFS is a congenital disease of the connective tissue that is caused by mutations in the ECM protein fibrillin-1 and that includes osteopenia as part of the phenotype (Ramirez and Dietz, 2007b). MFS mutations impair tissue integrity by interfering with the formation and/or stability of fibrillin-1 assemblies (microfibrils) as well as with the ability

of microfibrils to sequester and store latent TGFβ complexes and bone morphogenetic proteins (BMPs) in the ECM (Ramirez and Dietz, 2007a; Nistala et al., 2010a). Relevant to bone formation and remodeling, TGFβ is a key local regulator of osteogenic differentiation that promotes recruitment and proliferation of progenitor osteoblasts and inhibits differentiation of committed pre-osteoblasts (Roelen and Dijke, 2003). In this respect, locally released TGFβ signals are believed to participate in controlling the overall rate of bone formation by balancing the pool of mesenchymal progenitors that are committed to the osteoblast lineage and the pool of committed osteoblasts that are primed to undergo terminal differentiation (Nistala et al., 2010a). It follows that the effects of RAS activity on TGFβ signaling may be different depending on whether they are examined in cell cultures that represent mesenchymal progenitors or committed osteoblasts, as it was the case in the previous report of Ang II inhibition of calvarial osteoblast maturation.

The present study describes the characterization of AT1R as one of the gene products that drives osteogenic differentiation, in part, through the activation of TGFβ signaling in mesenchymal progenitor cells. AT1R identification was based on a high-throughput screening protocol that employed the expression of a small hairpin RNA (shRNA)-containing library in a multipotent murine cell line (W20-17) induced to differentiate into osteoblasts and that used mineral nodule formation as an informative read-out of in vivo bone formation. The results of our cell culture experiments are consistent with the notion that Ang II stimulates TGFβ production and progenitor osteoblast proliferation through the activity of AT1R. These findings shed new light on the controversial role of RAS activity in bone mass maintenance and implicitly, on the potential benefit of using anti-hypertensive drugs to improve low BMD in individuals afflicted with osteopenia/osteoporosis.

materials and methods

W20-17 culture conditions

W20-17 cell line was purchased from American Type Culture Collection (ATCC/LGC standards, Milan, Italy). Primary murine mesenchymal stem cells (mMSCs) were isolated from C57Bl/6 mice's bone marrow as described previously (Esposito et al., 2009). Human mesenchymal stem cells (hMSCs) were isolated from bone marrow of healthy adult donors after informed consent (Mariotti et al.,2008). mMSCs and W20-17 cells were seeded at a density of 6,000 cells/cm^2 and cultured in regular medium consisting of D-MEM (Euroclone, Siziano, Italy) supplemented with 10% fetal bovine serum (HyClone, Northumberland, UK) and 4 mM L-glutamine (Gibco, Paisley, UK) in absence of antibiotics. hMSCs were seeded at a density of 5,000 cells/cm^2 and cultured in regular medium consisting of D-MEM supplemented with 20% fetal bovine serum and 4 mM L-glutamine in absence of antibiotics. For osteoblastic differentiation induction, cells were cultured in D-MEM with 10% fetal bovine serum, 4 mM L-glutamine, 0.05 mM ascorbic acid 2-phosphate (Sigma, St. Louis, MO US), 10 mM glycerol 2-phosphate (Sigma, St. Louis, MO US), 10^{-6} M dexamethasone (Sigma, St. Louis, MO US). Cells were grown to 70%-90% confluence and then cultured in osteogenic medium for 21 days.

Alizarin red and alkaline phosphatase staining

For alizarin red staining, W20-17 cells were washed with PBS and fixed in 10% formaldehyde (Sigma-Aldrich, St. Louis, MO) for 1 hour; after rinsing with distilled water, they were incubated with 2% alizarin red S (Sigma-Aldrich, St. Louis, MO) at pH 4.1 for 10 minutes. Excess staining was removed using water (Digirolamo et al., 1999). After visual examination, the mineralized deposit-bound dye was extracted adding 100 µl of 4 M guanidine–hydrochloric acid (Sigma-Aldrich, St. Louis, MO) and incubating them over night at room temperature. A semi-quantitative assay was used to determine the amount of cell-bound dye assessing absorbance at 490 nm of a 10-times dilution of the resulting supernatant. For alkaline phosphatase (AP) staining, cells were washed after 14 days of osteogenic differentiation with PBS and fixed in 10% cold neutral formalin buffer (10% formalin, 0.1M Na_2HPO_4, 0.029M NaH_2PO_4, Sigma-Aldrich, St. Louis, MO).

Cells were then rinsed with distilled water and stained with a substrate solution containing 0.24 mM naphthol AS MX-PO_4 (Sigma-Aldrich, St. Louis, MO), 0.4% N,N-dimethylformamide (Sigma-Aldrich, St. Louis, MO), 1.6 μM red violet LB salt (Sigma-Aldrich, St. Louis, MO S) in 0.2 M Tris-HCl pH 8.3 (Carlo Erba Reagenti, Milano, Italy) for 45 minutes at room temperature. Excess staining was removed washing twice with distilled water and AP-positive cells visualized under microscope. Determination of AP activity was performed as previously described (Bruder et al., 1997). Briefly, cells were washed twice with Tyrode's balanced salt solution (50 mM Tris-HCl pH 7.4, 0.15 M NaCl) and then incubated with 5 mM p-nitrophenylphosphate (Sigma-Aldrich, St. Louis, MO) in 50 mM glycine (Sigma-Aldrich, St. Louis, MO US), 1 mM $MgCl_2$ (Carlo Erba Reagenti, Milano, Italy), pH 10.5 at 37°C for 15 minutes. The reaction was stopped with 3 M NaOH (Carlo Erba Reagenti, Milano, Italy). Relative activity was determined measuring absorbance at 405 nm.

shRNA-mediated screening

W20-17 cells were plated in 96-well plates at a density of 8,000 cells/cm^2 . Transfections of the different shRNA-expressing plasmids (Open Biosystem, Huntsville, AL) were performed using 0.2 μl of Lipofectamine 2000 (Invitrogen, Paisley, UK) with 200 ng of plasmid, according to manufacturer's instructions. Transfected cells were enriched with a 2 μg/ml puromycin selection in regular medium (Sigma, St. Louis, MO) for 3 days, then cultured in differentiation medium for 21 days, as previously described. Cells were stained with alizarin red to identify shRNAs that were able to interfere with the differentiation process. Wells completely or partially stained with alizarin red were scored as differentiated, whereas those not stained were considered positive and the relevant genes were further analyzed as potentially relevant for osteoblast differentiation.

Real-time PCR quantitation of osteoblast differentiation markers

Total RNA was extracted from W20-17 cultured in control or differentiation media at different time points using TriReagent (Sigma-Aldrich, St. Louis, MO) and then treated with DNase I (Ambion, Monza, Italy). Reverse transcription polymerase chain reaction (RT-PCR) was performed with M-MuLV Reverse Transcriptase (New England BioLabs, Beverly, MA) using 2 μg of total RNA with 5 μm random primers. Real time PCR was performed using the SYBR Green PCR master mixture in an ABI PRISM 7500 apparatus (Applera, Foster City, CA). Levels of target genes were quantified using specific oligonucleotide primers and normalized for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression.

Angiotensin II signaling blockade

Angiotensin II (Sigma, St. Louis, MO), PD123,319 (Sigma, St. Louis, MO) and losartan potassium (Fluka, St. Louis, MO) were added to the medium every day during differentiation protocols as described for each different experiment in the Results section.

Proliferation assays

Cells were seeded in 96-well plates and cultured for 4 days in osteogenic medium. Cells were then washed with PBS and cultured for 48 hours as described in the Results section. For rescue experiments, 5 or 10 ng/ml of human recombinant TGFβ1 (Sigma, St. Louis, MO) were added to the medium 1 hour after losartan treatment. Proliferation was determined with CellTiter 96 AQueous Non-Radioactive assay (Promega, Madison, WI) measuring absorbance at 490 nm, according to manufacturer's instructions.

Western blot assays

Cells were treated with lysis buffer (1mM EDTA, 70 mM NaCl, 1% Triton X-100, 50 mM Tris-HCl, pH 7.5), in presence of proteinase inhibitors (Complete Protease Inhibitor Cocktail, Roche) and phosphatase inhibitors (5 mM sodium fluoride and 1mM sodium orthovanadate). Proteins were quantified using Bradford solution (AppliChem, Darmstadt, Germany). Protein extracts were run on 10% SDS-PAGE gels, transferred on Immuno-Blot PVDF membranes (Bio-Rad, Segrate, Italy). Western blots were performed with the following antibodies: Phospho (p)Smad2/3 (monoclonal, Cell Signaling, Danvers, MA US), Smad2/3 (monoclonal, Cell Signaling, Danvers, MA),

pSmad1/5/8 (polyclonal, Cell Signaling, Danvers, MA US), Smad1 (polyclonal, Cell Signaling, Danvers, MA), AT1R (polyclonal, Santa Cruz, Heidelberg, Germany), GAPDH (monoclonal, Santa Cruz, Heidelberg, Germany) and secondary anti-mouse or anti-rabbit horseradish peroxidase-conjugated antibodies (Amersham Bioscience, Uppsala, Sweden). Detection was performed by chemiluminescence, according to manufacturer's protocols (ECL plus, GE Healthcare). Results were quantitated using Chemidoc (Biorad, Hercules, CA, USA), according to manufacturer's instructions.

Statistical analysis

Data are expressed as mean values ± standard deviation of 3 independent experiments. Two-tailed Student's t test was performed using GraphPad Prism 5.0 software (San Diego, CA, USA). A P-value <0.05 was considered statistically significant.

Results

shRNA-mediated identification of AT1R as a regulator of osteoblast differentiation

We have previously transfected mouse embryonic stem cells with a library of shRNA-expressing plasmids in order to identify genes that confer stemness (Parisi et al., 2008). The same library, that consists of 66,600 shRNAs covering the entire murine transcriptome, was used in the present study to identify genes involved in osteoblast differentiation. Briefly, until now 10,000 independent shRNAs were stably transfected in cells with features of immature mesenchymal progenitors (W20-17 cell line) under previously established conditions that ensure chromosomal integration of shRNA plasmids. Puromycin-resistant cell clones were then scored for the inability of forming mineral nodules 18-21 days after the addition of osteogenic medium compared to cells transfected with non-silencing shRNA used as controls (Fig. 1, panel A). Parallel W20-17 cells transfected with plasmids expressing shRNAs for known determinants of osteoblastogenesis, such as BMP1 and Smad5, served as reference cultures of failed ECM mineralization (Fig. 1, panels B and C). Such a screening protocol identified 650 shRNA-transfected cell clones that displayed a substantial paucity of mineralized nodules. The 650 shRNAs corresponded to 208 genes with unknown role at the time of the screening and to 442 known genes, which Gene Ontology segregated into 5 functionally separate groups (Fig. 1, panel D). A partial list representative of the different functional categories of candidate genes identified during the screening is reported in Suppl. Table 1. The present report focuses on the characterization of the gene encoding for AT1R; characterization of other genes potentially implicated in osteogenic differentiation will be described elsewhere along with additional details of the shRNA screen.

Following AT1R identification by the shRNA screening, we confirmed the relevance of this gene for proper osteoblast maturation with an additional silencing experiment with an independent shRNA against a different sequence of AT1R (Fig. 2). This conclusion was independently validated by the finding that losartan, a specific AT1R antagonist, reduced alizarin red staining of W20-17 cultures nearly to the same extent as AT1R silencing (Fig. 3, panels A through E). As expected, losartan treatment also brought down to base-line levels the AP activity of osteoinduced W20-17 cultures (Fig. 3, panel F). Furthermore, quantitative Real-time PCR (qPCR) revealed that AT1R is expressed earlier and at a higher level than AT2R, suggesting a possible involvement of the former gene product in the early stages of osteoblast differentiation (Fig. 3, panel G). In addition, we observed that ATR1 shRNA-mediated interference reduced RunX2 and AP levels during differentiation (Suppl. Fig. 1). Incidentally, treatment of W20-17 cultures with the AT2R inhibitor PD123,319 had virtually no effect on ECM mineralization, thus excluding a prominent role of this Ang II receptor in the differentiation process of osteoblast progenitors (Fig. 3, panels A through E).

AT1R signaling is mostly required for pre-osteoblast proliferation

The next set of experiments was designed to interrogate the stage in which AT1R activity is most relevant for the progression of W20-17 cell differentiation. Accordingly,

W20-17 cells were first treated with losartan or PD123,319 at the time of (day 0) or 7 or 14 days after addition of osteogenic medium and scored for mineral nodule formation at day 21 of differentiation (Fig. 4). This assay correlated most of the inhibitory activity of losartan on osteoblast maturation with the first week of cell culture when differentiating W20-17 cells are in the proliferative phase (Fig. 4, panel A-C and Suppl Fig. 2). Consistent with the above data (Fig. 3), PD123,319 treatment had no effect on W20-17 maturation, irrespective of the time of administration of the AT2R inhibitor (Fig. 4, panels D-F). In the second assay, cell proliferation was measured in osteoinduced or untreated W20-17 cells cultured in the presence of increasing Ang II concentrations. Unlike cells grown in regular medium, those cultured in osteogenic medium displayed a dose-dependent increase in proliferative activity in response to Ang II treatment (Fig. 5, panel A). Importantly, losartan (but not PD123,319) completely abrogated Ang II-induced stimulation of W20-17 cell proliferation (Fig. 5, panel B). Ang II treatment also stimulated proliferation of primary mMSCs, albeit to a minor extent compared to W20-17 cells; increase in proliferation was reversed by losartan also in mMSCs, thus further implicating the hormone and the AT1R receptor in the physiological regulation of bone formation (Fig. 5, panel B).

AT1R inhibition blocks SMAD-mediated induction of proliferation

TGFβ promotes proliferation of osteoblast progenitors and AT1R stimulates canonical TGFβ signaling in several cell types (Urano et al., 1999; Chon RD et al., 2007; Zhang et al., 2014). Therefore, we evaluated whether losartan inhibition of W20-17 cell proliferation was associated with a reduction of TGFβ signaling in these cells. Western blot analyses revealed a significant decrease in the relative ratio of pSmad2/3 to Smad2/3 in losartan-treated compared to untreated cells (Fig. 6, panels A and B). By contrast, no differences were noted in the relative ratio of pSmad1/5/8 to Smad1, which transduce BMP signals (Fig. 6, panels A and C). Taken at face value, these results suggested specificity of losartan inhibition of canonical TGFβ signaling. In accordance with recent evidence suggesting that Ang II stimulates TGFβ expression (Li and Zhuo, 2008), qPCR analyses revealed a substantial increase of TGFβ transcript levels in W20-17 cells cultured in osteogenic medium or in regular medium supplemented with Ang II (Fig. 6, panel D); in addition, osteogenic medium induced also a stimulation of Angiotensinogen expression, thus showing the presence of an autocrine loop and further corroborating Ang II relevance in this developmental process (Suppl Fig. 3). In both cases, TGFβ up-regulation was blunted by the addition of losartan, further confirming the causal relationship between Ang II and TGFβ signaling in these cells (Fig. 6, panel D). Moreover, addition of TGFβ to W20-17 cells cultured in presence of both Ang II and losartan restored cell proliferation to the same level as in cultures with only Ang II (Fig. 6, panel E). Lastly, we confirmed a decrease in pSmad2/3 accumulation in mMSCs cultured for 4 days in the presence of losartan; this observation further reiterates the physiological significance of our findings (Fig. 6, panels A-C). Collectively, these results were interpreted to indicate that Ang II stimulates TGFβ production and osteoblast progenitors proliferation through the activity of AT1R. This conclusion is consistent with the well-established role of TGFβ signaling in promoting proliferation of progenitor but not differentiated osteoblasts (Erlebacher and Derynck, 1996; Urano et al., 1999). Importantly, we were able to confirm these findings also in hMSCs. Indeed, losartan, but not PD123,319, completely abrogated Ang II-induced stimulation of proliferation of hMSCs pre-treated with osteogenic medium, whereas the addition of TGFβ counteracted losartan inhibitory effect and restored cell proliferation (Suppl Fig. 4). These findings show that, as in mouse, Ang II stimulates proliferation of osteoprogenitor cells even in human; this effect is AT1R-mediated and occurs, at least in part, through the activation of TGFβ pathway.

Discussion

In spite of suggestive clinical evidence and experimental data, RAS role in bone mass maintenance remains ill defined and, consequently, the potential benefits of anti-hypertensive drug therapies in osteopenia/osteoporosis have yet to be demonstrated.

The present study identified AT1R as one of the gene products that drive osteoblast differentiation, in part, through the stimulation of TGFβ production, which in turn participates in promoting progenitor cell proliferation. This conclusion was based on the findings that *At1r* gene silencing by shRNA or AT1R antagonism by losartan similarly impair maturation of multipotent W20-17 cells that are induced to differentiate into osteoblasts, and that Ang II stimulates *Tgfβ* gene expression and W20-17 cell proliferation in an AT1R-dependent manner. While our results are consistent with an earlier report by Hiruma et al. (1997) that implicated Ang II in promoting calvarial osteoblast proliferation via AT1R (Hiruma et al., 1997), they are also at odd with the finding by Hagirwara et al. (1998) that the hormone decreases AP activity and mineral nodule formation in the same cell culture system (Hagiwara et al., 1998). We believe that these discrepancies may simply reflect contextual differences in Ang II action between cells that represent distinct stages of osteoblast differentiation and/or different populations of differentiating osteoblasts. We support our contention by noting that TGFβ signaling promotes proliferation of osteoprogenitor cells, which were used in our study, but inhibits proliferation of differentiated osteoblasts, which were used in the Hagiwara et al. investigations (Erlebacher and Derynck, 1996; Urano et al., 1999).
Although relevant to the physiological condition, the analysis of Ang II impact on W20-17 cell differentiation was limited to the anabolic arm of bone remodeling without taking into account other important aspects operating in vivo. Among other factors, bone remodeling is driven by local coupling between the opposing activities of osteoblasts and osteoclasts, as well as by osteoblast regulation of osteoclast differentiation and activity through the production of positive (M-CSF and RANKL) and negative (osteoprotegerin) osteogenic factors. RAS regulation of bone homeostasis is similarly complex. Shimuzu et al. have shown that ACE inhibitors can block BMD decrease in ovariectomized spontaneously hypertensive rats by attenuating osteoclast activity (Shimizu et al., 2009). In a separate study, other investigators have concluded that Ang II promotes bone loss by increasing osteoblast production of RANKL through AT1R-mediated induction of the mitogen-activated protein kinases (MAPKs) (Asaba et al., 2009). There is also genetic evidence for a functional interaction between the two Ang II receptors with regard to osteoblast-supported osteoclastogenesis through RANKL production (Asaba et al., 2009). Ongoing investigations are examining how Ang II and cognate receptors influences the expression of pro- and anti-osteoclastogenic factors in our cell model of progenitor osteoblasts. Recent data have in fact demonstrated that Ang II-mediated activation of AT1R regulates osteoclast differentiation *in vitro* as well as in mice developing skeleton. The authors have shown that losartan administration increases bone mass and impairs osteoclastogenesis both *in vivo* and in a cellular model through the suppression of RANKL-mediated activation of MAPK pathway (Chen et al., submitted).
Moreover, it has recently been shown that Ang II could affect bone mass homeostasis also by regulating ECM turnover in osteoid by osteoblasts. Indeed, Nakai et al. demonstrated that Ang II administration was able to induce the production of matrix metalloproteinases (MMPs) -3 and -13 in ROS17/2.8 rat osteosarcoma cell line; this effect seems to be AT1R-mediated since losartan but not PD123,319 completely abrogated Ang II-induced MMPs expression (Nakai et al., 2013).
Pharmacological interventions both in cell cultures and rodents have recently connected AT1R activity with the stimulation of the canonical TGFβ signaling and ECM production. Accordingly, AT1R antagonism has been used in animal models as well as in patients with fibrotic conditions driven in part by TGFβ signaling, such as scleroderma (Kawaguchi et al., 2004), or with congenital abnormalities associated with improper TGFβ activation, such as aortic aneurysm in MFS (Brooke et al., 2008; Habashi et al., 2006). Our findings are consistent with these data in that they indicate that Ang II is a positive regulator of TGFβ production in osteoblasts. The following lines of evidence support this contention; (a) Ang II enhances the accumulation of *Tgfβ* transcripts in undifferentiated W20-17 cells; (b) AT1R antagonism blunts Ang II enhancement of *Tgfβ* expression in undifferentiated W20-17 cells and Smad2/3 activation during cell differentiation of W20-7 cells as well as mMSCs; (c) both TGFβ and Ang II stimulate

W20-17 cells and hMSCs proliferation in an AT1R-dependent manner; and (d) TGFβ can restore high proliferative activity in W20-17 cells and hMSCs treated with both Ang II and losartan. Based on these findings, it is therefore conceivable that ARB strategies might prove effective not only in normalizing aortic aneurysm but also osteopenia in MFS. However, Nistala et al. have shown that losartan administration was able to ameliorate aortic wall degeneration but not bone loss in a mouse model of MFS (Nistala et al., 2010b). This result could be due to the complex interplay between TGFβ and other local or systemic regulators of bone homeostasis that may be altered in MFS, such as the BMPs normally sequestered and store by fibrillin-1 assemblies in the ECM. Moreover, another possible explanation could be associated to the mouse model employed in the study and the pathogenic mechanism underlying osteopenia. Indeed, in the fibrillin-1 mutant mice (*Fbn1*$^{mgR/mgR}$) used by Nistala et al., osteoblasts develop normally but stimulate osteoclast activity *in vitro* more than control cells, suggesting a probable alteration in bone resorption rather than in bone formation. On the other end, in the tight skin (*Tsk/*+) mouse model of MFS, osteopenia seems to be correlated to a reduced rate of osteoblast maturation and, therefore, losartan treatment could be beneficial. In fact, while ARBs and ACE inhibitors represent promising new means to manage age-related osteopenia/osteoporosis, pharmacologic inhibition of AT1R signaling may also have unexpected consequences on BMD depending on the pathogenetic mechanism underlying disease progression. Decreased osteoblast proliferation may reduce RANKL-mediated stimulation of osteoclast proliferation with the net effect of normalizing bone metabolism. ARB therapy could also prove beneficial in diseases due to hyperproliferation of mesenchymal stem cells or progenitor osteoblasts. By contrast, ARBs may exacerbate bone loss in conditions in which there is an osteoblast-independent increase in bone resorption or characterized by an impaired osteoblast maturation.

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Figure legends

Figure 1. shRNA-mediated silencing allows identification of genes involved in osteoblastogenesis. W20-17 cells transfected with shRNAs interfering with control (scrambled) shRNA are able to differentiate into osteoblasts and produce mineralized deposits (panel A); on the other hand, cells transfected with shRNAs interfering with BMP1 (panel B) or SMAD5 (panel C), genes known to be relevant for osteoblastogenesis, are unable to produce mineralized nodules. Scale bar corresponds to 100 μm. Genes that, when interfered, produced absence of mineral deposits were classified for their ontology; most of the genes were of unknown function whereas a significant proportion were involved in signal transduction (panel D). AT1R belongs to the “signal transduction / G-protein-coupled receptor protein signaling pathway” group.

Figure 2. Silencing of AT1R gene reduces osteoblast-mediated calcium deposition after 21 days of differentiation. Cells transfected with shAT1r(1) (panel A) and shAT1r(2) (panel B) show absence of alizarin red staining whereas untransfected cells (panel C) and cells transfected with a non silencing shRNA (panel D) show mineral deposits. Scale bar corresponds to 100 μm. Microscopy data are confirmed by alizarin red dye quantification (panel E): differences in mineral deposition between cells where AT1R had been silenced and controls are highly significant (**$p<0.01$). Effective silencing of AT1R has been confirmed in W20-17 cells transfected with shAT1r(1) or shAT1r(2) that show absence of the protein product after western blot analysis with specific antibodies. GAPDH levels were included as loading control (panel F).

Figure 3. Pharmacological inhibition of AT1R reduces osteoblast-mediated calcium deposition after 21 days of differentiation. W20-17 cells treated with osteogenic medium (panel A) and the AT2R inhibitor PD 123,319 (panel C) show mineral deposits whereas cells treated with osteogenic medium and losartan show a significant reduction (*$p<0.05$) of alizarin red staining (panel B); untreated cells do not show a significant presence of mineral deposition (panel D). Scale bar corresponds to 100 μm. Microscopy data were confirmed by alizarin red dye quantification: cells treated with osteogenic medium and losartan or with regular medium showed a significant decrease in the absorbance at 490 nm (*$p<0.05$ and **$p<0.01$, respectively) compared to cells treated with osteogenic medium (panel E, results are reported as fold change compared to cells treated with osteogenic medium alone). Losartan-treated cells reduced also AP activity after 14 days of treatment with osteogenic medium (panel F, results are reported as fold change compared to cells treated with osteogenic medium alone). Expression of AT1R in the first days of differentiation (black bars) is compatible with a relevance of this gene in the early stages of differentiation; lower levels and delayed expression of AT2R (white bars) are compatible with a function of this gene in differentiated osteoblast (panel G, results are reported as ATR/GAPDH levels ratio).

Figure 4. Inhibition of AT1R in the early days of differentiation is able to reduce osteoblast-mediated calcium deposition after 21 days of differentiation. W20-17 cells were kept in osteogenic medium and stained after 21 days of differentiation. Losartan and PD 123,319 were added to the medium starting from day 0 (panel A and D, respectively), from day 7 (panel B and E) and from day 14 (panel C and F). Staining of

control W20-17 cells differentiated in osteogenic medium with no addition is shown in panel G. Scale bar corresponds to 100 μm. Only cells treated with losartan from day 0 (panel A) show reduction of alizarin red staining. Ratio of alizarin red absorbance in the treated cells versus control cells is reported in panel H: only cells treated with losartan from day 0 show a significant (**p<0.01) alteration of this ratio confirming the previous observations.

Figure 5. Ang II-mediated stimulation of AT1R is required for proliferation of W20-17 cells treated with osteogenic medium. Addition of Ang II to W20-17 prestimulated with osteogenic medium significantly (**p<0.01) increases proliferation in a dose-dependent (*p<0.05 and **p<0.01) fashion (panel A, black bars); naïve W20-17 cells show minimal increase of proliferation (panel A, white bars). Ang II-mediated stimulation of proliferation (*p<0.05) in differentiating W20-17 (black bars) and primary bone marrow mMSCs (white bars) is inhibited by losartan-mediated AT1R pharmacological blockade as well as by AT1R shRNA whereas PD123,319-mediated inhibition of AT2R has no effect (panel B).

Figure 6. Pharmacological inhibition of AT1R reduces SMAD2/3 phosphorylation through inhibition of TGFβ transcription. W20-17 cells treated with osteogenic medium (Control) show increase of phosphorylation of SMAD2/3, usually activated by TGFβ signaling, at the early time-points of differentiation and absence of pSMAD2/3 at the end of the mineralization process, whereas cells treated with osteogenic medium and losartan do not show an increase of SMAD2/3 phosphorylation (panel A); data were confirmed in differentiating primary mMSCs after 4 days of treatment (C: control cells and L: cells treated with losartan). Levels of pSMAD1/5/8 are not significantly modified in losartan-treated W20-17 and mMSC cells (panel A). GAPDH levels were included as loading control (panel A). Ratio between pSMAD2/3 and SMAD2/3 (panel B) and pSMAD1/5/8 and SMAD1 (panel C) were obtained after quantitation of the relative signals at the different time-points; the black and white bars refer to control and losartan-treated cells, respectively (panel B and C). Treatments with either osteogenic medium or Ang II induce a significant increase of TGFβ mRNA levels (*p<0.001) that in both cases is reversed by losartan (panel D, results are reported as TGFβ/GAPDH expression levels ratio); in addition, losartan-mediated inhibition of proliferation of cells treated with osteogenic medium or Ang II is counteracted by the addition of TGFβ in the medium (*p<0.05 and **p<0.01) (panel E).

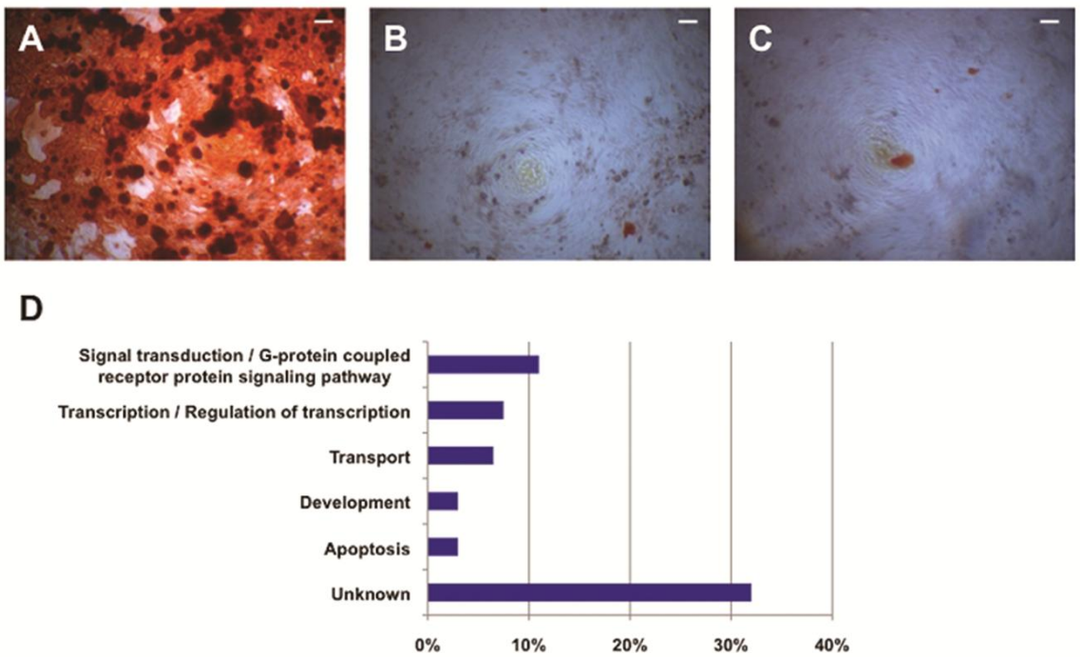

Figure 1

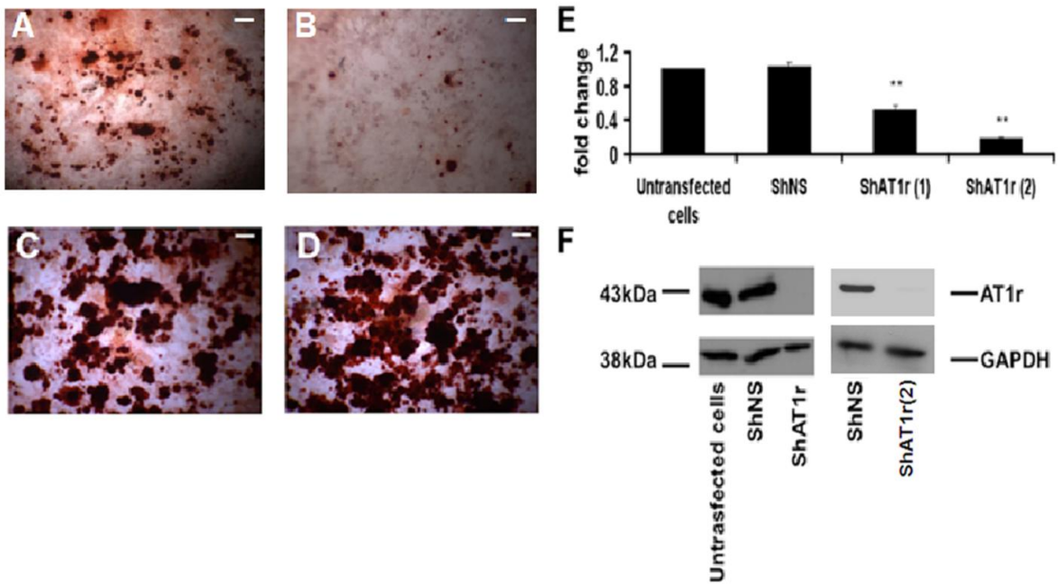

Figure 2

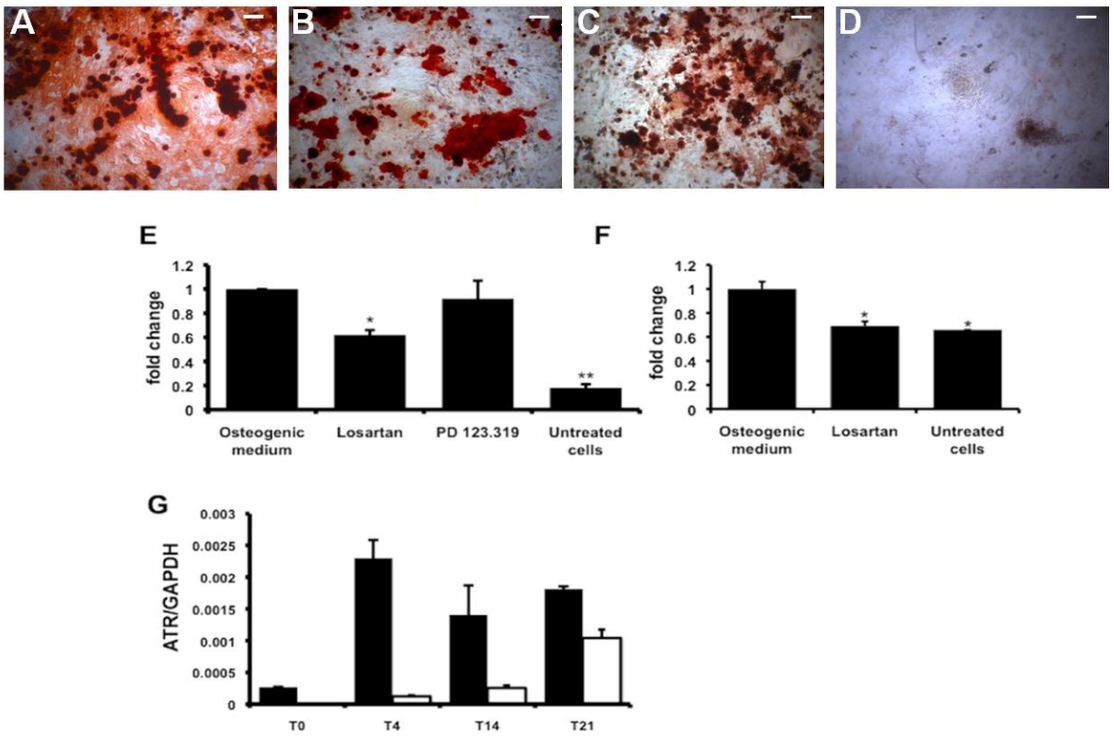

Figure 3

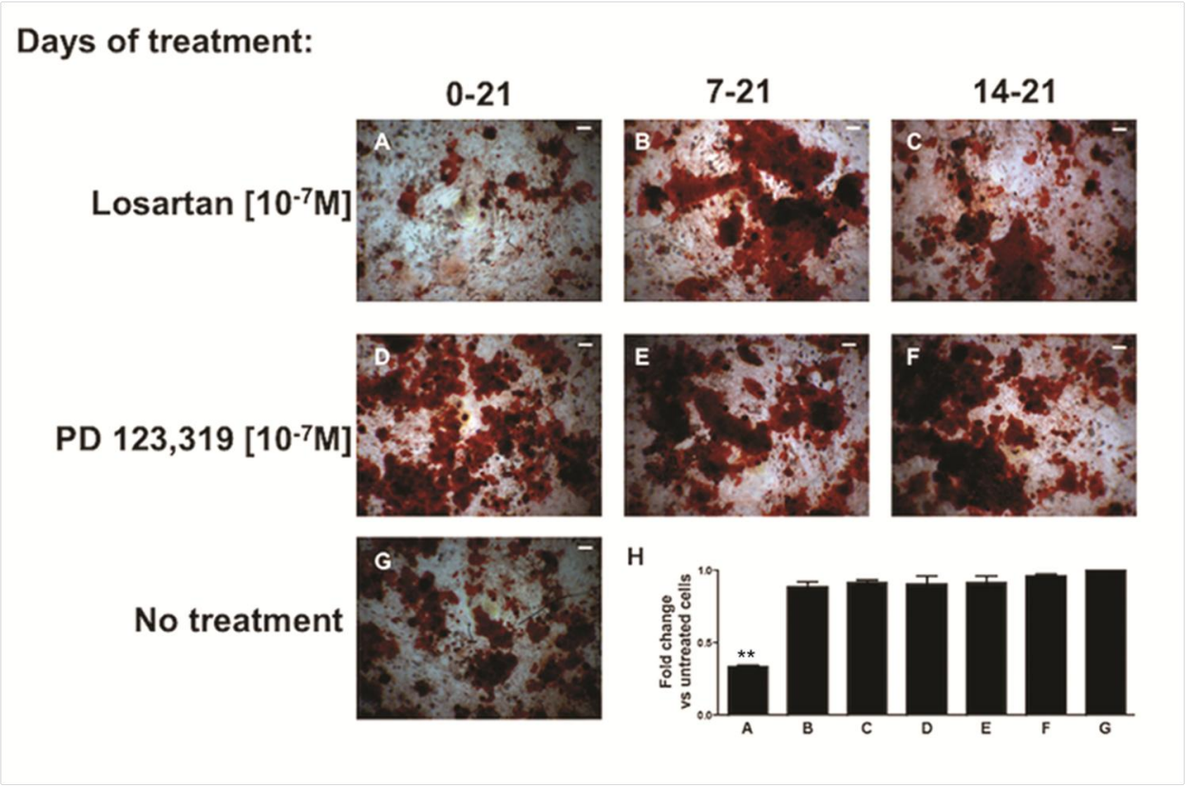

Figure 4

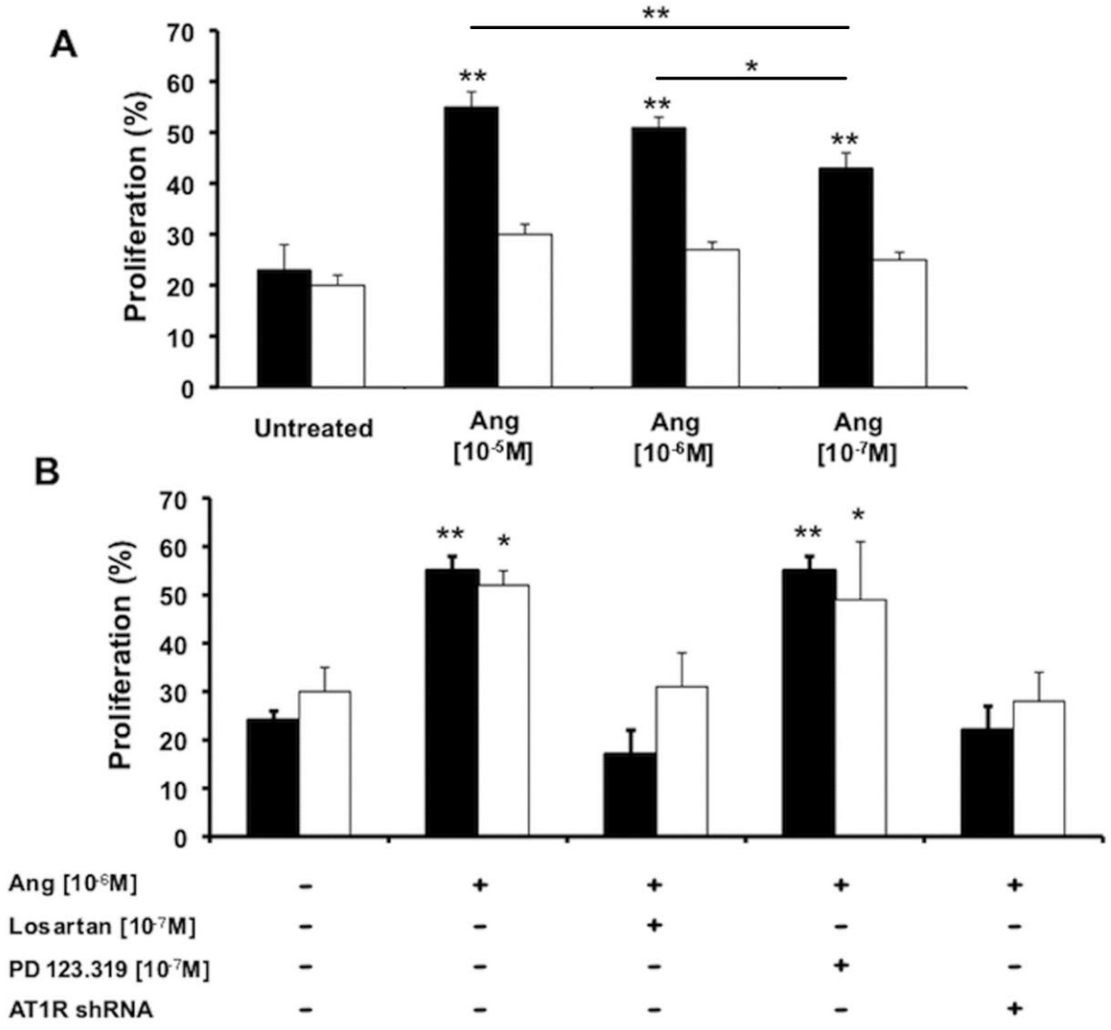

Figure 5

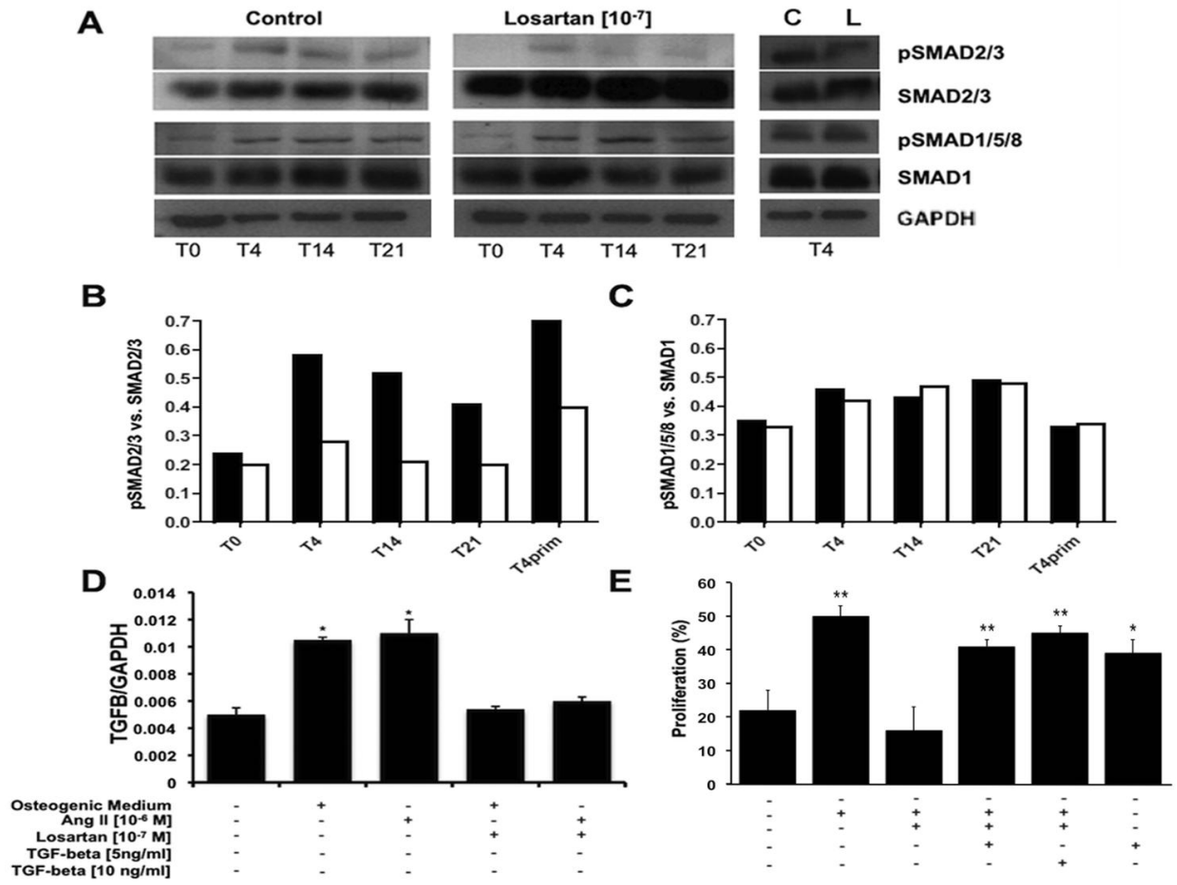

Figure 6