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4 A single two-day-pulse of Activin-A leads to a transient change in osteoblast gene expression and reduction in extracellular matrix mineralization

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ABSTRACT

Activins belong to the transforming growth factor-β superfamily and regulate bone formation by controlling osteoclast and osteoblast activity. Previously, we showed that Activin-A strongly inhibited matrix mineralization in osteoblast cultures, and that Activin A-signaling was most effective before the onset of mineralization.

The aim of the present study was to investigate the mechanism of action of Activin-A on human osteoblast mineralization, RNA and miRNA expression profile, by analyzing the effect of a 2-day-pulse of Activin-A in the premineralization period, and thereby to identify the cellular and molecular processes underlying the Activin-A effect.

A single 2-day-pulse of Activin-A at day 5 of osteoblast differentiation was sufficient to significantly reduce extracellular matrix mineralization at day 12. This effect was counteracted by SMAD-inhibitor. Moreover, SMAD3 phosphorylation reached the maximum 1 hour after Activin-A treatment. Gene expression profiling illustrated that the Activin A-treated osteoblasts responded with a transient change in osteoblast gene expression, in a 2-wave-fashion over time. The 38 genes differentially regulated during the first wave (within 8 hours after Activin A-start) were mainly involved in transcription regulation. In the second wave (1-2 days after Activin A-start), 65 genes were differentially regulated and related to extracellular matrix structure and cell-matrix adhesion. Both waves included differentially expressed genes that are associated to TGFβ signaling. Moreover, we identified which microRNAs that modulate osteoblast differentiation were regulated by Activin-A.

In summary, these findings demonstrate that two-day treatment with Activin-A within the pre-mineralization period reduced extracellular matrix mineralization 5-7 days later. These data give new insights into the mechanism by which Activin-A modulates osteoblast differentiation, by influencing osteoblast miRNA profile, gene transcription and eventually altering matrix mineralization. Modulation of Activin A-signaling might be useful for therapeutic purposes to control bone mineralization and thereby quality.

Keywords: Activin-A; osteoblasts; bone; mineralization

INTRODUCTION

Activins belong to the transforming growth factor beta (TGFβ) superfamily and are formed by dimerization of 2 inhibin β subunits [1, 2]. Activin-A binds to transmembrane serine/threonine kinase receptor type 2 (ACTR2A and ACTR2B), and leads to the recruitment and activation of receptor type 1B (ACVR1B=ALK4). The activated receptor induces a cytoplasmic signal transduction involving SMAD2/3, eventually recruiting the common mediator SMAD4. Only this phosphorylated complex (SMAD2/3/4) can then translocate into the nucleus and regulate target gene transcription [3-5]. The Activin-A signaling cascade modulates several biological functions, such as cell proliferation, differentiation and apoptosis [5, 6]. Activin-A signaling is modulated by several regulatory proteins, such as Activin receptors interacting proteins (ARIPs), and eventually shut off by SMAD ubiquitin regulatory factors (SMURF1 and SMURF2), that interact with inhibitory SMADs (SMAD6 and SMAD7), and mediate the ubiquitin-dependent degradation of the receptors [7]. SMAD2/3 belongs also to TGFβ-signaling, whereas bone morphogenic protein (BMP) signaling involves SMAD1,5,8 but it is transduced by receptor type 2, highlighting a crosstalk between these pathways [8, 9]. In addition, Activin-A also signals through SMAD-independent pathways, such as p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK1/2) and Jun N-terminal kinase (JNK) pathways, increasing the complexity of this intracellular signal [5, 10, 11]. Endogenous inhibitors such as Follistatin (FST), inhibins and betaglycan antagonizes Activin-A signaling [12].

The role of activins and inhibins in bone formation and metabolism has been extensively studied in the last years. They regulate skeletal metabolism, by acting on activins, TGFβs and BMPs in bone [13, 14]. However, the role of activins in bone metabolism is still not fully clear. Activin-A has been detected in human and bovine bone matrix [14-16]. It has been shown to be secreted by both osteoblasts and bone marrow cultures during osteoblastogenesis, and during bone matrix resorption by osteoclasts [16-18]. Activin-A has been shown to modulate bone-cell behavior and having a pro-osteoclastogenic effect *in vitro* [14, 18-20]. Despite this, its effect on osteoblasts is still controversial. Several studies report Activin-A to promote osteogenic differentiation *in vitro*, and bone formation and fracture healing in *in vivo* systems [15, 18, 21, 22]. However, we and others have shown the inhibitory effect of Activin-A on osteoblast as well as vascular smooth muscle cells-mediated mineralization *in vitro* [16, 23, 24]. These findings were also supported by *in vivo* studies, in which increased bone mass has been observed after blocking Activin-A in murine and primate models [25, 26]. These controversial findings might be related to differences in cell-culture system or in the species that were used [14, 23].

We have shown that Activin-A affected the expression of extracellular matrix (ECM)-related genes, influencing the ECM maturation phase. This resulted in an altered ECM protein composition that was unable to mineralize. This effect was stronger when Activin-A was present during the last days prior to the onset of extracellular matrix mineralization [16, 27]. In addition, Activin-A impaired matrix vesicle secretion at the onset of extracellular matrix mineralization [27]. This highlights the importance of Activin-A signaling in bone metabolism, but despite this, the molecular processes underlying Activin A-driven inhibition of osteogenic differentiation and mineralization are still unclear.

The aim of the present study was to investigate the impact of a temporary 2-day-treatment of Activin-A on osteoblast extracellular matrix mineralization, and to unravel the molecular mechanisms of Activin-A signaling during differentiation of human osteoblasts.

MATERIALS AND METHODS

Osteoblast cultures

Human SV-HFO cells (Simian virus 40-immortalized osteoblast precursors) were cultured (1 x 10^4 vital cells/cm^2) in alpha-Mem (pH 7.5, phenol-red free; GIBCO, Paisley, UK) supplemented with streptomycin/penicillin, 1.8 mM $CaCl_2$ (Sigma, St. Louis, MO, USA), HEPES and 2% charcoal-treated and heat inactivated fetal bovine serum (FBS; GIBCO). After 2 days, medium was supplemented with dexamethasone (100nM) and β-glycerophosphate (10mM β glycerophosphate; Sigma, St. Louis, MO, USA) to induce osteogenic differentiation. Activin-A (50ng/ml; R&D System, Minneapolis, MN, USA) was added at day 5 of osteogenic differentiation and removed after 2 days, keeping cultures in osteogenic medium. Medium was replaced every 2-3 days.

To assess the specificity of the Activin-A signaling, osteoblasts were treated with a SMAD inhibitor (SB-505124; Sigma-Aldrich, St. Louis, MO, USA). SB-505124 selectively inhibits ALK4 kinase activity, repressing Activin A- and TGFβ-induced SMAD2 and SMAD3 signaling [12]. SB-505124 (0,125μM in DMSO) was added 30 minutes before Activin-A, and removed at the same time. Osteoblasts treated only with SB-505124 were used as control.

Analysis of osteogenic differentiation and mineralization

Alkaline phosphatase (ALP) activity and extracellular matrix mineralization were measured in cell extracts of Activin-A treated and untreated osteoblasts at late stages of culture (day 10, 12 and 14, see Figure 1A), as previously described [16].

Phospho-flow cytometry of SMAD3 phosphorylation

Human SV-HFO cells were seeded in a density of 4210 vital cells/cm^2 and cultured as described above, and SMAD3 phosphorylation was measured by flow cytometry. Samples were collected from Activin A-treated and untreated osteoblasts, at 10 minutes, 20 minutes, 1 hour, 2 hours, 4 hours, 8 hours, 24 hours and 48 hours after the start of Activin-A pulse. Cell extracts were fixed with 4% paraformaldehyde (PFA) and permeabilized with 90% ice-cold Methanol. Samples were incubated with anti-SMAD3 antibody (anti-SMAD3, pS423+ S425; clone ab52903, Abcam, Cambridge, UK; secondary antibody: anti rabbit IgG, Alexa Fluor® 488 conjugated, #4412, Cell Signaling Technology) and SMAD3 phosphorylation was followed over time by flow cytometry (Becton Dickinson FACS-Canto and DIVA Flow Cytometry System [BD Bioscience]).

Illumina gene chip-based expression

Illumina HumanHT-12 v3 BeadChip (Illumina, Inc.) human whole-genome expression arrays were used to analyze gene expression of Activin A-treated and untreated osteoblasts. RNA (100 ng) was collected from 3 biological replicates for each condition (at 20 minutes, 1 hour, 2 hours, 4 hours, 8 hours, 1 day, 2 days, 3 days, 5 days, 7 days, and 9 days after starting of Activin A-pulse). RNA integrity was checked by RNA 6000 Nano assay on a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA was amplified by Illumina TotalPrep RNA amplification kit (Ambion, Austin, TX, USA), accordingly to manufacturer's instruction. Briefly, single stranded cDNA was generated by using T7oligo (dT) primer, then followed by second strand synthesis to generate double stranded cDNA. Biotin-labeled cRNA was synthesized by *in vitro* transcription using T7 RNA polymerase and column purified. cRNA quality was assessed on a Bioanalyzer and its concentration by NanoDrop (Thermo Fisher). A total of 750ng of cRNA were hybridized for each array and detected using standard Illumina protocol with Streptavidin-Cy3 (GE). Slides were scanned on iScan and analyzed by GenomeStudio v2010.1 (both from Illumina, Inc.)

Microarray analysis

Raw gene expression data was background subtracted using Genome Studio v2010 (gene expression module 1.6), and further processed using the Bioconductor R2.10 lumi package [28]. Data was transformed by variance stabilization and quantile normalized.

Probes that were present in at least 3 samples (Illumina detection *P* value <0.01) were considered to be expressed and further analyzed. Differentially expressed probes were identified using the Bioconductor package 'limma' [29] with adjusted *P* value (q-value) to reduce false discovery rate. Differentially expressed probes

in Activin A-treated samples relative to untreated samples at the same time point (q<0.01) were considered. Selected genes were further analyzed for enrichment of gene ontology (GO) terms using DAVID Bioinformatic Resource v6.7 [30], against the whole genome as background, and by QIAGEN's Ingenuity® Pathway Analysis (IPA®, QIAGEN Redwood City www.qiagen.com/ingenuity) using the Expression Analysis tool (Canonical Pathways, Upstream Analysis and Disease and Function).

Quantification of mRNA expression

Total RNA isolation, cDNA synthesis and quantitative polymerase chain reaction (qPCR) were performed as previously described [16]. Primer sequences (Sigma-Aldrich) are listed in Supplementary Table 1.

microRNA array and analysis

Total RNA and small RNA populations were isolated from Activin A-treated and untreated osteoblasts, at 4 hours and 1 day after the start of Activin-A pulse. RNA (250 ng) was collected from 2 biological replicates of each condition. Illumina microRNA Expression Profiling Assay for BeadChip array (Illumina, Inc.) was used to analyze microRNA profiling of Activin A-treated and untreated osteoblasts, following manufacturer's instructions.

Probes that were detected above background (detection P<0.01), in at least 1 time point, in both biological replicates, and annotated to a known miRNA (miRbase www.mirbase.org [31]), were considered for further analysis. Probes that were detected only in Activin A-treated or untreated osteoblasts and probes more than 2-fold upregulated or downregulated were further analyzed (Table 1). miRNAs were analyzed by using DIANA miRpath v3 [32], IPA and Targetscan Release 7.1 [33] as prediction tools. Human species and predicted interactions derived from TarBase v7.0 were used in DIANA miRpath v3. In Targetscan, all miRNAs were queried against human genome as background, if possible, otherwise mouse, and only predicted targets with conserved sites were considered.

Statistical analysis

Data for biochemical analysis were representative of independent experiments. All values were presented as average ± standard deviation (SD) of technical replicates. Significance was calculated by 2-way analysis of variance (ANOVA), followed by Bonferroni Post Hoc test, otherwise indicated elsewhere.

RESULTS

A single pulse of Activin-A is sufficient to reduce osteoblast mineralization

Previously, we have shown that Activin-A reduced matrix mineralization of osteoblasts that were continuously treated with Activin-A and that the inhibition of mineralization was most effective when Activin-A was present in the final 7 days in the premineralization period (considered up to day 10 of culture) [16]. Here we investigated if a short-term incubation with Activin-A from day 5 to 7 of culture in the premineralization period resulted in the same effect. Human osteoblasts were treated for 2 days with Activin-A starting at day 5 and ALP activity and extracellular matrix mineralization were analyzed at later stages, as represented in Figure 1A. Alkaline phosphatase activity was not affected by Activin-A pulse (Figure 1B). Nevertheless, a single 2-day-treatment with Activin-A was able to reduce the extracellular matrix mineralization at later stages of culture. Calcium deposition at day 12 of culture was 2–fold lower in Activin A-treated osteoblasts than untreated cells, as shown in Figure 1C ($P< 0.001$).

To assess the specificity of Activin A-signaling, osteoblasts were treated with the SMAD-signaling inhibitor SB-505124. SB-505124 counteracted the effect of Activin-A on mineralization. Matrix mineralization by osteoblasts treated with both Activin-A and SMAD inhibitor was significantly higher than in the cells treated with Activin-A only ($P<0.01$) and at comparable levels as that of untreated osteoblasts (Figure 1D). SMAD inhibitor did not affect differentiation of the cells, as osteoblasts cultured in the presence of SMAD inhibitor mineralize at similar extend than the untreated cells.

SMAD3 phosphorylation reached the maximum 1 hour after Activin-A treatment

As SMAD2/3 are downstream signal transducers of Activin-A, we analyzed SMAD3 phosphorylation by flow cytometry. Phosphorylation of SMAD3 was significantly higher in Activin A-treated osteoblasts than in untreated cells, as shown in Figure 1E. The Activin-A pulse increased SMAD3 phosphorylation, with a peak after 1 hour of treatment, which was almost 2-fold higher in Activin A-treated osteoblasts than in untreated cells ($P<0.001$). Western blot analysis in our lab also showed that SMAD2 phosphorylation was higher in Activin A-treated cells compared to the untreated ones (data not shown).

Activin-A pulse induced changes in gene expression in a 2-wave fashion

As a 2-day treatment of Activin-A at early stages of osteoblast differentiation was able to reduce extracellular matrix mineralization, we investigated the molecular mechanism by which Activin-A would affect osteoblast gene expression. We performed comparative gene expression profiling of Activin-A treated and untreated

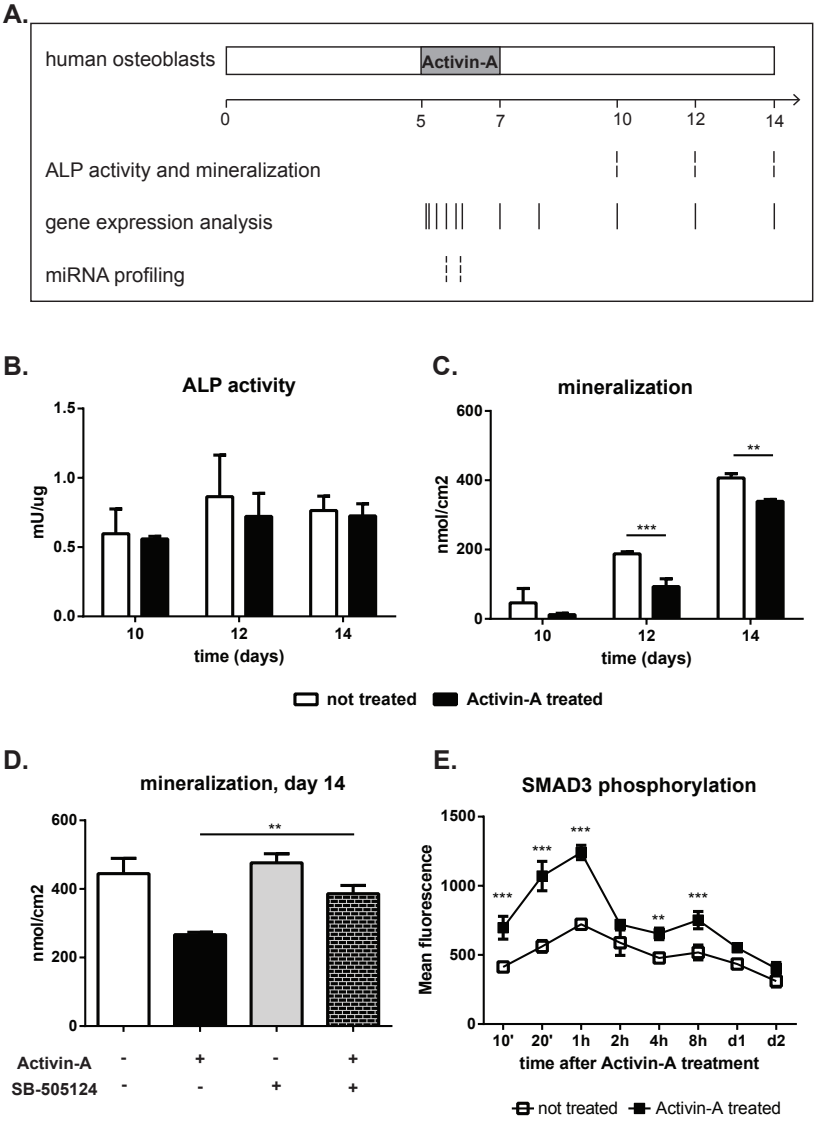

Figure 1. A single 2-day pulse of Activin-A reduced osteoblast mineralization. A) Schematic overview of culture conditions. Human SV-HFO cells were treated with Activin-A from day 5 to day 7 of osteogenic differentiation. ALP activity, mineralization, gene expression and miRNA profile were checked at the indicated time points. B) ALP activity of Activin A-treated and untreated osteoblasts at day 10, 12, 14 of culture. ALP activity was corrected for protein content at each time point. C) Calcium deposition by Activin A-treated and untreated osteoblasts at day 10, 12, 14 of culture (**, P<0.01; ***, P<0.001). D) Calcium deposition at day 14 of culture, in cell-extracts of untreated osteoblasts, osteoblasts treated with Activin-A, SMAD inhibitor (SB-505124), and Activin-A and SMAD inhibitor. Bars indicate Average ± SD. (**, P<0.01). E) Phosphorylation of SMAD3 followed by flow cytometry at the indicated time points, in Activin-A treated and untreated osteoblasts. Values: Average ± SD. (**, P<0.01; ***, P<0.001 relative to untreated cells).

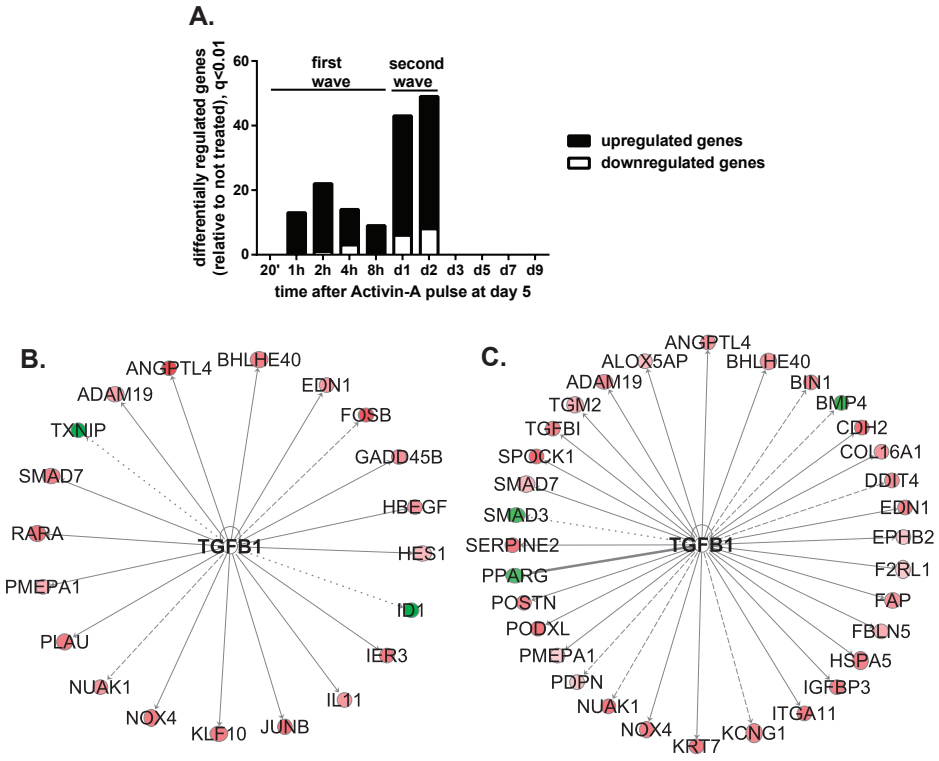

Figure 2. Activin-A pulse induced a transient change in osteoblast gene expression, in a 2-wave-fashion over time. A) Number of significantly differentially regulated genes in Activin A-treated osteoblasts compared to untreated cells at each time point (q<0.01). B) TGFβ is predicted to target 20 genes within those differentially regulated in the first wave, being one of the most enriched upstream regulators (IPA analysis). C) TGFβ is the most enriched upstream regulator, targeting 32 genes within those differentially regulated in the second wave of gene expression changes. In B and C: shades of red: upregulated genes by Activin-A (log ratio of Activin A-treated/untreated cells). Shades of green: downregulated genes. Solid line: predicted activation; thick solid line: predicted inhibition; dashed line: not predicted effect; dotted line: findings inconsistent with the state of downstream molecule by IPA.

osteoblasts at various time points using Illumina Human HT-12 v3 BeadChip array. Activin-A treatment induced a transient change in osteoblast gene expression in a 2-wave fashion over time, as shown in Figure 2A. The first wave consisted of 38 differentially regulated genes (q<0.01) and occurred from 1 hour till 8 hours after the start of Activin-A treatment (Supplementary Table 2). The second wave of differentially regulated genes occurred between 1 and 2 days after the Activin-A pulse (Supplementary Table 3). Moreover, differentially regulated genes were detected at each time point until day 2, but once Activin-A was replaced with control growth media, no gene expression differences were observed. The TGFβ signaling pathway was within the top 10 most enriched canonical pathways of the differentially regulated

genes in both waves (Supplementary Table 4 and 5). Moreover, TGFβ was within the most enriched upstream regulators in both first and second wave, as analyzed in IPA and shown in Figure 2B and 2C by the predicted target genes (Supplementary Table 6).

GO analysis of the 38 genes that are differentially regulated in the first wave showed that these changes were related to transcription regulation (GO:0045892) and vasculature development (GO:0001944) (P<0.05) (Figure 3A). In addition, these 38 genes were analyzed using IPA (Figure 3B). 'Binding of DNA', including genes such as SMAD7, JUNB and FOSB, was among the most significantly enriched terms (P=6.49*10^{-9}) (Figure 3B). Most of these genes were up-regulated by Activin-A. Furthermore 'Vasculogenesis' (SMAD7, JUNB and ANGPTL4) was identified to be significantly enriched (P=5.37*10^{-9}) among the regulated genes. Interestingly, 'differentiation of connective tissue cells', including genes such as SMAD7, KLF10 and JUNB, was significantly enriched (P=9.06*10^{-8}). As SMAD7, JUNB and KLF10 were involved in most of the identified pathways and have SMAD-responsive elements, their regulation was further confirmed by qPCR (Figure 3C).

In the second wave (1-2 days after Activin-A start), 65 genes were differentially regulated (q<0.01) (Supplementary Table 3). These genes are involved in vasculature development (GO:0001944), ECM structure (GO:0031012), cell migration (GO:0030334) and adhesion (GO:0007155) (Figure 4A). Ingenuity Pathway analysis of these 65 genes confirmed our findings by DAVID, as shown in Figure 4B. The functional categories Vasculogenesis (P=8.85*10^{-8}) including genes such as IGFBP3, FBLN5 and SMAD3, but also Adhesion of connective tissue cells (P=2.47*10^{-5}) (POSTN, TGFBI and BMP4) and Differentiation of osteoblasts (P=7.56*10^{-6}) (SMAD3, PPARG, POSTN, TGFBI and CTHRC1), were significantly enriched. ECM-related genes involved in these pathways such as POSTN, TGFB1, upregulated by Activin-A, and the downregulated BMP4 were further confirmed by qPCR (Figure 4C).

Activin-A pulse modified the microRNA profile of osteoblasts

In addition to the mRNA changes that occurred upon Activin-A treatment we analyzed the microRNA gene expression profile in osteoblasts, 4 hours and 1 day after addition of Activin-A at day 5 of culture. Assuming miRNAs as 'upstream' regulators of mRNA expression, and focusing on the second wave of gene expression changes (day 1 and day 2 after Activin-A addition), we chose 4 hours and day 1 respectively to assess the miRNA profiles. A detailed flowchart of the miRNA profile analysis is presented in Supplementary Figure 1.

Four hours after starting the Activin-A pulse, 12 of the 561 miRNAs were uniquely detected in the Activin A-treated osteoblasts (Figure 5A). Three miRNAs (miR-18b, miR-142-3p and miR-590-3p) were >2-fold upregulated in Activin A-treated osteo-

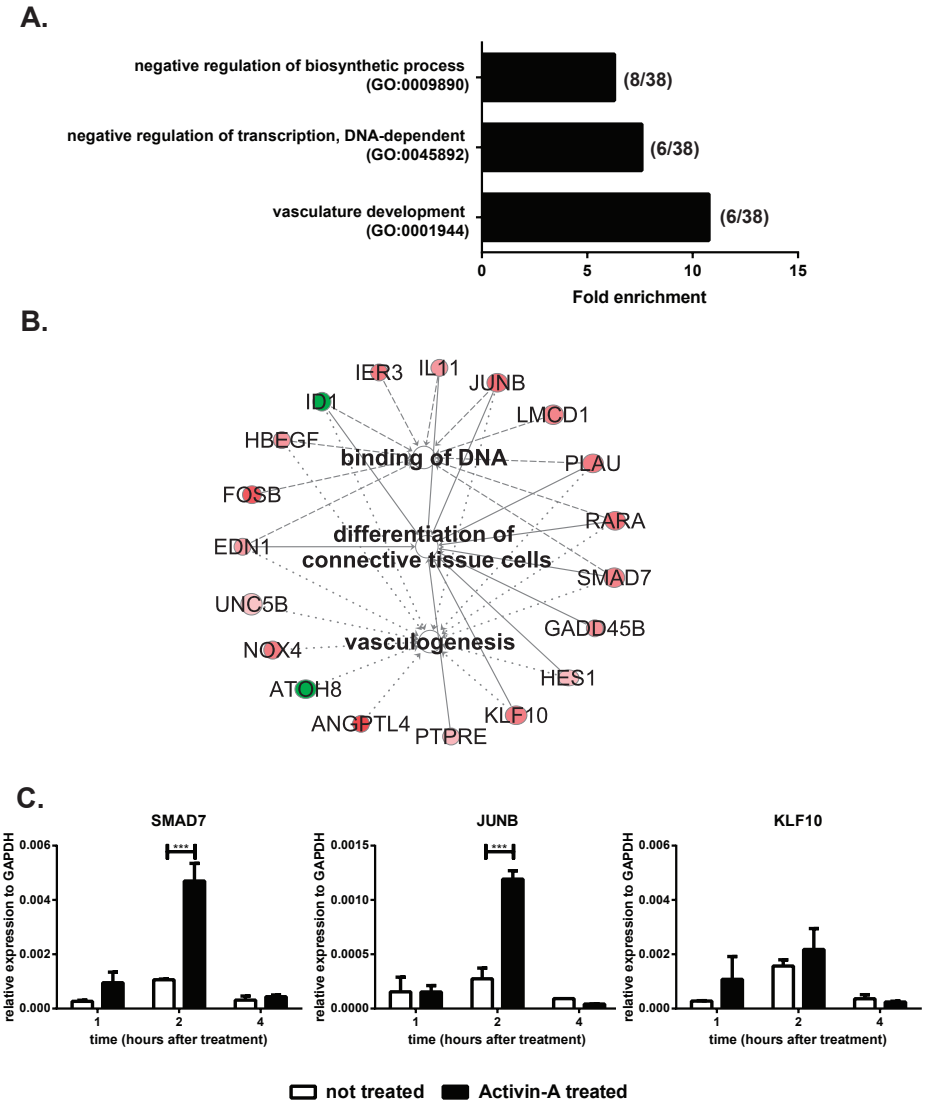

Figure 3. Activin-A pulse induced changes in gene transcription. A) GO analysis of the 38 genes of the first wave of gene expression changes (1-8 hours after Activin A-pulse start), that were differentially regulated in Activin A-treated cells compared to untreated ones. Only significantly enriched GO terms are shown (Benjamini $P<0.05$). In brackets: number of genes for each GO term. B) Pathway analysis of these 38 genes: vasculogenesis, binding of DNA and differentiation of connective tissue cells were significantly enriched. Shades of red: upregulated genes by Activin-A (log ratio of Activin A-treated/untreated cells). Shades of green: downregulated genes. C) qPCR analysis of selected transcription factors in Activin-A treated and untreated osteoblasts at the indicated time points. Bars indicate Average ± SD. (***, $P<0.001$).

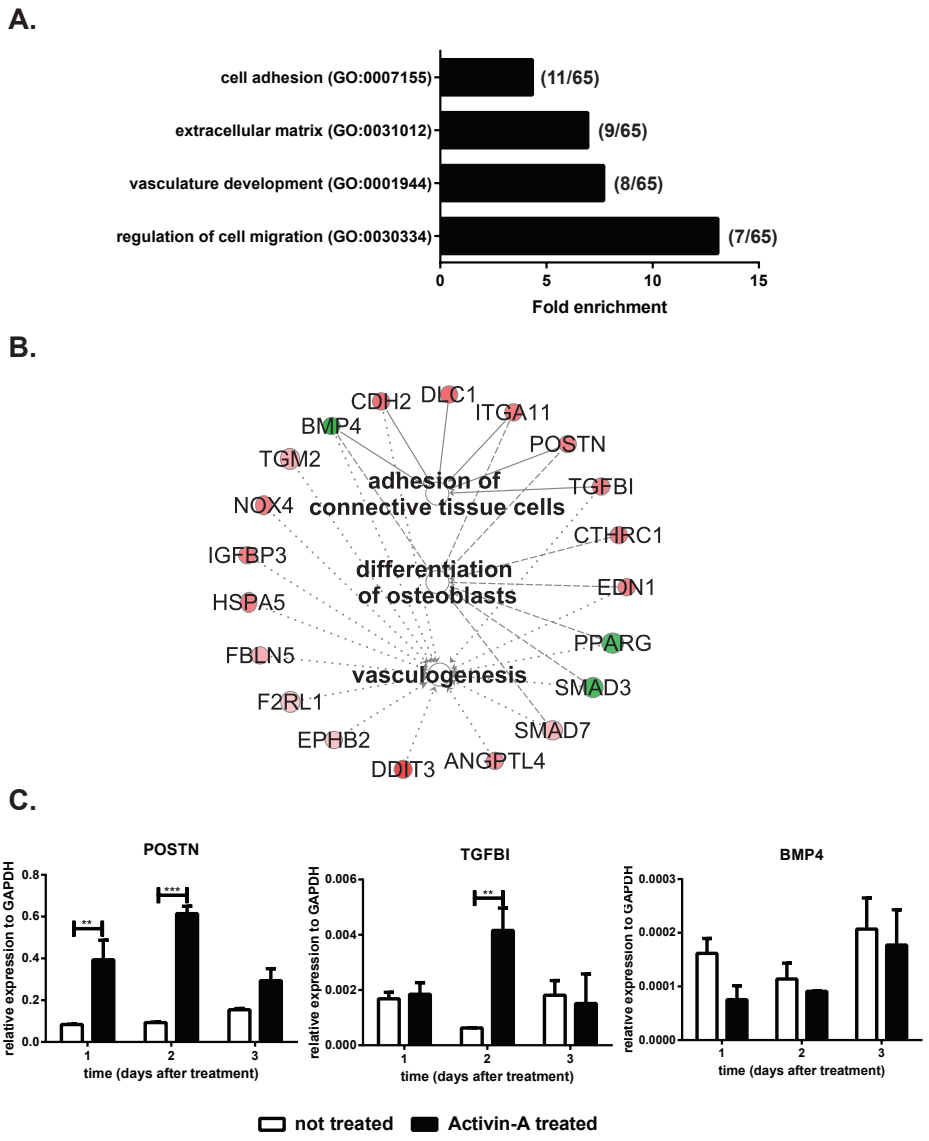

Figure 4. Activin A-pulse induced changes in ECM composition. A) GO analysis of the 65 genes that were differentially regulated in the second wave of gene expression (1 and 2 days after Activin A-pulse start) in Activin A-treated cells. Only significantly enriched GO terms are shown (Benjamini *P*<0.05). The number of genes for each enriched GO term are reported in brackets. B) Significantly enriched pathways of the IPA analysis of the 65 genes of second wave. Shades of red: upregulated genes by Activin-A (log ratio of Activin A-treated/untreated cells). Shades of green: downregulated genes. C) qPCR analysis of selected ECM proteins in Activin A-treated and untreated osteoblasts at 1, 2 and 3 days after the start of Activin-A pulse. Bars indicate Average ± SD. (**, *P*<0.01; ***, *P*<0.001).

blasts compared to the untreated cells (Figure 5B, Table 1). Furthermore, Activin-A decreased the expression of some miRNAs. Of these, 10 miRNAs were absent in the Activin A-treated osteoblasts, and 5 miRNAs (miR-19a, miR-32, miR-33a, miR-150 and miR-486-3p) were >2-fold downregulated in these cells compared to untreated cells (Figure 5B; Table 1). The miRNAs that were uniquely detected in each condition displayed very low expression. We analyzed in which pathways the targets of these miRNAs were involved using Diana pathway analysis [32]. Within the enriched pathways, we focused on TGFβ signaling, as it is known to guide osteoblast differentiation and as shown in Figure 2 gene expression analyses identified this signaling pathway. TGFβ signaling was significantly enriched (P=1.54x10^{-6}), as 9 of the 15

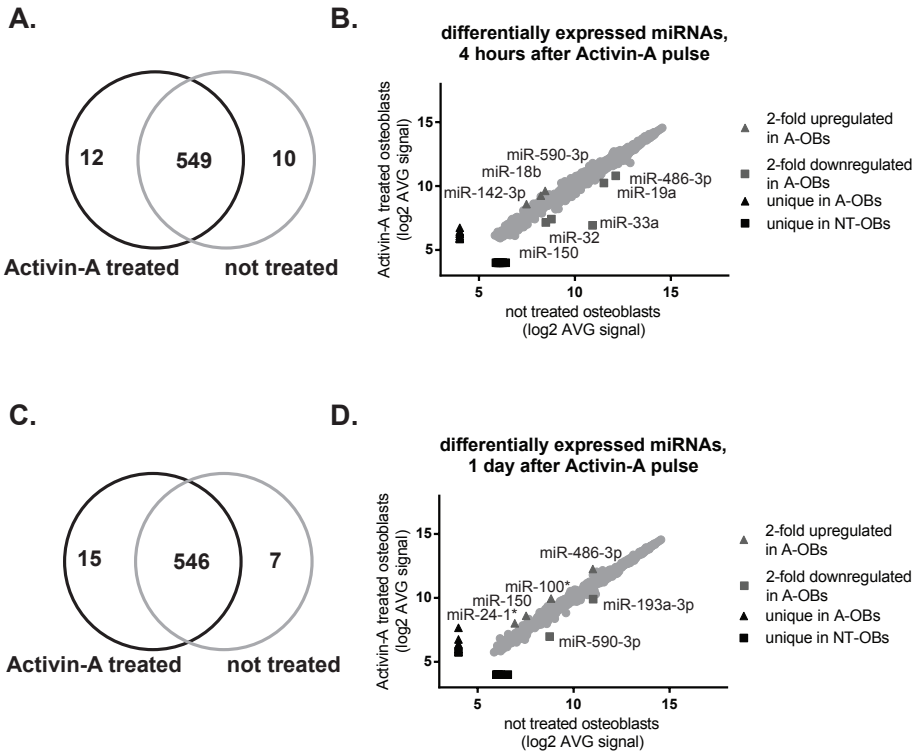

Figure 5. Osteoblast miRNA expression profiles were modulated by Activin-A treatment. A) 12 miRNAs were uniquely detected in Activin A-treated osteoblasts, 10 miRNAs uniquely in the untreated cells and 549 were detected in both conditions, 4 hours after the start of Activin-A pulse. B) miRNAs uniquely detected in Activin A-treated osteoblasts, untreated osteoblasts, and with more than 2-fold difference in detection, 4 hours after the start of Activin-A pulse. C) One day after the start of Activin-A pulse, 15 miRNAs were uniquely detected in Activin A-treated osteoblasts, 7 miRNAs uniquely in the untreated ones and 546 in both conditions. D) Number of miRNAs uniquely detected in Activin A-treated osteoblasts, untreated, and with more than 2-fold difference in detection, 1 day after the start of Activin-A pulse. Only probes detected above background and annotated to known miRNAs are shown (P<0.01). (A-OBs: Activin A-treated osteoblasts, NT-OBs: Not treated osteoblasts).

Table 1. miRNAs modulated by Activin-A in osteoblasts, 4 hours and 1 day after the start of Activin-A pulse. (A-OBs: Activin-A treated osteoblasts, NT-OBs: Non-treated osteoblasts). Intensities are indicated as AVG signal.

	Mature miRNAs	A-OBs (AVG signal)	NT-OBs (AVG signal)	Mature miRNAs	A-OBs (AVG signal)	NT-OBs (AVG signal)
4 hours	**>2-fold upregulated in A-OBs**			**>2-fold downregulated in A-OBs**		
	miR-590-3p	790.81	351.31	miR-486-3p	1788.62	4489.87
	miR-142-3p	386.24	178.37	miR-19a	1209.74	2925.18
	miR-18b	609.81	300.37	miR-33a	122.18	1942.43
				miR-32	171.06	439.93
				miR-150	142.06	358.49
	detected only in A-OBs			**not detected in A-OBs**		
	miR-432*	106.87		miR-573		84.24
	miR-337:9.1	88.12		miR-483-5p		76.06
	miR-33b*	84.37		miR-1263		75.37
	miR-96	77.87		miR-371-3p		70.37
	miR-592	76.87		miR-186*		69.06
	miR-744*	68.56		miR-765		66.74
	miR-338-3p*	67.99		miR-198		65.06
	miR-517a, b	61.68		miR-563		63.81
	miR-20b	60.93		miR-10b		62.18
	miR-645	59.31		miR-891a		59.18
	miR-580	58.93				
	miR-744*	58.68				
1 day	**>2-fold upregulated in A-OBs**			**>2-fold downregulated in A-OBs**		
	miR-486-3p miR-100*	4929.56 993.68	2055.87 453.87	miR-193a-3p miR-590-3p	963.43 126.06	2079.74 432.18
	miR-150	390.12	184.31			
	miR-24-1*	258.74	122.43			
	detected only in A-OBs			**not detected in A-OBs**		
	miR-18b	203.18		miR-220b		94.87
	miR-217	107.74		let-7f-2*		74.37
	miR-548o	83.43		miR-431*		69.99
	miR-589*	79.99		miR-610		67.62
	miR-548f	75.74		miR-518e		66.06
	miR-181d	71.93		miR-632		65.74
	miR-218-2	66.56		miR-520d-5p		63.06
	miR-1208	64.37				
	miR-921	64.31				
	miR-432*	63.56				
	miR-563	61.74				
	miR-559	57.43				
	miR-645	55.68				
	miR-517a,b	54.31				
	miR-1236	54.06				

miRNAs upregulated by Activin-A targeted 35 genes involved in TGFβ signaling. This is supported by the observation that also 17 mRNAs targeted by 8 miRNAs downregulated by Activin-A, were involved in the TGFβ pathway (Table 2). Thus, target genes that are involved in TGFβ signaling were targets of miRNAs that were both upregulated and downregulated within 4 hours of treatment by Activin-A.

One day after the start of Activin-A treatment, 15 miRNAs were detected being unique in Activin A-treated osteoblasts, and 546 were shared with the untreated cells (Figure 5C). Of these 546, 2 were more than 2-fold downregulated in Activin A-treated osteoblasts (Figure 5D; Table 1). In line with the 4-hour-treatment data, TGFβ signaling was significantly enriched (*P*=0.0001), as Activin-A upregulated 8 miRNAs that targeted 17 genes involved in this pathway, as well as downregulating 5 miRNAs that target 19 genes related to this signaling pathway (Table 2). Overall, within 1 day of treatment, Activin-A modulated miRNAs that are predicted to target genes involved in TGFβ signaling.

Table 2. Activin-A modulated miRNAs that are predicted to target genes involved in TGFβ signaling pathway (DIANA miRpath v3). (*P*<0.01). (A-OBs: Activin A-treated osteoblasts).

	Time after Activin-A pulse	KEGG pathway	*P* value	# involved miRNAs	# involved genes
miRNAs unique and upregulated in A-OBs	4 hours	TGFβ signaling	$1.54*10^{-5}$	9(/15)	35
miRNAs absent and downregulated in A-OBs			$5.30*10^{-5}$	8(/15)	29
miRNAs unique and upregulated in A-OBs	1 day		$1.15*10^{-4}$	8(/19)	17
miRNAs absent and downregulated in A-OBs			$2.70*10^{-2}$	5(/9)	19

Next, we combined the data of miRNA and mRNA profiling, hypothesizing that miRNAs that were upregulated by Activin A-treatment (4 hours and 1 day), would target mRNAs that were downregulated after 1 and 2 days in the Activin A-treated osteoblasts (for analysis scheme see Supplementary Figure 1). As Activin-A also downregulated some miRNAs, we checked if they could target genes found upregulated in Activin A-treated osteoblasts.

We considered the genes differentially regulated in the second wave of gene expression, and that were involved in the top 10 most enriched canonical pathways (Supplementary Table 5). Within these pathways, we analyzed whether the genes that were downregulated by Activin-A were also predicted targets of miRNAs upregulated by Activin-A (Supplementary Table 7). Indeed, we identified 2 miRNAs (miR-142-3p, miR-432*) that were upregulated by Activin-A and were predicted to target the downregulated gene BMP4 (Table 3). In addition, 7 miRNAs (miR-24-1*, miR-181d, miR-548f, miR-559, miR-589*, miR-645 and miR-1236) were identified that downregulated the expression of SMAD3 (Table 3).

Similarly, we investigated if the genes that were upregulated by Activin-A in the second wave of gene expression, were also predicted targets of miRNAs downregulated by Activin-A (Supplementary Table 8). Interestingly, Activin-A downregulated miRNAs targeted genes being upregulated in Activin A-treated osteoblasts (Table 4). For example, miR-32, miR-486-3p, miR-573 and miR-1263 were downregulated by Activin-A, and are predicted to target SMAD7, which was upregulated by Activin A treatment.

Table 3. Genes downregulated by Activin-A in the second wave of gene expression changes (day 1, day 2) and targets of the indicated miRNAs upregulated by Activin-A (IPA, Targetscan analysis).

Downregulated genes	Target of miRNAs 4 hours	Target of miRNAs 1day
BMP4	miR-142-3p miR-432*	miR-432*
PPARG	miR-590-3p	miR-559
SMAD3		miR-24-1* miR-181d miR-548f miR-559 miR-589* miR-645 miR-1236

Table 4. Genes upregulated by Activin-A (day 1, day 2) and predicted as target of the indicated miRNAs that are downregulated by Activin-A (IPA, Targetscan analysis).

Upregulated genes	Target of miRNAs 4 hours	Target of miRNAs 1day
DDIT4		miR-590-3p
EDN1	miR-19a miR-33a miR-486-3p miR-765	miR-590-3p
HSPA5		miR-590-3p
IGFBP3	miR-371b-3p miR-563	
NOX4	miR-10b miR-32 miR-33a	miR-590-3p
PMEPA1	miR-10b miR-23 miR-186* miR-765	
SMAD7	miR-32 miR-486-3p miR-573 miR-1263	

DISCUSSION

In this study, we demonstrated that a short-term Activin-A treatment for only two days in the osteoblast differentiation period preceding mineralization inhibits matrix mineralization 5-7 days later. Activin-A exerts this by affecting mRNA expression in a biphasic manner as well as regulating miRNA expression within these two days.

We took advantage of our previous work in which we demonstrated that Activin A-inhibition of matrix mineralization was most effective when Activin-A was present in the final 7 days of premineralization period [16]. In line with this, in this study we provided evidence that Activin A-treatment only between day 5 and 7 of osteoblast cultures reduced mineralization, highlighting the importance of timing of Activin-A presence during osteoblast differentiation and bone formation. ALP activity was not affected by Activin-A at the time points that were analyzed, in contrast with our previous findings [27]. Possibly, the timing of Activin-A treatment should be earlier during differentiation in order to influence ALP activity. Nevertheless, mineralization was significantly reduced by this Activin-A treatment regimen implicating that the effect is independent of changes in ALP activity. This is supported by earlier observations of Eijken et al [16], that the Activin-A treatment leads to a change in extracellular matrix composition.

The Activin-A pulse altered osteoblast gene expression in a biphasic fashion, by modulating genes involved in transcription regulation and ECM structure. Between 1 to 8 hours after the treatment, SMAD-responsive transcription factors, such as ID1, KLF10, JUNB and SMAD7 were regulated, highlighting the specificity of Activin A-signaling. The inhibitory SMAD7 was upregulated by Activin-A, in line with our previous findings [16]. As SMAD7 is a known inhibitor of BMP- and TGFβ-signaling, and was shown to reduce mouse osteoblast mineralization, confirming our findings in human osteoblast cultures functional findings [2, 34]. SMAD7 was also predicted as target of 4 miRNAs upregulated by Activin-A, thus representing an interesting candidate for functional analyses of auto-regulation by Activin-A signaling. Unexpectedly, other SMAD-responsive transcription factors were upregulated by Activin-A. KLF10 and JUNB, which are known inducers of osteogenic differentiation [35-38], were upregulated in human osteoblasts that were continuously treated with Activin-A [16]. Yet an explanation is unclear, however, it is tempting to speculate that these are part of an intricate regulatory network of genes of which the concerted action eventually leads to the inhibition of mineralization. In line, Activin-A was shown to act on many different proteins, revealing the complexity of its signaling [27], thus maybe explaining these findings.

In the second wave of gene expression changes we detected genes involved in osteoblast differentiation and ECM composition. Interestingly, Activin-A upregulated

matricellular proteins such as POSTN, SPARC/osteonectin (SPOCK1), and growth factors such as TGFβ, that promote osteoblast adhesion and bone structure [39-41], in line with previous findings showing that Activin-A induced ECM related genes [16, 27]. Activin-A might stimulate initial stages of osteogenesis, but creating an ECM compartment that fails to induce mineralization at later stages. In line with this, the osteogenic stimulator BMP4 was downregulated by Activin-A and also predicted as target of miR-142 and miR-432*, that were upregulated by Activin-A. Moreover, Heat shock protein family A member 5 (HSPA5) was upregulated in Activin A-treated osteoblasts and predicted as target of Activin A-modulated miRNAs. HSPA5 is involved in unfolded protein response (UPR) to counteract endoplasmic reticulum (ER) stress. In physiological conditions ER stress is elevated in osteoblasts, and UPR has been related to osteoblast differentiation, playing a role during bone homeostasis and skeletal disorders [42]. However, further studies are needed to investigate the impact of Activin-A in matrix secretion and its relation to osteoblast management of stress response.

Genes involved in vasculogenesis, such as ANGPTL4, NADPH oxidase 4 (NOX4) and endothelin 1 (EDN1), were upregulated by Activin-A in both waves of gene expression changes, indicating consistent regulation over time. ANGPTL4 is a target of TGFβ and Hypoxia inducible factor (HIF), and it mediates HIF-driven bone resorption in the angiogenic-osteogenic coupling [43]. Conversely, it also promotes osteoblast differentiation in fracture repair and stimulates Vascular endothelial growth factor (VEGF) expression [44]. Activin-A is considered as commitment factor for the differentiation of erythroid progenitors [45]. Therefore, the role of Activin-A in vasculogenesis needs further investigation for future clinical applications in fracture repair or control of metastatic bone diseases, also based on the importance of Erythropoietin (EPO) on bone formation [46], but also on the HIF-mediated EPO production by osteoblasts that contributes to erythropoiesis and hematopoietic stem cell expansion [47]. Our findings showed that Activin-A upregulated miRNAs involved in erythropoiesis. For instance, miR-486-3p was upregulated in untreated osteoblasts 4 hours after the treatment, but became very abundant in Activin A-treated osteoblasts 1 day after the treatment. miR-486-3p was shown to regulate γ-globin expression in erythroid cells, thus maybe representing and interesting candidate for Activin-A involvement in vasculogenesis [48]. miR-24 was shown to directly target ALK4 modulating Activin-mediated erythropoiesis [49], and indeed miR-24-1* was upregulated in Activin A-treated osteoblasts.

Activin-A was shown to modulate miRNA profile in human prostate cancer cell lines and human embryonic stem cells (hESCs) [50, 51]. Our study showed that Activin-A modified also the miRNA profile of osteoblasts. For instance, one day after the start of the treatment, miR-217 was upregulated by Activin-A. miR-217 was also upregu-

lated by Activin-A in hESCs [51] and it reduced murine osteoblast mineralization by targeting RUNX2 [52], thus representing an important target for Activin A-mediated inhibition of mineralization. Also other miRNAs, such as miR-20b, were upregulated by Activin-A, but were shown to enhance mineralization [53]. The number of miRNAs that were altered by Activin-A in osteoblasts reflects the complexity of Activin-A signaling. A miRNA profile at even more time points than the ones we selected may help us unravelling Activin-A mechanism in more detail and describe the Activin A-induced intracellular regulatory network.

In summary, we showed that a single two-day-pulse of Activin-A between day 5 to 7 of osteoblast differentiation was able to reduce extracellular matrix mineralization 5-7 days later. Activin-A altered osteoblast gene expression profile in a biphasic fashion, firstly acting at transcription level, and subsequently altering ECM-related genes. Moreover, Activin A-pulse was able to modify the miRNA profile of osteoblasts that could be linked to changes in mRNA expression. The results gave further insights into the mechanism by which Activin-A modulates osteoblast behaviour and matrix mineralization. Activin-A and/or its mRNA and miRNA targets identified represent potential targets for stimulation of bone formation and future clinical treatment of bone-related diseases to control bone formation and bone quality, but also conditions of ectopic calcification such as atherosclerosis [16].

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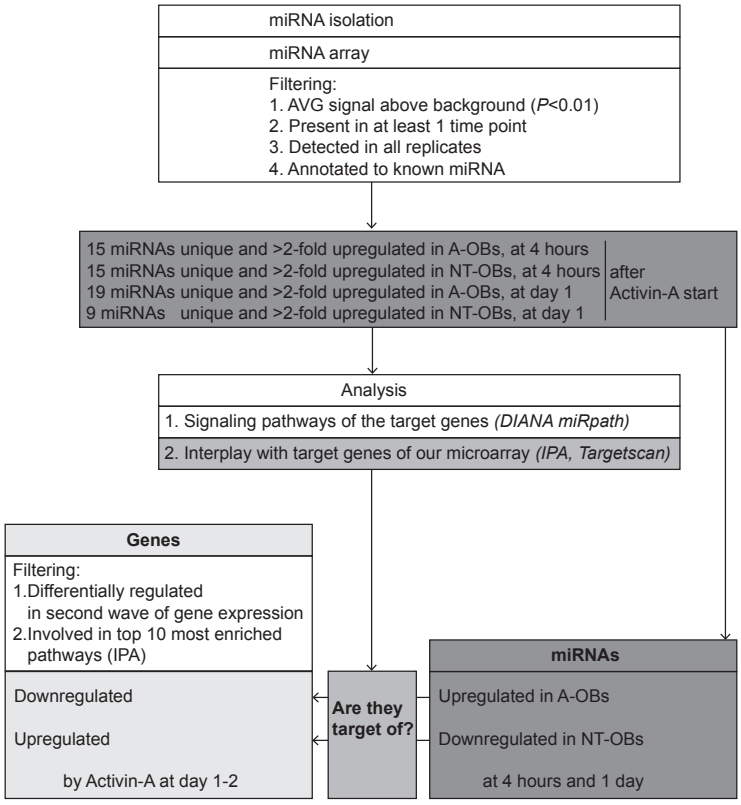

Supplementary Figure 1. Flow chart of miRNA data analysis. miRNAs were analyzed by Illumina microRNA Expression Profiling Assay for BeadChip array. Only probes that were detected above background (P<0.01), in at least 1 time point, in all replicates (N=2), and annotated to known miRNAs, were further analyzed. Four groups of miRNAs were considered: miRNAs uniquely detected in Activin A-treated osteoblasts and more than 2-fold upregulated than in untreated cells at each time point, and miRNA uniquely detected in untreated osteoblasts and more than 2-fold upregulated in these cells compared to the Activin-A-treated ones, at each time point. Firstly, we analyzed the pathways in which the genes that are predicted targets of the selected miRNAs were involved, by DIANA miRpath. Secondly, we combined the microRNA analysis with our gene expression data, to have a complete picture of the mechanism of action of Activin-A on osteoblast gene expression. As Activin-A induced a gene expression change in a 2-wave fashion, we were interested in the second wave (day 1 and 2 after Activin-A start) and miRNAs are 'upstream' of gene modulation, we thus choose 4 hours and 1 day to assess miRNA profiles. We assumed that Activin-A would modulate the miRNA profile in 4 hours, and that these miRNAs would target the mRNAs 1 day later. And on this line, miRNAs that were modulated by Activin-A after 1 day, would target the mRNAs at day 2. Thus, only genes that were differentially regulated in the second wave were considered, and belonging to the top10 most enriched pathways by IPA. We next checked if these genes were target of the selected miRNAs by Targetscan. Considering the timing, we checked whether genes downregulated by Activin-A at day 1, were targets of miRNAs upregulated by Activin-A at 4 hours. We also analyzed if miRNAs downregulated by Activin-A at 4 hours, were responsible for the upregulation of genes at day 1. The same analysis was performed also with the selected miRNAs at day 1 and the differentially regulated genes at day 2. (A-OBs: Activin A-treated osteoblasts, NT-OBs: Not-treated osteoblasts).

Supplementary Table 1. List of primer sets used for qPCR in this study. All the genes were quantified using SYBR.

Gene symbol	Forward primer	Reverse primer	pmol/ reaction
GAPDH	GCTCCTCCTGTTCGACAGTCA	ACCTTCCCCATGGTGTCTGA	2.5
KLF10	GACCAAACGAGTCTGGACAGT	AATCATTTCCATTCTTTCCTCCATT	2.5
SMAD7	CTTAGCCGACTCTGCGAACT	GCATCTGGACAGTCAGTTGGT	2.5
JUNB	CCACCTCCCGTTTACACCAA	GAGGTAGCTGATGGTGGTCG	2.5
POSTN	TGTGGACAGAAAACGACTGTGTTA	CGATGCCCAGAGTGCCATA	10
TGFB1	CTACATTTGGAGCCTGGACA	CCGGGTTATGCTGGTTGTA	1.25
BMP4	GCTTCCACCACGAAGAACAT	AAAGAGGAAACGAAAAGCAGA	1.25

Supplementary Table 2. List of 38 genes differentially regulated in the first wave (1-8 hours after adding Activin-A). Genes are indicated as Gene IDs and alphabetically ordered. Direction indicates upregulation or downregulation in Activin A-treated osteoblasts.

Gene ID	Direction	Gene ID	Direction
ADAM19	upregulated	LFNG	upregulated
ANGPTL4	upregulated	LMCD1	upregulated
APCDD1L	upregulated	LOC440160	upregulated
ATOH8	downregulated	NOX4	upregulated
AXUD1	upregulated	NPTX1	upregulated
BHLHB2	upregulated	NUAK1	upregulated
C13ORF15	downregulated	PLAU	upregulated
EDN1	upregulated	PMEPA1	upregulated
FOSB	upregulated	PRICKLE2	upregulated
GADD45B	upregulated	PTPRE	upregulated
HBEGF	upregulated	RARA	upregulated
HES1	upregulated	SLC46A3	upregulated
HS.369398	upregulated	SMAD7	upregulated
HS.549989	upregulated	SNF1LK	upregulated
ID1	downregulated	TNS1	upregulated
IER3	upregulated	TRIB1	upregulated
IL11	upregulated	TXNIP	downregulated
JUNB	upregulated	UNC5B	upregulated
KLF10	upregulated	ZSWIM4	upregulated

Supplementary Table 3. List of 65 genes differentially regulated in the second wave (day 1, day 2 after adding Activin-A). Genes are indicated as Gene names. Direction of transcriptional regulation in Activin A-treated osteoblasts is indicated.

Gene ID	Direction
ADAM19	upregulated
ALOX5AP	upregulated
ANGPTL4	upregulated
APCDD1L	upregulated
ARID1A	downregulated
AXUD1	upregulated
BHLHB2	upregulated
BIN1	upregulated
BMP4	downregulated
C13ORF15	downregulated
C5ORF46	upregulated
CD248	downregulated
CDH2	upregulated
CNKSR3	downregulated
COL16A1	upregulated
CTHRC1	upregulated
DACT1	upregulated
DDIT3	upregulated
DDIT4	upregulated
DKFZP586H2123	downregulated
DLC1	upregulated
DUSP5	downregulated
EDN1	upregulated
EPHB2	upregulated
F2RL1	upregulated
FAP	upregulated
FBLN5	upregulated
FBXO32	upregulated
FHOD3	upregulated
GALNTL1	upregulated
HERPUD1	upregulated
HNT	upregulated
HS.143018	upregulated
HS.554507	upregulated
HS.557431	upregulated
HSPA5	upregulated
IGFBP3	upregulated
ITGA11	upregulated
KCNG1	upregulated
KRT7	upregulated
LOC646723	upregulated
NOX4	upregulated
NPTX1	upregulated
NUAK1	upregulated
PCDH10	upregulated
PDPN	upregulated
PID1	upregulated
PMEPA1	upregulated
PODXL	upregulated
POSTN	upregulated
PPARG	downregulated
PRICKLE2	upregulated
PSCD1	upregulated
PTPRE	upregulated
SERPINE2	upregulated
SHROOM2	downregulated
SLC29A1	upregulated
SLC46A3	upregulated
SMAD3	downregulated
SMAD7	upregulated
SNF1LK	upregulated
SPOCK1	upregulated
TGFBI	upregulated
TGM2	upregulated
TMEM132D	downregulated

Supplementary Table 4. Significantly enriched canonical pathways of differentially regulated genes of first wave (IPA). Grey background indicates the top 10 most enriched canonical pathways.

Ingenuity Canonical Pathways	-log(*P* value)	Ratio	Genes
Notch Signaling	2.78E+00	5.26E-02	LFNG, HES1
TGF-β Signaling	2.07E+00	2.30E-02	SMAD7, PMEPA1
Hepatic Cholestasis	1.57E+00	1.25E-02	RARA, IL11
GADD45 Signaling	1.53E+00	5.26E-02	GADD45B
Hepatic Fibrosis / Hepatic Stellate Cell Activation	1.47E+00	1.09E-02	EDN1, SMAD7
RAR Activation	1.44E+00	1.05E-02	RARA, SMAD7
IL-8 Signaling	1.41E+00	1.02E-02	NOX4, HBEGF
Osteoarthritis Pathway	1.36E+00	9.52E-03	SMAD7, HES1
Circadian Rhythm Signaling	1.29E+00	3.03E-02	BHLHE40
Oncostatin M Signaling	1.28E+00	2.94E-02	PLAU
Coagulation System	1.27E+00	2.86E-02	PLAU
Netrin Signaling	1.22E+00	2.56E-02	UNC5B
Role of IL-17F in Allergic Inflammatory Airway Diseases	1.17E+00	2.27E-02	IL11
Role of Oct4 in Mammalian Embryonic Stem Cell Pluripotency	1.15E+00	2.17E-02	RARA
Hematopoiesis from Pluripotent Stem Cells	1.14E+00	2.13E-02	IL11
Retinoic acid Mediated Apoptosis Signaling	1.03E+00	1.61E-02	RARA
PCP pathway	1.01E+00	1.54E-02	JUNB
Glioma Invasiveness Signaling	9.76E-01	1.43E-02	PLAU
Hypoxia Signaling in the Cardiovascular System	9.53E-01	1.35E-02	EDN1
BMP signaling pathway	9.42E-01	1.32E-02	SMAD7
VDR/RXR Activation	9.32E-01	1.28E-02	HES1
ATM Signaling	9.21E-01	1.25E-02	GADD45B
Neuregulin Signaling	8.83E-01	1.14E-02	HBEGF
G Beta Gamma Signaling	8.83E-01	1.14E-02	HBEGF
Factors Promoting Cardiogenesis in Vertebrates	8.65E-01	1.09E-02	NOX4
Acute Myeloid Leukemia Signaling	8.60E-01	1.08E-02	RARA
ErbB Signaling	8.39E-01	1.02E-02	HBEGF
CDK5 Signaling	8.35E-01	1.01E-02	FOSB
FAK Signaling	8.35E-01	1.01E-02	TNS1
Mouse Embryonic Stem Cell Pluripotency	8.08E-01	9.43E-03	ID1
Axonal Guidance Signaling	7.92E-01	4.42E-03	UNC5B, ADAM19
p53 Signaling	7.89E-01	9.01E-03	GADD45B
Rac Signaling	7.68E-01	8.55E-03	NOX4
HIF1Œ± Signaling	7.65E-01	8.47E-03	EDN1
Pancreatic Adenocarcinoma Signaling	7.65E-01	8.47E-03	HBEGF
fMLP Signaling in Neutrophils	7.49E-01	8.13E-03	NOX4
Role of Tissue Factor in Cancer	7.49E-01	8.13E-03	HBEGF
FXR/RXR Activation	7.39E-01	7.94E-03	RARA
HMGB1 Signaling	7.18E-01	7.52E-03	IL11

Ingenuity Canonical Pathways	-log(*P* value)	Ratio	Genes
Adipogenesis pathway	7.15E-01	7.46E-03	TXNIP
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	7.06E-01	7.30E-03	IL11
Aryl Hydrocarbon Receptor Signaling	6.98E-01	7.14E-03	RARA
Human Embryonic Stem Cell Pluripotency	6.90E-01	6.99E-03	SMAD7
Hereditary Breast Cancer Signaling	6.87E-01	6.94E-03	GADD45B
Ovarian Cancer Signaling	6.87E-01	6.94E-03	EDN1
Phagosome Maturation	6.76E-01	6.76E-03	NOX4
Wnt/Œ≤-catenin Signaling	6.19E-01	5.81E-03	RARA
Endothelin-1 Signaling	5.82E-01	5.26E-03	EDN1
NRF2-mediated Oxidative Stress Response	5.76E-01	5.18E-03	JUNB
Role of NFAT in Cardiac Hypertrophy	5.76E-01	5.18E-03	IL11
LPS/IL-1 Mediated Inhibition of RXR Function	5.26E-01	4.52E-03	RARA
EIF2 Signaling	5.26E-01	4.52E-03	NOX4
Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	5.07E-01	4.29E-03	IL11
Signaling by Rho Family GTPases	4.84E-01	4.03E-03	NOX4
Glucocorticoid Receptor Signaling	4.32E-01	3.47E-03	PLAU
Molecular Mechanisms of Cancer	3.43E-01	2.66E-03	SMAD7
Protein Kinase A Signaling	3.27E-01	2.53E-03	PTPRE

Supplementary Table 5. Significantly enriched canonical pathways of differentially regulated genes of second wave. The top 10 most enriched pathways (grey background) were considered for miRNA analysis.

Ingenuity Canonical Pathways	-log(*P* value)	Ratio	Genes
TGF-β Signaling	3.78E+00	4.60E-02	BMP4,SMAD3,SMAD7,PMEPA1
Hepatic Fibrosis / Hepatic Stellate Cell Activation	3.57E+00	2.76E-02	COL16A1,EDN1,SMAD3,IGFBP3,SMAD7
Unfolded protein response	3.18E+00	5.56E-02	PPARG,DDIT3,HSPA5
Adipogenesis pathway	3.14E+00	3.12E-02	PPARG,BMP4,DDIT3,SMAD3
Endoplasmic Reticulum Stress Pathway	2.70E+00	9.52E-02	DDIT3,HSPA5
RAR Activation	2.54E+00	2.14E-02	ARID1A,SMAD3,IGFBP3,SMAD7
Osteoarthritis Pathway	2.43E+00	2.00E-02	PPARG,DDIT4,SMAD3,SMAD7
Human Embryonic Stem Cell Pluripotency	2.00E+00	2.14E-02	BMP4,SMAD3,SMAD7
BMP signaling pathway	1.64E+00	2.70E-02	BMP4,SMAD7
EIF2 Signaling	1.55E+00	1.44E-02	NOX4,DDIT3,HSPA5
Factors Promoting Cardiogenesis in Vertebrates	1.49E+00	2.25E-02	NOX4,BMP4
Cardiomyocyte Differentiation via BMP Receptors	1.23E+00	5.26E-02	BMP4
Glucocorticoid Receptor Signaling	1.22E+00	1.06E-02	ARID1A,SMAD3,HSPA5
GŒ±12/13 Signaling	1.20E+00	1.55E-02	CDH2,F2RL1
Polyamine Regulation in Colon Cancer	1.19E+00	4.76E-02	PPARG
Antiproliferative Role of TOB in T Cell Signaling	1.10E+00	3.85E-02	SMAD3
Circadian Rhythm Signaling	1.00E+00	3.03E-02	BHLHE40
RhoGDI Signaling	9.91E-01	1.17E-02	CDH2,DLC1
Molecular Mechanisms of Cancer	9.53E-01	8.13E-03	BMP4,SMAD3,SMAD7
Regulation of the Epithelial-Mesenchymal Transition Pathway	9.42E-01	1.09E-02	CDH2,SMAD3
Thyroid Cancer Signaling	9.34E-01	2.56E-02	PPARG
Protein Kinase A Signaling	9.24E-01	7.89E-03	PTPRE,DUSP5,SMAD3
Axonal Guidance Signaling	7.78E-01	6.74E-03	BMP4,EPHB2,ADAM19
Huntington's Disease Signaling	7.63E-01	8.44E-03	TGM2,HSPA5
Cell Cycle: G1/S Checkpoint Regulation	7.41E-01	1.59E-02	SMAD3
Signaling by Rho Family GTPases	7.38E-01	8.13E-03	NOX4,CDH2
PCP pathway	7.35E-01	1.56E-02	CTHRC1
Eicosanoid Signaling	7.35E-01	1.56E-02	ALOX5AP
Basal Cell Carcinoma Signaling	7.06E-01	1.45E-02	BMP4
Caveolar-mediated Endocytosis Signaling	6.95E-01	1.41E-02	ITGA11
Hypoxia Signaling in the Cardiovascular System	6.89E-01	1.39E-02	EDN1
Ephrin B Signaling	6.84E-01	1.37E-02	EPHB2
VDR/RXR Activation	6.63E-01	1.30E-02	IGFBP3

Ingenuity Canonical Pathways	-log(*P* value)	Ratio	Genes
Regulation of IL-2 Expression in Activated and Anergic T Lymphocytes	6.63E-01	1.30E-02	SMAD3
Role of BRCA1 in DNA Damage Response	6.59E-01	1.28E-02	ARID1A
Growth Hormone Signaling	6.39E-01	1.22E-02	IGFBP3
HIPPO signaling	6.21E-01	1.16E-02	SMAD3
PEDF Signaling	6.17E-01	1.15E-02	PPARG
PPAR Signaling	5.96E-01	1.09E-02	PPARG
Chronic Myeloid Leukemia Signaling	5.54E-01	9.71E-03	SMAD3
Mouse Embryonic Stem Cell Pluripotency	5.47E-01	9.52E-03	BMP4
IGF-1 Signaling	5.47E-01	9.52E-03	IGFBP3
Androgen Signaling	5.33E-01	9.17E-03	SMAD3
Neuroprotective Role of THOP1 in Alzheimer's Disease	5.30E-01	9.09E-03	FAP
Paxillin Signaling	5.30E-01	9.09E-03	ITGA11
p53 Signaling	5.27E-01	9.01E-03	SERPINE2
HIF1α± Signaling	5.11E-01	8.62E-03	EDN1
Rac Signaling	5.11E-01	8.62E-03	NOX4
p38 MAPK Signaling	5.11E-01	8.62E-03	DDIT3
PTEN Signaling	5.08E-01	8.55E-03	CNKSR3
Pancreatic Adenocarcinoma Signaling	5.05E-01	8.47E-03	SMAD3
Role of Tissue Factor in Cancer	5.01E-01	8.40E-03	F2RL1
Role of NANOG in Mammalian Embryonic Stem Cell Pluripotency	4.98E-01	8.33E-03	BMP4
RhoA Signaling	4.96E-01	8.26E-03	DLC1
fMLP Signaling in Neutrophils	4.93E-01	8.20E-03	NOX4
FXR/RXR Activation	4.84E-01	8.00E-03	PPARG
Type II Diabetes Mellitus Signaling	4.81E-01	7.94E-03	PPARG
p70S6K Signaling	4.70E-01	7.69E-03	F2RL1
Aryl Hydrocarbon Receptor Signaling	4.57E-01	7.41E-03	TGM2
D-myo-inositol (1,4,5,6)-Tetrakisphosphate Biosynthesis	4.52E-01	7.30E-03	DUSP5
D-myo-inositol (3,4,5,6)-tetrakisphosphate Biosynthesis	4.52E-01	7.30E-03	DUSP5
Phagosome Maturation	4.49E-01	7.25E-03	NOX4
Hereditary Breast Cancer Signaling	4.44E-01	7.14E-03	ARID1A
Ovarian Cancer Signaling	4.39E-01	7.04E-03	EDN1
Epithelial Adherens Junction Signaling	4.37E-01	6.99E-03	CDH2
IL-12 Signaling and Production in Macrophages	4.35E-01	6.94E-03	PPARG
3-phosphoinositide Degradation	4.21E-01	6.67E-03	DUSP5
D-myo-inositol-5-phosphate Metabolism	4.09E-01	6.45E-03	DUSP5
eNOS Signaling	3.99E-01	6.25E-03	HSPA5
Tight Junction Signaling	3.89E-01	6.06E-03	CNKSR3

Ingenuity Canonical Pathways	-log(*P* value)	Ratio	Genes
Aldosterone Signaling in Epithelial Cells	3.85E-01	5.99E-03	HSPA5
PPARŒ±/RXRŒ± Activation	3.83E-01	5.95E-03	SMAD3
Germ Cell-Sertoli Cell Junction Signaling	3.83E-01	5.95E-03	CDH2
Wnt/Œ≤-catenin Signaling	3.81E-01	5.92E-03	CDH2
Ephrin Receptor Signaling	3.75E-01	5.81E-03	EPHB2
NF-kB Signaling	3.73E-01	5.78E-03	BMP4
Agranulocyte Adhesion and Diapedesis	3.71E-01	5.75E-03	PODXL
Endothelin-1 Signaling	3.51E-01	5.41E-03	EDN1
NRF2-mediated Oxidative Stress Response	3.43E-01	5.26E-03	HERPUD1
mTOR Signaling	3.38E-01	5.18E-03	DDIT4
3-phosphoinositide Biosynthesis	3.38E-01	5.18E-03	DUSP5
IL-8 Signaling	3.34E-01	5.13E-03	NOX4
Clathrin-mediated Endocytosis Signaling	3.31E-01	5.08E-03	EPHB2
ERK/MAPK Signaling	3.31E-01	5.08E-03	PPARG
AMPK Signaling	3.28E-01	5.03E-03	ARID1A
Leukocyte Extravasation Signaling	3.19E-01	4.88E-03	DLC1
Integrin Signaling	3.11E-01	4.76E-03	ITGA11
Phospholipase C Signaling	2.93E-01	4.48E-03	TGM2
Superpathway of Inositol Phosphate Compounds	2.88E-01	4.41E-03	DUSP5
Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	2.87E-01	4.39E-03	BMP4
Colorectal Cancer Metastasis Signaling	2.69E-01	4.13E-03	SMAD3
Protein Ubiquitination Pathway	2.45E-01	3.80E-03	HSPA5
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	2.11E-01	3.34E-03	F2RL1

Supplementary Table 6. A) List of top 10 most enriched upstream regulators of 38 genes differentially regulated in first wave. Bold: terms highlighted in Figure 2B. B) List of top 10 most enriched upstream regulators of genes that were differentially regulated in second wave of gene expression. Bold: terms highlighted in Figure 2C.

A)

#	Upstream Regulator	Predicted Activation State	Activation z-score	*P* value of overlap	Target genes in dataset
1	PDGF BB	Activated	2.189	4.24E-14	BHLHE40,EDN1,FOSB,HBEGF,HES1,IER3,JUNB,KLF10,NOX4,PLAU,TRIB1,UNC5B
2	**TGFB1**	**Activated**	**3.507**	**5.69E-14**	**ADAM19,ANGPTL4,BHLHE40,EDN1,FOSB,GADD45B,HBEGF,HES1,ID1,IER3,IL11,JUNB,KLF10,NOX4,NUAK1,PLAU,PMEPA1,RARA,SMAD7,TXNIP**
3	Tgf beta	Activated	2.734	6.02E-14	ANGPTL4,BHLHE40,EDN1,GADD45B,ID1,JUNB,KLF10,NOX4,PLAU,PMEPA1,SMAD7
4	IL1B	Activated	2.107	1.04E-12	ANGPTL4,CSRNP1,EDN1,FOSB,GADD45B,HBEGF,HES1,IER3,IL11,JUNB,KLF10,PLAU,RARA,SMAD7,TXNIP
5	forskolin	Activated	2.21	1.21E-11	BHLHE40,EDN1,FOSB,GADD45B,ID1,IER3,IL11,JUNB,PLAU,PTPRE,SMAD7,TRIB1,TXNIP
6	TNF	Activated	3.157	1.44E-11	ANGPTL4,BHLHE40,EDN1,FOSB,GADD45B,HBEGF,HES1,ID1,IER3,IL11,JUNB,KLF10,LFNG,NUAK1,PLAU,RARA,SMAD7,TXNIP
7	U0126	Inhibited	-2.943	1.42E-10	ANGPTL4,BHLHE40,EDN1,FOSB,GADD45B,HBEGF,IER3,JUNB,KLF10,PLAU,TRIB1
8	beta-estradiol		1.015	2.68E-10	BHLHE40,EDN1,FOSB,GADD45B,HBEGF,HES1,ID1,IER3,JUNB,KLF10,LMCD1,LOC102724428/SIK1,NPTX1,PLAU,RARA,TRIB1,TXNIP
9	ERK1/2	Activated	2.605	6.28E-10	EDN1,FOSB,HBEGF,ID1,IL11,JUNB,NOX4,PLAU,PTPRE
10	ERBB2	Activated	3.121	7.99E-10	ADAM19,ANGPTL4,BHLHE40,EDN1,HBEGF,HES1,ID1,JUNB,NOX4,PLAU,PMEPA1,SMAD7

B)

#	Upstream Regulator	Predicted Activation State	Activation z-score	*P* value of overlap	Target genes in dataset
1	**TGFB1**	**Activated**	**4.82**	**6.41E-20**	**ADAM19,ALOX5AP,ANGPTL4,BHLHE40,BIN1,BMP4,CDH2,COL16A1,DDIT4,EDN1,EPHB2,F2RL1,FAP,FBLN5,HSPA5,IGFBP3,ITGA11,KCNG1,KRT7,NOX4,NUAK1,PDPN,PMEPA1,PODXL,POSTN,PPARG,SERPINE2,SMAD3,SMAD7,SPOCK1,TGFBI,TGM2**
2	TNF	Activated	3.899	7.06E-11	ALOX5AP,ANGPTL4,BHLHE40,BMP4,CDH2,COL16A1,DDIT3,DUSP5,EDN1,EPHB2,F2RL1,FBXO32,IGFBP3,NUAK1,PCDH10,PDPN,POSTN,PPARG,SERPINE2,SMAD3,SMAD7,SPOCK1,TGM2
3	ERBB2		1.804	2.65E-10	ADAM19,ANGPTL4,BHLHE40,CDH2,DDIT3,EDN1,EPHB2,IGFBP3,KRT7,NOX4,PMEPA1,PPARG,SMAD3,SMAD7,SPOCK1,TGFBI
4	Tgf beta	Activated	3.055	1.07E-09	ANGPTL4,BHLHE40,CDH2,EDN1,IGFBP3,NOX4,PMEPA1,POSTN,SMAD7,TGFBI
5	HIF1A	Activated	2.756	1.38E-09	ALOX5AP,ANGPTL4,BHLHE40,CDH2,DDIT4,EDN1,HSPA5,IGFBP3,NOX4,PCDH10,SLC29A1,SMAD7
6	PDGF BB		0.825	1.62E-09	BHLHE40,BMP4,DDIT3,DUSP5,EDN1,FBLN5,NOX4,POSTN,PPARG,SLC29A1,TGM2
7	D-glucose		0.396	2.00E-09	BMP4,DDIT3,DDIT4,EDN1,FBXO32,HERPUD1,HSPA5,NOX4,NUAK1,PODXL,PPARG,SLC29A1,TGM2
8	dexamethasone		0.937	2.80E-09	ALOX5AP,ANGPTL4,BHLHE40,DDIT3,DDIT4,DUSP5,EDN1,F2RL1,FBXO32,HERPUD1,HSPA5,IGFBP3,KRT7,PODXL,POSTN,PPARG,SERPINE2,SLC46A3,SPOCK1,TGFBI,TGM2
9	Jnk	Activated	2.253	4.78E-09	CDH2,DDIT3,EDN1,F2RL1,FBXO32,HSPA5,NOX4,POSTN,TGM2
10	deferoxamine		1.294	5.79E-09	ANGPTL4,BHLHE40,CYTH1,DDIT3,DUSP5,IGFBP3,PPARG,SERPINE2,SMAD7

Supplementary Table 7. List of genes responsible for the enrichment of the top 10 canonical pathways, and predicted as target of the indicated miRNAs upregulated by Activin-A. Genes downregulated by Activin-A are highlighted in grey.

#	canonical pathway	-log (*P* value)	differentially regulated genes		target of miRNAs 4 hours	target of miRNAs 1 day
1	TGF-ß Signaling	3.78E+00	BMP4	downregulated	miR-142-3p miR-432*	miR-432*
			SMAD3	downregulated		miR-1236 miR-181d miR-24-1* miR-548f miR-559 miR-589* miR-645
			SMAD7	upregulated		
			PMEPA1	upregulated		
2	Hepatic Fibrosis / Hepatic Stellate Cell Activation	3.57E+00	SMAD3	downregulated		miR-1236 miR-181d miR-24-1* miR-548f miR-559 miR-589* miR-645
			COL16A1	upregulated		
			EDN1	upregulated		
			SMAD7	upregulated		
			IGFBP3	upregulated		
3	Unfolded protein response	3,18E00	PPARG	downregulated	miR-590-3p	miR-559
			DDIT3	upregulated		
			HSPA5	upregulated		
4	Adipogenesis pathway	3,14E00	PPARG	downregulated	miR-590-3p	miR-559
			BMP4	downregulated	miR-142-3p miR-432*	miR-432*
			SMAD3	downregulated		miR-1236 miR-181d miR-24-1* miR-548f miR-559 miR-589* miR-645
			DDIT3	upregulated		
5	Endoplasmic Reticulum Stress Pathway	2,70E00	DDIT3	upregulated		
			HSPA5	upregulated		
6	RAR Activation	2,54E00	SMAD3	downregulated		miR-1236 miR-181d miR-24-1* miR-548f miR-559 miR-589* miR-645
			ARID1A	downregulated	not predicted target of miRNAs in our list	
			SMAD7	upregulated		
			IGFBP3	upregulated		

#	canonical pathway	-log (*P* value)	differentially regulated genes		target of miRNAs 4 hours	target of miRNAs 1 day
7	Osteoarthritis Pathway	2.43E+00	SMAD3	downregulated		miR-1236 miR-181d miR-24-1* miR-548f miR-559 miR-589* miR-645
			PPARG	downregulated	miR-590-3p	miR-559
			DDIT4	upregulated		
			SMAD7	upregulated		
8	Human Embryonic Stem Cell Pluripotency	2,00E00	BMP4	downregulated	miR-142-3p miR-432*	miR-432*
			SMAD3	downregulated		miR-1236 miR-181d miR-24-1* miR-548f miR-559 miR-589* miR-645
			SMAD7	upregulated		
9	BMP signaling pathway	1,64E00	BMP4	downregulated	miR-142-3p miR-432*	miR-432*
			SMAD7	upregulated		
10	EIF2 Signaling	1.55E+00	DDIT3	upregulated		
			HSPA5	upregulated		
			NOX4	upregulated		

Supplementary Table 8. List of genes involved in the top 10 canonical pathways, and predicted targets of the indicated miRNAs downregulated by Activin-A. Grey: genes upregulated by Activin-A.

#	canonical pathway	-log(*P* value)	differentially regulated genes		target of miRNAs 4 hours	target of miRNAs 1 day
1	TGF-ß Signaling	3.78E+00	BMP4	downregulated		
			SMAD3	downregulated		
			SMAD7	upregulated	miR-573 miR-1263 miR-486-3p miR-32	
			PMEPA1	upregulated	miR-186* miR-765 miR-10b miR-23	
2	Hepatic Fibrosis / Hepatic Stellate Cell Activation	3.57E+00	SMAD3	downregulated		
			COL16A1	upregulated	not predicted target of miRNAs in our list	
			EDN1	upregulated	miR-19a miR-33a miR-765 miR-486-3p	miR-590-3p
			SMAD7	upregulated	miR-573 miR-1263 miR-486-3p miR-32	
			IGFBP3	upregulated	miR-563 miR-371b-3p	
3	Unfolded protein response	3,18E00	PPARG	downregulated		
			DDIT3	upregulated	not predicted target of miRNAs in our list	
			HSPA5	upregulated		miR-590-3p
4	Adipogenesis pathway	3,14E00	PPARG	downregulated		
			BMP4	downregulated		
			SMAD3	downregulated		
			DDIT3	upregulated	not predicted target of miRNAs in our list	
5	Endoplasmic Reticulum Stress Pathway	2,70E00	DDIT3	upregulated	not predicted target of miRNAs in our list	
			HSPA5	upregulated		miR-590-3p
6	RAR Activation	2,54E00	SMAD3	downregulated		
			ARID1A	downregulated		
			SMAD7	upregulated	miR-573 miR-1263 miR-486-3p miR-32	
			IGFBP3	upregulated	miR-563 miR-371b-3p	

#	canonical pathway	-log(*P* value)	differentially regulated genes		target of miRNAs 4 hours	target of miRNAs 1 day
7	Osteoarthritis Pathway	2.43E+00	SMAD3	downregulated		
			PPARG	downregulated		
			DDIT4	upregulated		miR-590-3p
			SMAD7	upregulated	miR-573 miR-1263 miR-486-3p miR-32	
8	Human Embryonic Stem Cell Pluripotency	2,00E00	BMP4	downregulated		
			SMAD3	downregulated		
			SMAD7	upregulated	miR-573 miR-1263 miR-486-3p miR-32	
9	BMP signaling pathway	1,64E00	BMP4	downregulated		
			SMAD7	upregulated	miR-573 miR-1263 miR-486-3p miR-32	
10	EIF2 Signaling	1.55E+00	DDIT3	upregulated	not predicted target of miRNAs in our list	
			HSPA5	upregulated		miR-590-3p
			NOX4	upregulated	miR-33a miR-10b miR-32	miR-590-3p

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