


ORIGINAL RESEARCH ARTICLE

Two-day-treatment of Activin-A leads to transient change in SV-HFO osteoblast gene expression and reduction in matrix mineralization

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Abstract

Activins regulate bone formation by controlling osteoclasts and osteoblasts. We investigated Activin-A mechanism of action on human osteoblast mineralization, RNA and microRNA (miRNA) expression profile. A single 2-day treatment of Activin-A at Day 5 of osteoblast differentiation significantly reduced matrix mineralization. Activin A-treated osteoblasts responded with transient change in gene expression, in a 2-wave-fashion. The 38 genes differentially regulated during the first wave (within 8 hr after Activin A start) were 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. Differentially expressed genes in both waves were associated to transforming growth factor beta signaling. We identified which microRNAs modulating osteoblast differentiation were regulated by Activin-A. In summary, 2-day treatment with Activin-A in premineralization period of osteoblast cultures influenced miRNAs, gene transcription, and reduced matrix mineralization. Modulation of Activin A signaling might be useful to control bone quality for therapeutic purposes.

KEYWORDS

Activin-A, bone, extracellular matrix, mineralization, osteoblasts

1 | INTRODUCTION

Activins belong to the transforming growth factor- β (TGF- β) superfamily and are formed by dimerization of two inhibin β subunits (Massague, 1998; Vale et al., 2004). Activin-A binds to transmembrane serine/threonine kinase receptor type 2 (ACVR2A and ACVR2B), 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 (Chen et al., 2006; Derynck, 1998). The Activin-A signaling cascade modulates several biological functions, such as cell proliferation, differentiation, and apoptosis (Chen et al., 2006; Chen, Lui, Lin, Lee, & Ying, 2002). 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),

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and mediate the ubiquitin-dependent degradation of the receptors (Inoue & Imamura, 2008). SMAD2/3 belongs also to TGF- β -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 (Harrison et al., 2004; Tsuchida et al., 2009). 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 (Chen et al., 2006; de Guise et al., 2006). Endogenous inhibitors such as Follistatin (FST), inhibins and β -glycan antagonize Activin-A signaling (Harrison, Gray, Vale, & Robertson, 2005).

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, TGF- β s and BMPs in bone (Nicks, Perrien, Akel, Suva, & Gaddy, 2009; Perrien et al., 2006). However, the role of activins in bone metabolism is still not fully clear. Activin-A has been detected in human and bovine bone matrix (Eijken et al., 2007; Nicks et al., 2009; Ogawa et al., 1992). It has been shown to be secreted by both osteoblasts and bone marrow cultures during osteoblastogenesis, and during bone matrix resorption by osteoclasts (Eijken et al., 2007; Funaba et al., 1996; Gaddy-Kurten, Coker, Abe, Jilka, & Manolagas, 2002). Activin-A has been shown to modulate bone-cell behavior and having a pro-osteoclastogenic effect *in vitro* (Fuller, Bayley, & Chambers, 2000; Gaddy-Kurten et al., 2002; Nicks et al., 2009; Sakai, Eto, Ohtsuka, Hirafuji, & Shinoda, 1993). 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 (Gaddy-Kurten et al., 2002; Ogawa et al., 1992; Sakai et al., 2000; Sakai, Miwa, & Eto, 1999). 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* (Eijken et al., 2007; Hashimoto et al., 1992; Ikenoue, Jingushi, Urabe, Okazaki, & Iwamoto, 1999). 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 (Lotinun et al., 2010; Pearsall et al., 2008). These controversial findings might be related to differences in cell-culture system or in the species that were used (Ikenoue et al., 1999; Nicks et al., 2009).

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 before the onset of extracellular matrix mineralization (Alves, Eijken, Bezstarosti, Demmers, & van Leeuwen, 2013; Eijken et al., 2007). In addition, Activin-A impaired matrix vesicle secretion at the onset of extracellular matrix mineralization (Alves et al., 2013). 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.

2 | MATERIALS AND METHODS

2.1 | Osteoblast cultures

Human SV-HFO cells (Simian virus 40-immortalized osteoblast precursors) were cultured (1×10^4 vital cells/cm²) in α -MEM (pH 7.5, phenol-red free; GIBCO, Paisley, UK) supplemented with streptomycin/penicillin, 1.8 mM CaCl₂ (Sigma, St. Louis, MO), HEPES, and 2% charcoal-treated and heat inactivated fetal bovine serum (GIBCO). After 2 days, medium was supplemented with dexamethasone (100 nM) and β -glycerophosphate (10 mM; Sigma, St. Louis, MO) to induce osteogenic differentiation. Activin-A (50 ng/ml; R&D System, Minneapolis, MN) 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). SB-505124 selectively inhibits ALK4 kinase activity, repressing Activin A- and TGF- β -induced SMAD2 and SMAD3 signaling (Harrison et al., 2005). SB-505124 (0.125 μ M in dimethyl sulfoxide) was added 30 min before Activin-A, and removed at the same time. Osteoblasts treated only with SB-505124 were used as control.

2.2 | 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 1 a), as previously described (Eijken et al., 2007).

2.3 | Phospho-flow cytometry of SMAD3 phosphorylation

Human SV-HFO cells were seeded in a density of 4,210 vital cells/cm² 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 min, 20 min, 1 hr, 2 hr, 4 hr, 8 hr, 24 hr, and 48 hr after the start of Activin-A treatment. Cell extracts were fixed with 4% paraformaldehyde, 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]).

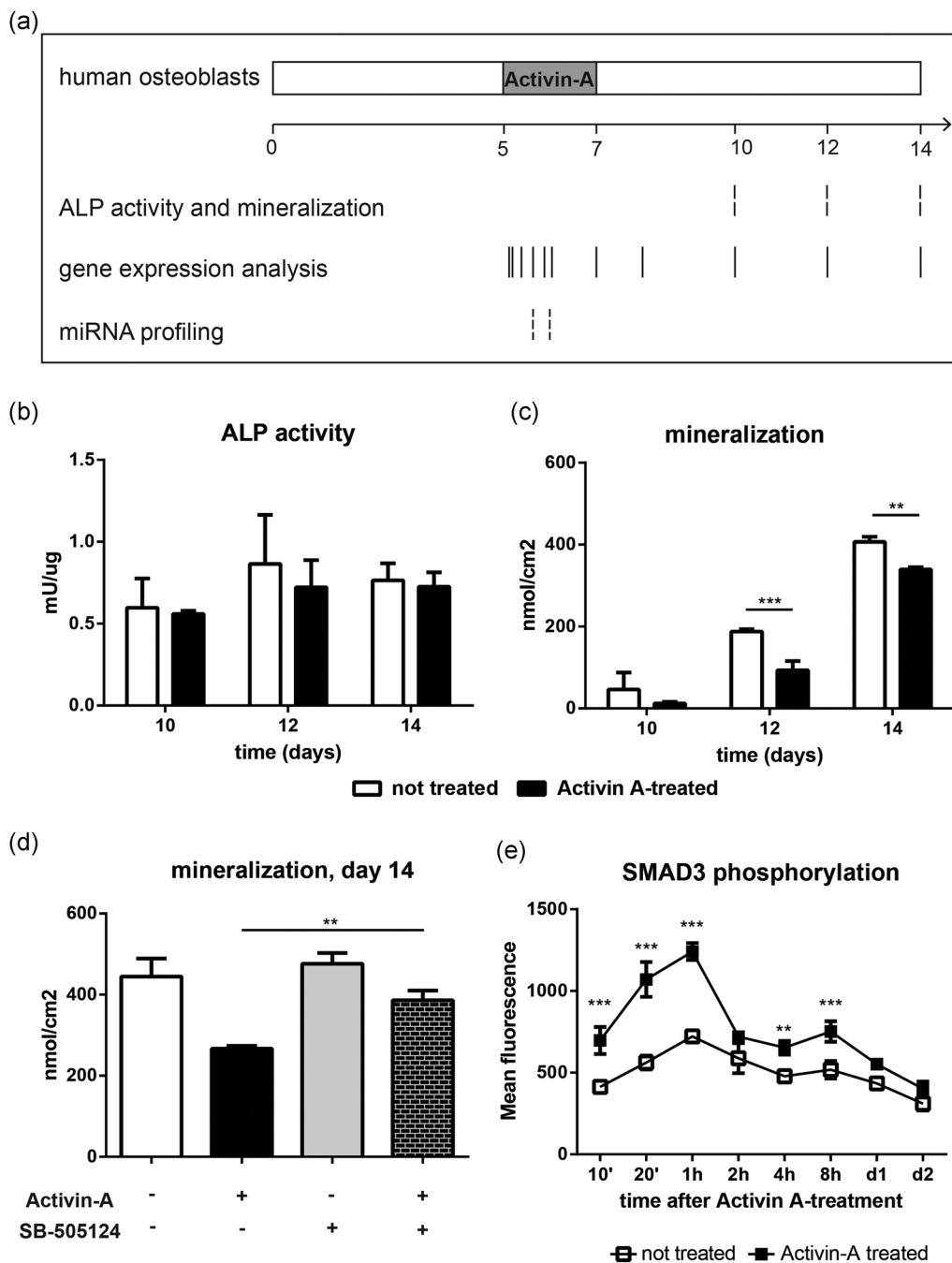


FIGURE 1 A single 2-day pulse of Activin-A reduced osteoblast mineralization. (a) Schematic overview of culture conditions. Human SV-HFO cells were treated with Activin-A from Day 5 to Day 7 of osteogenic differentiation. ALP activity, mineralization, gene expression, and miRNA profile were checked at the indicated time points. (b) ALP activity of Activin A-treated and untreated osteoblasts at Day 10, 12, and 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, and 14 of culture. (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 mean \pm SD. (** p <.01; *** p <.001). (e) Phosphorylation of SMAD3 followed by flow cytometry at the indicated time points, in Activin-A treated and untreated osteoblasts. Values: mean \pm SD. (** p <.01; *** p <.001 relative to untreated cells). ALP, alkaline phosphatase; miRNA, microRNA; SD, standard deviation

2.4 | 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 three biological replicates for each condition (at 20 min, 1 hr, 2 hr, 4 hr, 8 hr, 1 day, 2 days, 3 days, 5 days, 7 days, and 9 days after starting of Activin A treatment). RNA integrity was checked by RNA 6000 Nano assay on a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). RNA was amplified by Illumina

TotalPrep RNA amplification kit (Ambion, Austin, TX), according to manufacturer's instruction. Briefly, single stranded complementary DNA (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 Scientific). A total of 750 ng of cRNA was 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.)

2.5 | 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 (Du, Kibbe, & Lin, 2008). Data was transformed by variance stabilization and quantile normalized.

Probes that were present in at least three samples (Illumina detection $p < .01$) were considered to be expressed and further analyzed. Differentially expressed probes were identified using the Bioconductor package "limma" (Smyth, 2004) 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 ($q < .01$) were considered. Selected genes were further analyzed for enrichment of gene ontology (GO) terms using Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resource v6.7 (Huang da, Sherman, & Lempicki, 2009), against the whole human 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).

2.6 | Quantification of mRNA expression

Total RNA isolation, cDNA synthesis and quantitative polymerase chain reaction (qPCR) were performed as previously described (Eijken et al., 2007). Primer sequences (Sigma-Aldrich) are listed in Table S1.

2.7 | microRNA array and analysis

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

A-treated and untreated osteoblasts, following manufacturer's instructions.

Probes that were detected above background (detection $p < .01$), in at least one time point, in both biological replicates and annotated to a known miRNA (miRbase www.mirbase.org; Griffiths-Jones, Grocock, van Dongen, Bateman, & Enright, 2006), were considered for further analysis. Probes that were detected only in Activin A-treated or untreated osteoblasts and probes more than two-fold upregulated or downregulated were further analyzed (Table 1). miRNAs were analyzed by using DIANA miRpath v3 (Vlachos et al., 2015), IPA and Targetscan Release 7.1 (Agarwal, Bell, Nam, & Bartel, 2015) 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.

2.8 | Statistical analysis

Data for biochemical analysis were representative of independent experiments. All values are presented as mean \pm (SD) of technical replicates. Significance was calculated by two-way analysis of variance (ANOVA), followed by Bonferroni Post Hoc test, otherwise indicated elsewhere.

3 | RESULTS

3.1 | A single pulse of Activin-A is sufficient to reduce SV-HFO 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; Eijken et al., 2007). 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 SV-HFO 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 1 a. ALP activity was not affected by Activin-A (Figure 1 b). 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 two-fold lower in Activin A-treated osteoblasts than untreated cells, as shown in Figure 1 c ($p = .0003$).

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 SV-HFO osteoblasts treated with both Activin-A and SMAD inhibitor was significantly higher than in the cells treated with Activin-A

TABLE 1 miRNAs modulated by Activin-A in osteoblasts, 4 hr and 1 day after the start of Activin-A treatment. 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 hr	>2-Fold upregulated in A-OBs			>2-Fold downregulated in A-OBs		
	miR-590-3p	790.81	351.31	miR-486-3p	1,788.62	4,489.87
	miR-142-3p	386.24	178.37	miR-19a	1,209.74	2,925.18
	miR-18b	609.81	300.37	miR-33a	122.18	1,942.43
				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		4,929.56	2,055.87	miR-193a-3p	963.43	2,079.74
miR-100*		993.68	453.87	miR-590-3p	126.06	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					

Abbreviations: A-OBs, Activin-A treated osteoblasts; miRNAs, microRNAs; NT-OBs, non-treated osteoblasts.

only ($p = .004$) and at comparable levels as that of untreated osteoblasts (Figure 1d). SMAD inhibitor did not affect differentiation of the cells, as SV-HFO osteoblasts cultured in the presence of SMAD inhibitor mineralize at similar extent than the untreated cells.

3.2 | SMAD3 phosphorylation reached the maximum 1 hr 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 SV-HFO osteoblasts than in untreated cells, as shown in Figure 1e. The Activin-A pulse increased SMAD3 phosphorylation, with a peak after 1 hr of treatment, which was almost 2-fold higher in Activin A-treated osteoblasts than in untreated cells ($p < .001$).

3.3 | Activin-A treatment induced changes in gene expression in a two-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 osteoblasts at various time points using Illumina Human HT-12 v3 BeadChip array. Activin-A treatment induced a transient change in SV-HFO osteoblast gene expression in a two-wave fashion over time, as shown in Figure 2a. The first wave consisted of 38 differentially regulated genes ($q < .01$) and occurred from 1 hr till 8 hr after the start of Activin-A treatment (Table S2). The second wave of differentially regulated genes

occurred between 1 and 2 days after the Activin-A pulse (Table S3). 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 TGF- β signaling pathway was within the top 10 most enriched canonical pathways of the differentially regulated genes in both waves (Tables S4 and S5). Moreover, TGF- β was within the most enriched upstream regulators in both first and second wave, as analyzed by IPA and shown in Figure 2b,c by the predicted target genes (Table S6).

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; $p < .05$; Figure 3a). In addition, these 38 genes were analyzed using IPA (Figure 3b and Table S7). "Binding of DNA," including genes such as *SMAD7*, *JUNB*, and *FOSB*, was among the most significantly enriched terms ($p = 6.49 \times 10^{-2}$; Figure 3b). Most of these genes were upregulated by Activin-A. Furthermore "Vasculogenesis" (*SMAD7*, *JUNB*, and *ANGPTL4*) was identified to be significantly enriched ($p = 5.37 \times 10^{-9}$)

among the regulated genes. Interestingly, "differentiation of connective tissue cells," including genes such as *SMAD7*, *KLF10*, and *JUNB*, was significantly enriched ($p = 9.06 \times 10^{-8}$). 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 ($q < .01$; Table S3). 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 and Table S8. The functional categories Vasculogenesis ($p = 8.85 \times 10^{-8}$) including genes such as *IGFBP3*, *FBLN5*, and *SMAD3*, but also Adhesion of connective tissue cells ($p = 2.47 \times 10^{-5}$) (*POSTN*, *TGFBI*, and *BMP4*) and Differentiation of osteoblasts ($p = 7.56 \times 10^{-6}$) (*SMAD3*, *PPARG*, *POSTN*, *TGFBI*, and *CTHRC1*), were significantly enriched. ECM-related genes involved in these pathways such as *POSTN*, *TGFBI*, upregulated by Activin-A, and the downregulated *BMP4* were further confirmed by qPCR (Figure 4c).

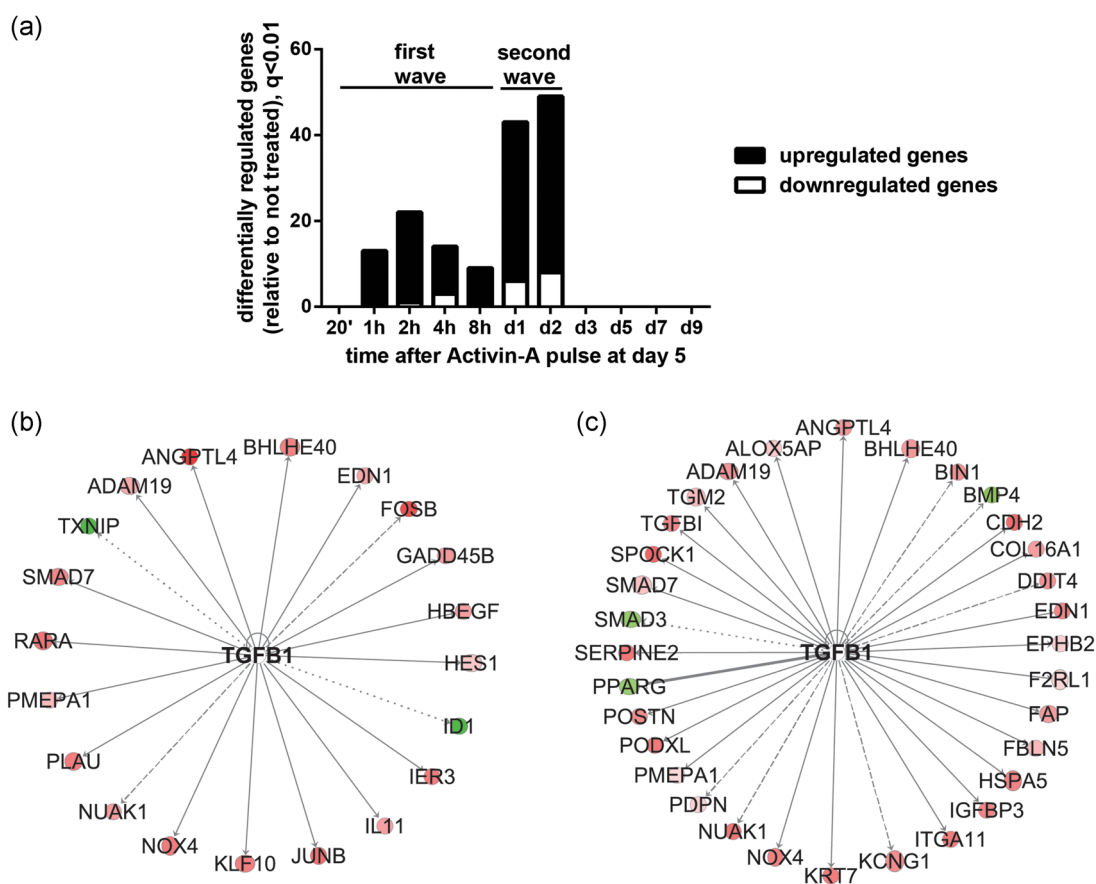


FIGURE 2 Activin-A treatment induced a transient change in osteoblast gene expression, in a two-wave-fashion over time. (a) Number of significantly differentially regulated genes in Activin A-treated osteoblasts compared to untreated cells at each time point ($q < .01$). (b) TGF- β is predicted to target 20 genes within those differentially regulated in the first wave (IPA analysis). (c) TGF β 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. IPA, Ingenuity® Pathway Analysis; TGF- β , transforming growth factor- β

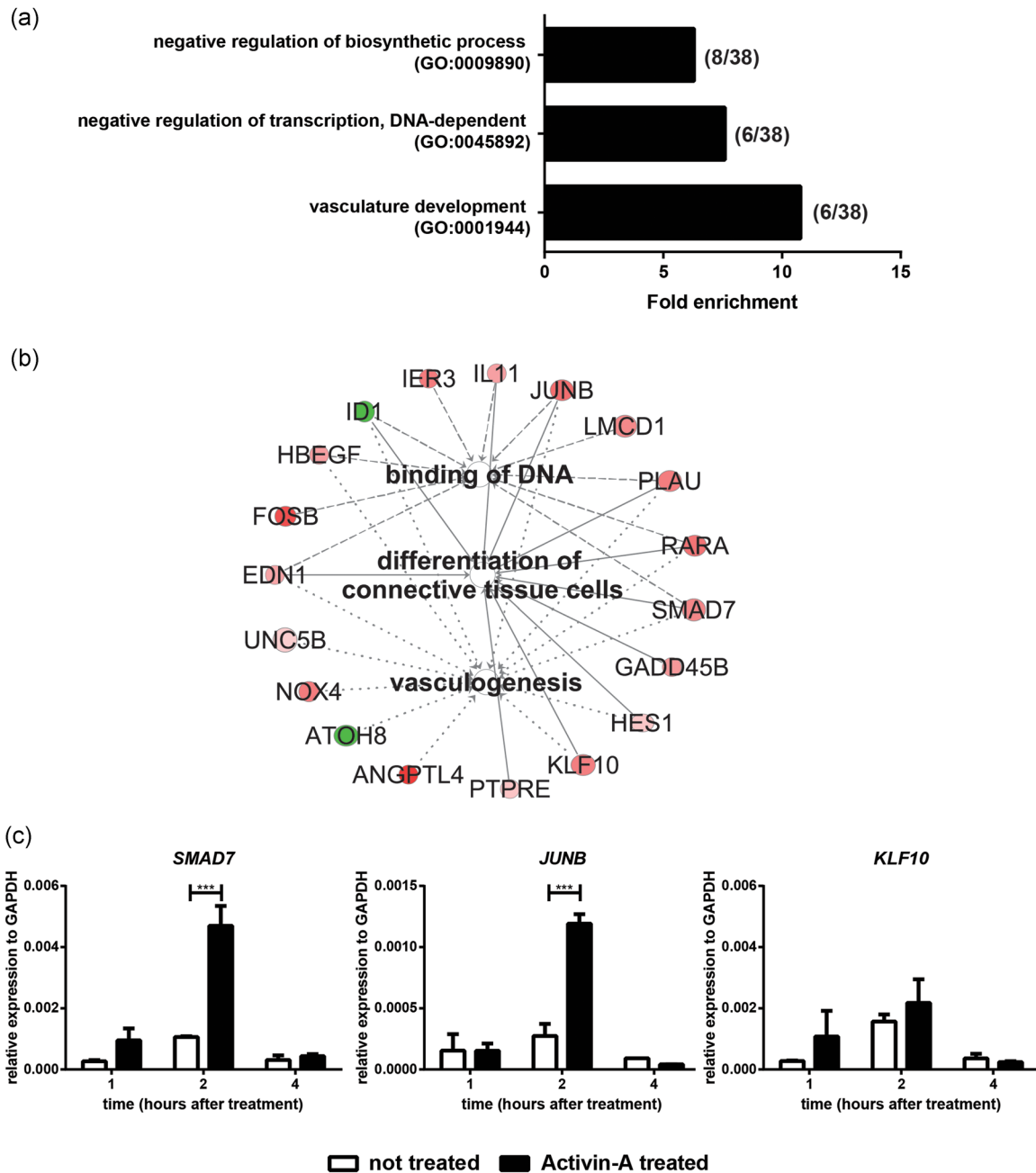


FIGURE 3 Activin-A pulse induced changes in gene transcription. (a) GO analysis of the 38 genes of the first wave of gene expression changes (1–8 hr after Activin A treatment start), that were differentially regulated in Activin A-treated cells compared to untreated ones. Only significantly enriched GO terms are shown (Benjamini $p < .05$). 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 mean \pm SD. GO, Gene Ontology; SD, standard deviation. (***) $p < .001$

3.4 | Activin-A treatment modified the microRNA profile of SV-HFO osteoblasts

In addition to the mRNA changes that occurred upon Activin-A treatment we analyzed the microRNA gene expression profile in SV-HFO osteoblasts, 4 hr 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 hr and Day 1 respectively to assess the miRNA profiles. A detailed flowchart of the miRNA profile analysis is presented in Figure S1.

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)

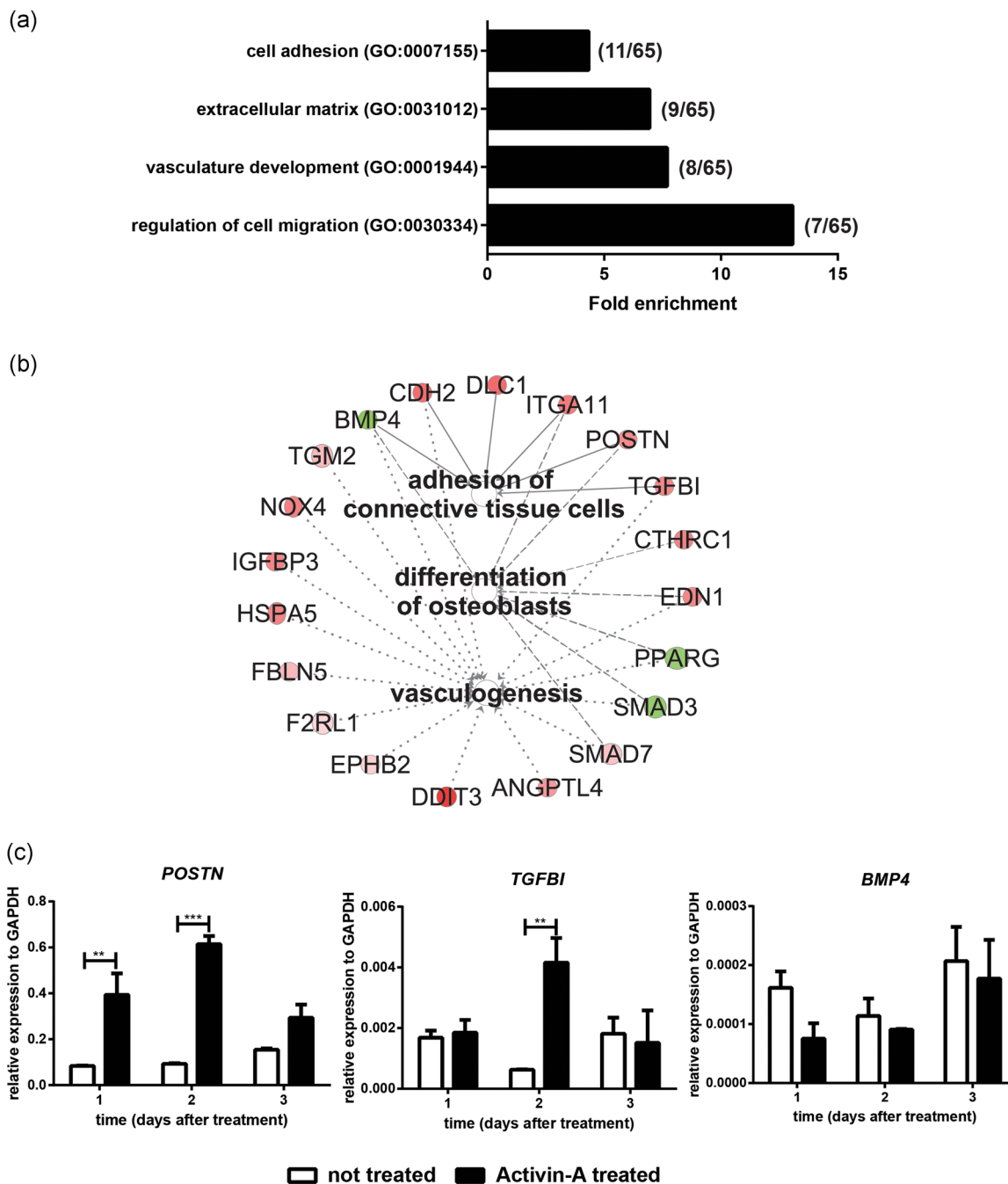


FIGURE 4 Activin A pulse induced changes in ECM composition. (a) GO analysis of the 65 genes that were differentially regulated in the second wave of gene expression (1 and 2 days after Activin A pulse start) in Activin A-treated cells. Only significantly enriched GO terms are shown (Benjamini $p < .05$). 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 treatment. Bars indicate mean \pm SD. ECM, extracellular matrix; IPA, Ingenuity® Pathway Analysis; GO, Gene Ontology; qPCR, quantitative polymerase chain reaction; SD, standard deviation. (** $p < .01$; *** $p < .001$)

were > 2-fold upregulated in Activin A-treated osteoblasts compared with 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 five miRNAs (miR-19a, miR-32, miR-33a, miR-150, and miR-486-3p) were > 2-fold

downregulated in these cells compared with 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 (Vlachos et al., 2015). Within the enriched

pathways, we focused on TGF- β signaling, as it is known to guide osteoblast differentiation and as shown in Figure 2 gene expression analyses identified this signaling pathway. TGF- β signaling was significantly enriched ($p = 1.54 \times 10^{-6}$), as 9 of the 15 miRNAs upregulated by Activin-A targeted 35 genes involved in TGF- β signaling. This is supported by the observation that also 29 mRNAs targeted by eight miRNAs downregulated by Activin-A, were involved in the TGF- β pathway (Table 2). Thus, target genes that are involved in TGF- β signaling were targets of miRNAs that were both upregulated and downregulated by Activin-A within 4 hr of treatment.

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, two were more than two-fold downregulated in Activin A-treated osteoblasts (Figure 5d; Table 1). In line with the 4-hr-treatment data, TGF- β signaling was significantly enriched ($p = .0001$), 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 TGF β signaling.

Next, we combined the data of miRNA and mRNA profiling, hypothesizing that miRNAs that were upregulated by Activin A treatment (4 hr and 1 day), would target mRNAs that were downregulated after 1 and 2 days in the Activin A-treated osteoblasts (for analysis scheme see Figure S1). 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 (Table S5). Within these pathways, we analyzed whether the genes that were downregulated by Activin-A were also predicted targets of miRNAs upregulated by Activin-A (Table S9). Indeed, we identified two miRNAs (miR-142-3p and miR-432*) that were upregulated by Activin-A and were predicted to target the downregulated gene *BMP4* (Table 3). In addition, seven miRNAs (miR-24-1*, miR-181d, miR-548f, miR-559, miR-589*,

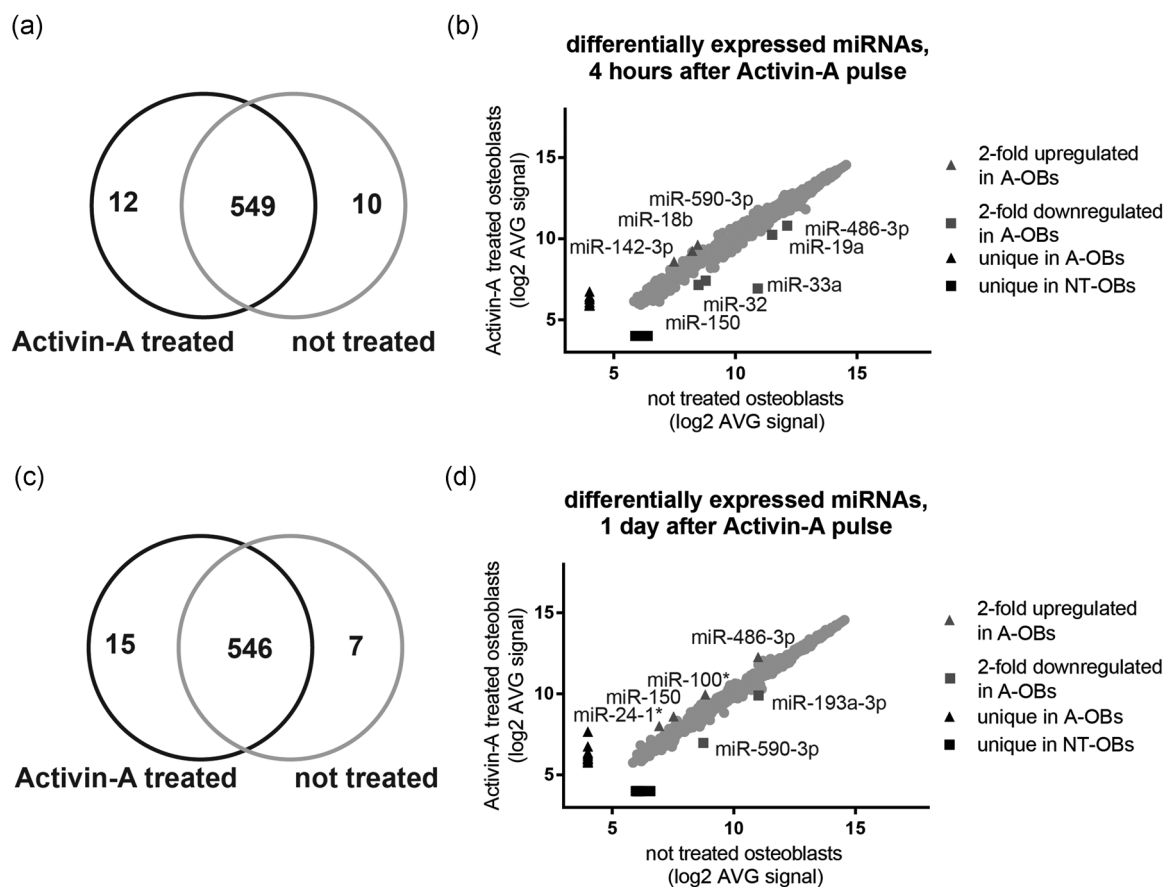


FIGURE 5 Osteoblast miRNA expression profiles were modulated by Activin-A treatment. (a) 12 miRNAs were uniquely detected in Activin A-treated osteoblasts, 10 miRNAs uniquely in the untreated cells, and 549 were detected in both conditions, 4 hr after the start of Activin-A treatment. (b) miRNAs uniquely detected in Activin A-treated osteoblasts, untreated osteoblasts, and with more than 2-fold difference in detection, 4 hr after the start of Activin-A treatment. (c) One day after the start of Activin-A treatment, 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. Only probes detected above background and annotated to known miRNAs are shown ($p < .01$). A-OBs, Activin A-treated osteoblasts; miRNAs, microRNAs; NT-OBs, not treated osteoblasts

TABLE 2 Activin-A modulated miRNAs that are predicted to target genes involved in TGF- β signaling pathway (DIANA miRpath v3; $p < .01$)

	Time after Activin-A pulse	KEGG pathway	<i>p</i> value	# Involved miRNAs	# Involved genes
miRNAs unique and upregulated in A-OBs	4 hr	TGF β 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

Abbreviations: A-OBs, Activin-A treated osteoblasts; KEGG, Kyoto Encyclopedia of Genes and Genomes; miRNAs, microRNAs; TGF- β , transforming growth factor- β .

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 (Table S10). Interestingly, Activin-A downregulated miRNAs that 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.

4 | DISCUSSION

In this study, we demonstrated that a short-term Activin-A treatment for only 2 days in the SV-HFO 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 2 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 (Eijken et al., 2007). In line with this, in this study we provided evidence that Activin A treatment only

TABLE 3 Genes downregulated by Activin-A in the second wave of gene expression changes (Day 1 and Day 2) and targets of the indicated miRNAs upregulated by Activin-A (IPA, TargetsScan analysis)

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

Abbreviation: miRNAs, microRNAs.

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 (Alves et al., 2013). Possibly, the timing of Activin A treatment should be earlier during differentiation 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. (2007), that the Activin-A treatment leads to a change in ECM 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 and 8 hr 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 (Eijken et al., 2007). As *SMAD7* is a known inhibitor of BMP- and TGF β -signaling, and was shown to reduce mouse osteoblast mineralization (Massague, 1998; Yano et al., 2012), this confirms our findings in human osteoblast cultures. *SMAD7* was also predicted as target of four 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 (Kenner et al., 2004; Long, 2012; Subramaniam et al., 2005), were upregulated in human osteoblasts that were continuously treated with Activin-A (Eijken et al., 2007). 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 (Alves et al., 2013), 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 TGF- β , that promote osteoblast adhesion and bone structure (Delany, Kalajzic, Bradshaw, Sage, & Canalis, 2003; Janssens, ten Dijke, Janssens, & Van Hul, 2005; Merle & Garnero,

TABLE 4 Genes upregulated by Activin-A (Day 1 and 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 hr	Target of miRNAs 1 day
<i>DDIT4</i>		miR-590-3p
<i>EDN1</i>	miR-19a miR-33a miR-486-3p miR-765	miR-590-3p
<i>HSPA5</i>		miR-590-3p
<i>IGFBP3</i>	miR-371b-3p miR-563	
<i>NOX4</i>	miR-10b miR-32 miR-33a	miR-590-3p
<i>PMEPA1</i>	miR-10b miR-23 miR-186* miR-765	
<i>SMAD7</i>	miR-32 miR-486-3p miR-573 miR-1263	

Abbreviation: miRNAs, microRNAs.

2012), in line with previous findings showing that Activin-A induced ECM-related genes (Alves et al., 2013; Eijken et al., 2007). 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 (Horiuchi, Tohmonda, & Morioka, 2016). 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 TGF β and Hypoxia inducible factor (HIF), and it mediates HIF-driven bone resorption in the angiogenic-osteogenic coupling (Knowles, Cleton-Jansen, Korsching, & Athanasou, 2010). Conversely, it also promotes osteoblast differentiation in fracture repair and stimulates vascular endothelial growth factor expression (Wilson, Wong, Toupadakis, & Yellowley, 2015). Activin-A is considered as commitment factor for the differentiation of erythroid progenitors (Yu,

Shao, Vaughan, Vale, & Yu, 1989). 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 (Shiozawa et al., 2010), but also on the HIF-mediated EPO production by osteoblasts that contributes to erythropoiesis and hematopoietic stem cell expansion (Rankin et al., 2012). Our findings showed that Activin-A upregulated miRNAs involved in erythropoiesis. For instance, miR-486-3p was upregulated in untreated osteoblasts 4 hr 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 an interesting candidate for Activin-A involvement in vasculogenesis (Lulli et al., 2013). miR-24 was shown to directly target *ALK4* modulating Activin-mediated erythropoiesis (Wang et al., 2008), 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) (Ottley, Nicholson, & Gold, 2016; Tsai et al., 2010). Our study showed that Activin-A modified also the miRNA profile of SV-HFO osteoblasts. For instance, 1 day after the start of the treatment, miR-217 was upregulated by Activin-A. miR-217 was also upregulated by Activin-A in hESCs (Tsai et al., 2010) and it reduced murine osteoblast mineralization by targeting *RUNX2* (Zhang et al., 2012), 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 (He et al., 2010). 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 unraveling 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 and 7 of SV-HFO osteoblast differentiation was able to reduce extracellular matrix mineralization 5–7 days later. Activin-A altered osteoblast gene expression profile in a biphasic fashion, first acting at transcription level, and subsequently altering ECM-related genes. Moreover, Activin A pulse was able to modify the miRNA profile of SV-HFO osteoblasts that could be linked to changes in mRNA expression. The results gave further insights into the mechanism by which Activin-A modulates osteoblast behavior and matrix mineralization. Activin-A and/or its mRNA and miRNA targets represent potential candidates 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 (Eijken et al., 2007).

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Study design and conduct: M. B., K. D., M. E., J. P., and J. L. Data collection: M. B. and K. D. Data analysis and interpretation: M. B., K. D., B. E., M. E., J. P., and J. L. Drafting manuscript, revising manuscript content and approving final version of manuscript: M. B., J. P., B. E., and J. L.

DATA AVAILABILITY STATEMENT

Data available on request to the authors.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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