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Authors
Gilham, Dean
Tsujikawa, Laura M
Sarsons, Christopher D
et al.

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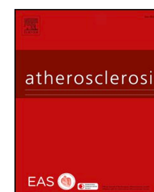

Apabetalone downregulates factors and pathways associated with vascular calcification

Dean Gilham[a], Laura M. Tsujikawa[a], Christopher D. Sarsons[a], Christopher Halliday[a], Sylwia Wasiak[a], Stephanie C. Stotz[a], Ravi Jahagirdar[a], Michael Sweeney[b], Jan O. Johansson[b], Norman C.W. Wong[a], Kamyar Kalantar-Zadeh[c], Ewelina Kulikowski[a,*]

[a] *Resverlogix Corp., Calgary, Canada*
[b] *Resverlogix Inc., San Francisco, USA*
[c] *University of California, Irvine, USA*

HIGHLIGHTS

- Bromodomain and extraterminal (BET) proteins are implicated in VSMC transdifferentiation and calcification.
- Apabetalone, a BET inhibitor, prevents calcification of VSMCs by regulating expression of key factors.
- BET protein BRD4 may cooperate with 7 specific transcription factors (TFs) to promote transdifferentiation and calcification.
- Apabetalone is a promising therapeutic for pathological vascular calcification.

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ABSTRACT

Background and aims: Apabetalone is an inhibitor of bromodomain and extraterminal (BET) proteins. In clinical trials, apabetalone reduced the incidence of major adverse cardiac events (MACE) in patients with cardiovascular disease and reduced circulating factors that promote vascular calcification (VC). Because VC contributes to MACE, effects of apabetalone on pro-calcific processes were examined.
Methods and results: Apabetalone inhibited extracellular calcium deposition and opposed induction of transdifferentiation markers in human coronary artery vascular smooth muscle cells (VSMCs) under osteogenic culture conditions. Tissue-nonspecific alkaline phosphatase (TNAP) is a key contributor to VC, and apabetalone suppressed osteogenic induction of the mRNA, protein and enzyme activity. The liver is a major source of circulating TNAP, and apabetalone also downregulated TNAP expression in primary human hepatocytes. BRD4, a transcriptional regulator and target of apabetalone, has been linked to calcification. Osteogenic transdifferentiation of VSMCs resulted in disassembly of 100 BRD4-rich enhancers, with concomitant enlargement of remaining enhancers. Apabetalone reduced the size of BRD4-rich enhancers, consistent with disrupting BRD4 association with chromatin. 38 genes were uniquely associated with BRD4-rich enhancers in osteogenic conditions; 11 were previously associated with calcification. Apabetalone reduced levels of BRD4 on many of these enhancers, which correlated with decreased expression of the associated gene. Bioinformatics revealed BRD4 may cooperate with 7 specific transcription factors to promote transdifferentiation and calcification.
Conclusions: Apabetalone counters transdifferentiation and calcification of VSMCs via an epigenetic mechanism involving specific transcription factors. The mechanistic findings, combined with evidence from clinical trials, support further development of apabetalone as a therapeutic for VC.

1. Introduction

Vascular calcification (VC) is aberrant deposition of calcium phosphate and hydroxyapatite in blood vessels, leading to pathological vascular stiffness [1]. VC is prevalent in chronic kidney disease (CKD), and the extent of VC predicts cardiovascular risk [2,3]. VC arising in CKD patients occurs in the medial layer of the vessel wall, an elastic region comprised of vascular smooth muscle cells (VSMCs), in a process

* Corresponding author. Resverlogix Corp., 300 – 4820 Richard Road SW, Calgary, AB T3E 6L1, Canada.
E-mail address: Ewelina@resverlogix.com (E. Kulikowski).

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that can occur independently of hypercholesterolemia and atherosclerosis [4]. During this process, VSMCs transdifferentiate into osteoblast-like cells that deposit calcium-rich hydroxyapatite and express osteoblast markers [5,6]. Elevated serum levels of inorganic phosphate, proinflammatory cytokines, oxidized LDL and uremia that accompany renal impairment promote transdifferentiation of VSMCs [7]. Therapies for VC include phosphate binders, calcimimetics and pyrophosphate analogs that alter mineral imbalances arising from impaired kidney function [8]. However, these agents do not target VSMCs directly, and a high rate of cardiovascular complications persist in patients with renal impairment and VC.

Bromodomain and extraterminal (BET) proteins BRD2, BRD3 and BRD4 are epigenetic readers that bind acetylated lysines on histone tails and transcription factors (TFs) via bromodomains (BD) 1 and 2 [9]. BET proteins act as molecular scaffolds between acetylation-dependent binding sites and transcriptional machinery to regulate gene expression. Apabetalone is an oral small molecule inhibitor of BET bromodomains (BETi) that preferentially targets BD2, a characteristic that differentiates it from pan-BET inhibitors that bind BD1 and BD2 with equal affinity [10]. In clinical trials, apabetalone reduced major adverse cardiac events in patients with cardiovascular disease (CVD), resulting in 44% relative risk reduction on top of standard of care [11]. Further, apabetalone beneficially affected levels of dysregulated proteins and biological pathways that drive vascular disease and pro-calcific processes. In CVD patients, apabetalone increased high density lipoprotein (HDL) [11], which is known to counter calcification of VSMC induced by IL-6 or IL-1β *in vitro* [12]. In CKD patients, apabetalone had favorable effects on kidney function evaluated by estimated glomerular filtration rate [13]. Apabetalone also reduced circulating alkaline phosphatase (ALP), a robust and independent predictor of all-cause mortality that contributes to VC progression [13,14]. Notably, a single dose provided to CKD patients rapidly resulted in reduction of several inflammatory cytokines, including IL-6 [15]. Canonical pathway analysis of plasma proteomics predicted downregulation of IL-6 and bone morphogenetic protein signaling pathways, which both promote VC [15]. Processes involved in VC are similar to the mineralization of bone, and therefore strategies to alleviate VC could have effects on bone metabolism. However, preclinical models have demonstrated that BETi do not diminish bone structure or mechanical properties, and may increase bone volume and restore mechanical strength [16–18]. Although apabetalone improved cardiovascular outcomes and beneficially regulated factors that promote VC in clinical trials, it remains unknown whether it has direct effects on mineralization of VSMCs.

This report shows for the first time that transdifferentiation and matrix mineralization of primary coronary artery VSMCs involves factors that are regulated by BET proteins. Apabetalone opposes calcification of VSMCs through an epigenetic mechanism that governs expression of pro-calcific genes. We demonstrate that BRD4 is redistributed on chromatin during transdifferentiation, and that BRD4 chromatin occupancy is apabetalone sensitive. Bioinformatics of chromatin occupancy with and without apabetalone suggest specific TFs cooperate with BRD4 to perpetuate transdifferentiation. Based on this data, apabetalone may downregulate pathological cellular responses that lead to VC. This may contribute to the positive outcomes observed clinically in CVD patients treated with apabetalone.

2. Materials and methods

2.1. Materials

Apabetalone and the pan-BETi JQ1 were synthesized as described previously by NAEJA Pharmaceuticals (Edmonton, Canada) or IRIX Pharmaceuticals (Florence, SC) [19,20]. The inhibitor of tissue-non-specific ALP (TNAPi) was from Calbiochem (Billerica, MA #613810). Cell culture reagents were from Life Technologies (Burlington, ON) and chemicals from Sigma-Aldrich (St. Louis, MO).

2.2. Cell culture

Primary human coronary artery VSMCs (VSMCs; PromoCell, Heidelberg, Germany) were cultured as described previously [21]. Cells were grown in Smooth Muscle Cell Growth Medium 2 (PromoCell), and used in studies between passages 3 and 8. After reaching confluence, cells were switched to Dulbecco's Modified Eagle's Media supplemented with 10% FBS and penicillin-streptomycin as basal media. Osteogenic conditions included the basal media plus 10 nM dexamethasone, 10 mM β-glycerophosphate and 100 μM ascorbic acid. Media were changed three times per week. Protocols for culture of primary human hepatocytes and A7r5 vascular smooth muscle cells appear in Supplemental Materials.

2.3. mRNA quantification

Relative mRNA expression was determined by TaqMan based real-time PCR [22]. TaqMan ID numbers are listed in Supplemental Table 1.

2.4. Imaging

Micrographs were obtained using a Leica DM IL microscope.

2.5. Alizarin red staining

VSMCs were fixed with 4% paraformaldehyde for 15 min, washed with PBS, and stained with 2% alizarin red S in 5 mM Tris pH 4.1 for 30 min. Cells were destained with 0.2% acetic acid prior to photographing.

2.6. Quantification of calcium deposition

Extracellular calcium was measured as previously described [23], and outlined in Supplemental Materials.

2.7. TNAP activity

VSMCs were harvested in PBS containing 1% Nonidet® P40 and 0.1% sodium dodecyl sulfate (SDS). Cells were lysed by brief sonication and insoluble material removed by centrifugation at 10,000 *g* for 3 min. TNAP enzyme activity in the lysate was determined in a colorimetric assay (Biovision, K412-500), while protein concentration was determined using the DC protein assay (Bio-Rad, Hercules, CA). TNAP activity was normalized to total protein.

2.8. Immunoblots

Cell lysates were prepared in PBS containing 1% Nonidet® P40, 0.5% sodium deoxycholate, 0.1% SDS, 60 mM sodium fluoride, 1 mM sodium orthovanadate, 5 mM sodium pyrophosphate and protease inhibitors (Sigma-Aldrich). Cells were sonicated at 75% output for 15 s with a Branson SLPt sonicator (Branson Ultrasonics, Danbury, CT), then incubated on ice for 15 min. Insoluble material was removed by centrifugation at 10,000 g at 4 °C for 3 min. Lysates containing equal amounts of protein were resolved on NuPAGE gels and nitrocellulose membranes were incubated with antibodies for TNAP 12 ng/mL (Abcam, Cambridge, MA, ab108337) or β-actin 1:40,000 (Sigma-Aldrich, A3854).

2.9. ELISA

Human TNAP ELISA was performed using a kit from Abbexa Ltd. (Cambridge, United Kingdom, abx575206).

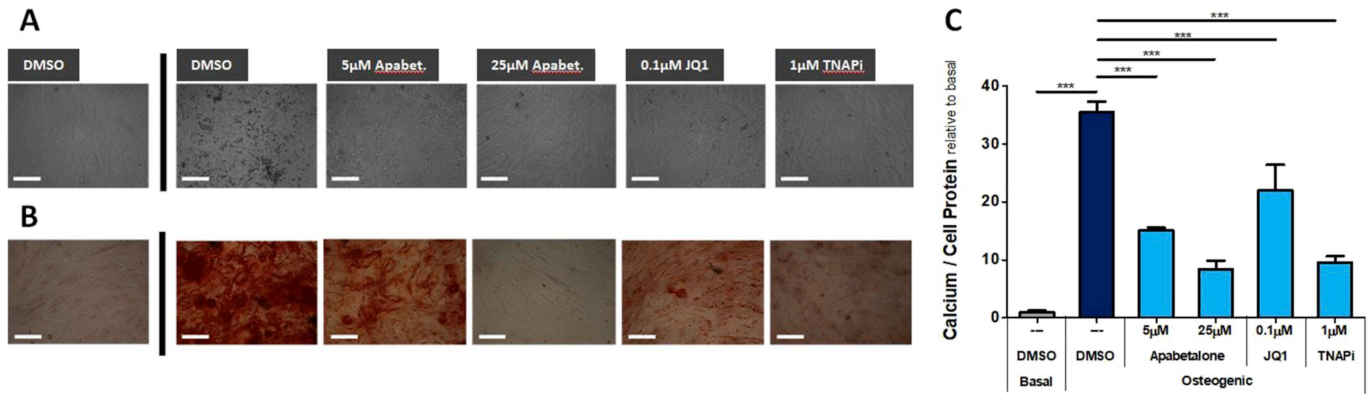

Fig. 1. BETi opposes osteogenic calcification of VSMCs.
VSMCs were cultured for 12 days in basal conditions or osteogenic conditions with or without apabetalone (Apabet.), JQ1 or an inhibitor of tissue-nonspecific alkaline phosphatase (TNAPi). (A) Representative light microscope images. (B) Calcification detected by alizarin red S–staining. Scale bar: 100 μm. C: Quantification of eluted calcium: mean ± SD of 4 independent replicates/group. ***$p < 0.001$, one-way ANOVA followed by Dunnett's Multiple Comparison Test.

2.10. Chromatin immunoprecipitation combined with deep sequencing (ChIP-seq)

VSMCs were cultured for 12 days in basal or osteogenic conditions. Active Motif (Carlsbad, CA) prepared chromatin, performed ChIP-seq reactions, alignments with the human genome and basic data examination. Details of the ChIP-seq protocol appear in Supplemental Materials.

2.11. ChIP-seq analysis

BRD4 peak locations across the genome were determined using the MACS algorithm (v1.4.2) with a cutoff p-value of 10^{-7} [24]. Active Motif's proprietary analysis assigned peak metrics, peak locations and gene annotations. “Intervals” were defined as genomic regions with local BRD4 enrichment. To compare peak metrics between samples, overlapping intervals were grouped into “active regions.” Active regions were used because the locations and lengths of intervals were not the same between samples. The area of BRD4 active regions was calculated as total fragments in the interval. NCBI-annotated genes within 10,000 base pairs (bp) were associated with that active region. BRD4-rich enhancers were recognized using a two-step algorithm. First, MACS peaks were merged if their inner distance was less than 12,500 bp. Second, the merged peak regions with the strongest signals (top 5%) were identified as BRD4-rich enhancers. The gene annotation margin for BRD4-rich enhancers was 25,000 bp.

2.12. ChIP-sequencing bioinformatic analysis

Ingenuity® Pathway Analysis (IPA®) software (QIAGEN, Redwood City, CA; www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis) was used to identify upstream regulators of transcription in NCBI-annotated genes associated with ≥1.3 fold change in the area of BRD4 active regions *versus* controls. When multiple active regions were associated with the same gene, the largest one appearing in the control group was used.

2.13. Genomic co-localization of BRD4 with transcription factor binding sites

Enrichment of motifs overlapping with chromatin associated BRD4 was determined using Peak-motifs software (http://rsat.sb-roscoff.fr). The top ten enriched motifs of 6–8 bases were then queried using the JASPAR core nonredundant vertebrates (2018) database (http://jaspar.genereg.net) to identify consensus TF binding sites. This *de novo* motif discovery protocol identified putative TF binding sites enriched in regions of BRD4 chromatin association, and were considered significant when the number of similarly aligned sequences expected to be returned at random (e-value) was less than 10^{-10}.

2.14. Statistical analysis

Two-tailed Student's t-test or one-way ANOVA followed by Dunnett's Multiple Comparison Test were applied and indicated in figure legends; $p < 0.05$ was considered significant.

3. Results

3.1. Effect of apabetalone on matrix mineralization in calcifying human coronary artery VSMCs

We first examined the role of BET proteins in transdifferentiation and matrix mineralization of primary human coronary artery VSMCs. When grown for 15 days in osteogenic conditions, VSMCs developed dark foci (extracellular deposits) that were not apparent in basal conditions (Fig. 1A). These foci were verified by alizarin red S staining to be calcium-rich deposits (Fig. 1B). Compared to basal conditions, extracellular calcium levels were more than 35-fold higher under osteogenic conditions ($p < 0.001$; Fig. 1C). BETi with distinct chemical scaffolds were applied during the 15-day osteogenic transdifferentiation period, including the BD2-selective apabetalone, or the pan-BETi JQ1 that targets both bromodomains [19]. TNAPi, also known as MLS-0038949, was previously shown to oppose calcification of VSMCs [25] and utilized as a positive control. Like TNAPi, apabetalone and JQ1 reduced alizarin red S staining, which was consistent with reduction of foci (Fig. 1A-B). Quantitatively, apabetalone impeded calcium accumulation in a dose dependent manner, an effect also observed with JQ1 and TNAPi (Fig. 1C). Thus, calcium deposition was dependent on TNAP activity and was BETi sensitive.

3.2. Induction of TNAP in calcifying VSMC is countered by apabetalone

Because pharmacological inhibition of TNAP reduced mineralization of VSMCs (Fig. 1), we examined BETi regulation of TNAP protein levels and enzyme activity. A 4-fold increase in TNAP protein was detected by immunoblot in osteogenic *versus* basal conditions (Fig. 2A). The increase was suppressed by either apabetalone or JQ1 (Fig. 2B). The BETi mediated reduction in TNAP protein was verified using an ELISA assay (Fig. 2C). Correspondingly, cell associated TNAP enzyme activity increased approximately 4-fold in osteogenic conditions (Fig. 2D; $p < 0.001$), mirroring the change in protein levels (Fig. 2B and C). Apabetalone or JQ1 reduced cell associated TNAP enzyme

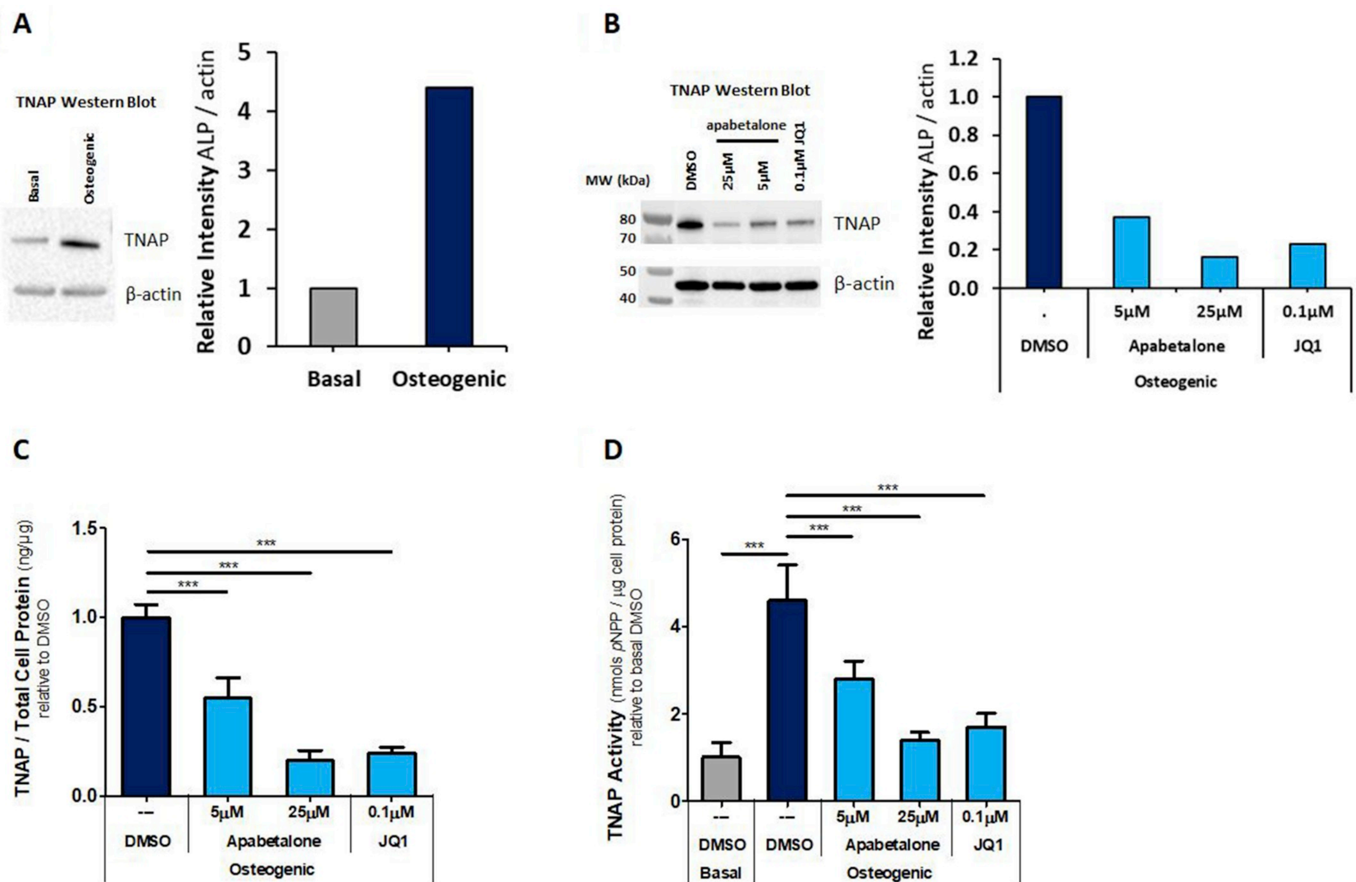

Fig. 2. Osteogenic conditions induce TNAP protein and activity levels, which are blocked by BETi.
VSMCs were cultured in basal or osteogenic conditions ± apabetalone or JQ1 for 15 days.
(A and B) Representative immunoblots of TNAP and quantification by densitometry. β-Actin served as a loading control. (C) TNAP ELISA: mean ± SD of 3 independent replicates/group. (D) Cell associated TNAP activity. Mean of 6 independent replicates/group. ***$p < 0.001$, one-way ANOVA followed by Dunnett's Multiple Comparison Test.

activity (Fig. 2D), consistent with lower amounts of TNAP protein observed by immunoblot and ELISA. Notably, in biochemical assays with purified recombinant human TNAP, apabetalone did not inhibit TNAP enzyme activity (data not shown), indicating apabetalone affects cellular TNAP production or degradation rather than modifying the enzyme's function.

3.3. Apabetalone regulates TNAP gene expression in PHH

The liver is a major source of TNAP in serum [26]. Therefore, regulation of TNAP expression (gene symbol *ALPL*) through BETi was investigated in primary human hepatocytes (PHH). As shown in Supplemental Fig. 1A, *ALPL* expression was reduced by apabetalone by 24 h of treatment, and this suppression was sustained through 72 h. Results were verified in PHH from another donor (Supplemental Fig. 1B). JQ1 also reduced *ALPL* expression, indicating regulation of hepatic *ALPL* expression involves BET proteins.

3.4. Apabetalone regulates osteogenic gene expression in VSMCs

To dissect the mechanisms for BETi mediated prevention of matrix mineralization, the expression of genes with established roles in osteogenic reprogramming, as well as markers of the transdifferentiation process, were measured including *ALPL* [26], runt-related transcription factor 2 (*RUNX2*) [8], wingless-type MMTV integration site family member 5A (*WNT5A*) [27] and matrix Gla protein (*MGP*) [5]. Expression of these genes was induced in VSMCs under osteogenic conditions in a time dependent manner (Fig. 3A). Induction was suppressed by apabetalone or JQ1 (Fig. 3A–B). Downregulation of *Alpl* expression by apabetalone in osteogenic conditions was confirmed in rat A7r5 VSMCs (Supplemental Fig. 1C). These data support a role for BET proteins in expression of genes that drive transdifferentiation and contribute to VC.

In addition to the osteogenic conditions used in this study, inflammatory cytokines, leptin, periostin, oxidized low density lipoproteins (oxLDL) via NFATC1 activation, and non-canonical WNT5A-receptor tyrosine kinase-like orphan receptor 2 (ROR2) signaling also induce calcification of VSMCs [5]. Leptin and periostin expression were decreased in osteogenic *versus* basal conditions, and further suppressed with apabetalone treatment (Supplemental Fig. 2). In contrast, *NFATC1*, Activin A Receptor Type 2A (*ACVR2A*; a transforming growth factor beta [TGF-β] receptor) and *ROR2* were induced in osteogenic conditions, but were suppressed by apabetalone or JQ1. Thus, multiple independent pathways that promote calcification of VSMCs may be inhibited by BETi.

3.5. BRD4 is redistributed on chromatin during transdifferentiation of VSMCs, and is displaced by apabetalone

BRD4, a target of BETi [19,28], has been linked to calcification [16,29]. Therefore, BRD4 association with chromatin during VSMC transdifferentiation and the effects of BETi were investigated by ChIP-seq. A flow chart of the analysis is provided in Fig. 4A. Osteogenic conditions resulted in ∼50% reduction in the number of peaks and BRD4-rich enhancers (defined in Materials and methods). Apabetalone

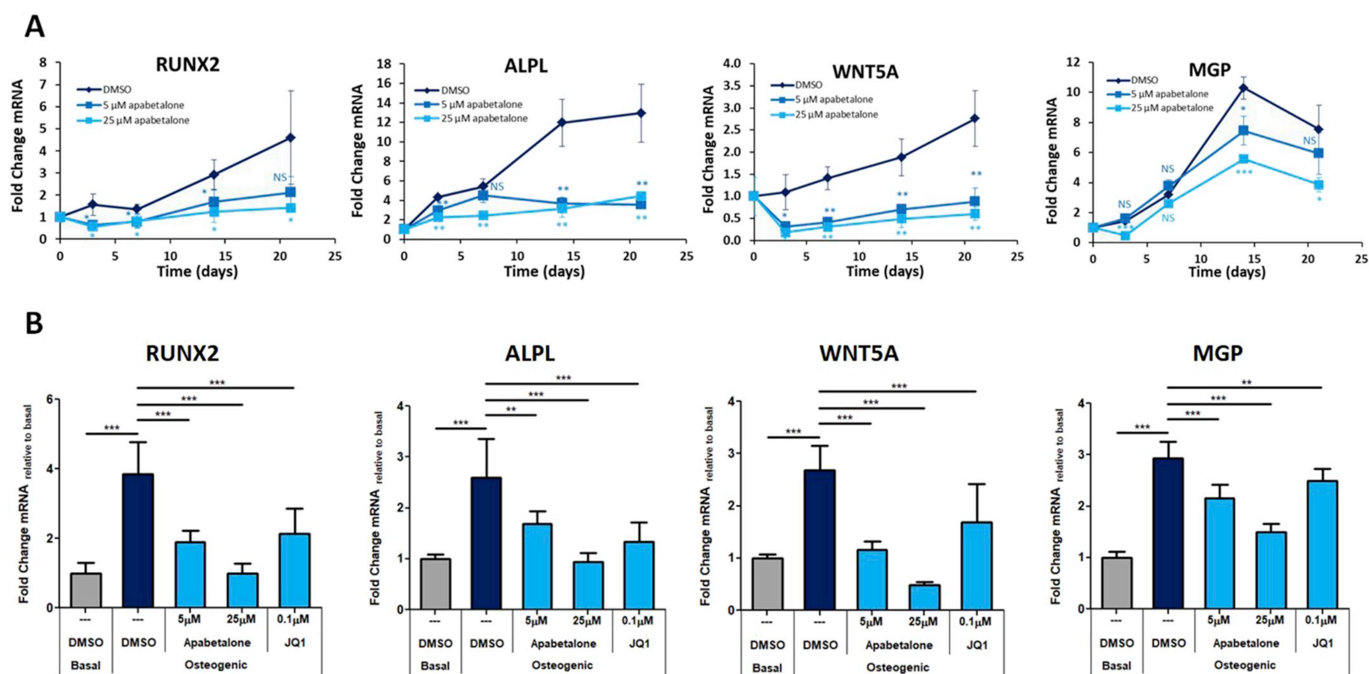

Fig. 3. BETi mediated downregulation of critical factors in VSMC transdifferentiation and mineralization.
(A) VSMCs were cultured in osteogenic conditions ± apabetalone for 3, 7, 14, or 21 days. Expression of genes associated with osteogenic calcification was measured by real-time PCR. Mean ± SD of 3 independent replicates/group. NS = not significant, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, Student's t-test *versus* DMSO treated cells at the same time point. (B) VSMCs were cultured in basal or osteogenic conditions ± apabetalone or JQ1 for 12 days, followed by real-time PCR analysis. Data represent mean ± SD of 6 independent replicates/group. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, one-way ANOVA followed by Dunnett's Multiple Comparison Test.

and JQ1 treatment caused further decrease in BRD4 peaks, which was dose dependent for apabetalone (Fig. 4B). Compared to basal conditions, the percentage of BRD4 peaks in promoters and exons were reduced under osteogenic conditions, with a concomitant increase in introns and intergenic regions (Fig. 4B). BRD4-rich enhancers were also impacted; although fewer in number (Fig. 5A), enhancers in osteogenic conditions were longer than those in basal conditions, and larger with more fragments per interval (Fig. 5B and C; $p < 0.05$). BRD4 enhancers became shorter and smaller in cells treated with BETi. Clearly genome wide redistribution of BRD4 occurs during transdifferentiation, and a portion of BRD4 chromatin association is BETi sensitive.

Genes in proximity to BRD4-rich enhancers were assessed. There were 753 NCBI-annotated genes within 25,000bp of a BRD4-rich enhancer in basal conditions and 236 in osteogenic (Supplemental File 1). Of these 236 genes, 198 (84%) were also identified in basal conditions (Fig. 5D). Intriguingly, 38 genes were uniquely associated with BRD4-rich enhancers under osteogenic conditions (Fig. 5D and Supplemental Table 2). Several of these genes are associated with atherosclerosis or calcification (references in Supplemental Table 2), including *C6*, *IL1R1*, *TIMP4*, *PKDCC* and *ZBTB16*. The expression of these genes was elevated under osteogenic *versus* basal conditions (Fig. 5E), in accordance with a newly formed BRD4-rich enhancer in proximity. Apabetalone dose dependently reduced the size of these enhancers (Fig. 5E and F). However, apabetalone only decreased the expression of *C6*, *IL1R1* and *TIMP4* (Fig. 5E). Treatment with JQ1 generated similar results as apabetalone, indicating both compounds target BRD4 binding in regulatory regions to modify expression of select genes.

3.6. Putative BRD4-TF associations

Using ChIP-seq, we identified 7,975 genes within 10,000bp of BRD4 active regions in VSMCs. Bioinformatics was used to predict TFs that may cooperate with BRD4 to drive calcification. In osteogenic conditions, 25 µM apabetalone altered the size of BRD4 active regions by ≥ 1.3 fold in proximity to 3,952 genes (decrease in 3,261; increase in 691). IPA® software organized these genes into transcriptional networks using upstream regulator analysis. The output highlighted TFs that may coregulate gene expression with BRD4 in osteogenic conditions and were sensitive to BETi. There were 154 upstream regulators of transcription predicted to be affected by 25 µM apabetalone ($p < 0.05$; Supplemental File 2). Twenty-three have recognized roles in calcification (Supplemental Table 3).

To further the analysis, TF binding motifs that overlap with BRD4 assemblies on chromatin were identified by *de novo* motif discovery (http://rsat.sb-roscoff.fr). Four consensus binding motifs and 16 putative TFs were disproportionately overrepresented with BRD4 in osteogenic *versus* basal conditions ($e < 10^{-10}$; Supplemental Table 4). TFs that bind these motifs may cooperate with BRD4 to facilitate transdifferentiation. Next, we examined BRD4-TF associations that were BETi sensitive. In osteogenic culture conditions, 23 TF consensus sequences showed enrichment with BRD4 that was significantly reduced by BETi ($e < 10^{-10}$; Supplemental Table 5).

Combining IPA® and *de novo* motif discovery analyses generated 7 TFs that may interact with BRD4 in VSMCs in osteogenic conditions in a BETi-dependent manner (Table 1). Potentially, these TFs work cooperatively with BRD4 to regulate osteogenic gene transcription.

4. Discussion

VC is common in patients with renal impairment, where progressive mineralization of the vessel leads to arterial stiffness and cardiovascular complications [30]. Primary human coronary artery VSMCs can be stimulated to transdifferentiate to a calcifying phenotype and model calcification affecting the artery. Our data demonstrate for the first time that VSMC calcification is mediated by BET proteins and is sensitive to BETi.

Elevated serum ALP is associated with vascular stiffening and cardiac events [31,32]. Apabetalone was recently shown to reduce serum

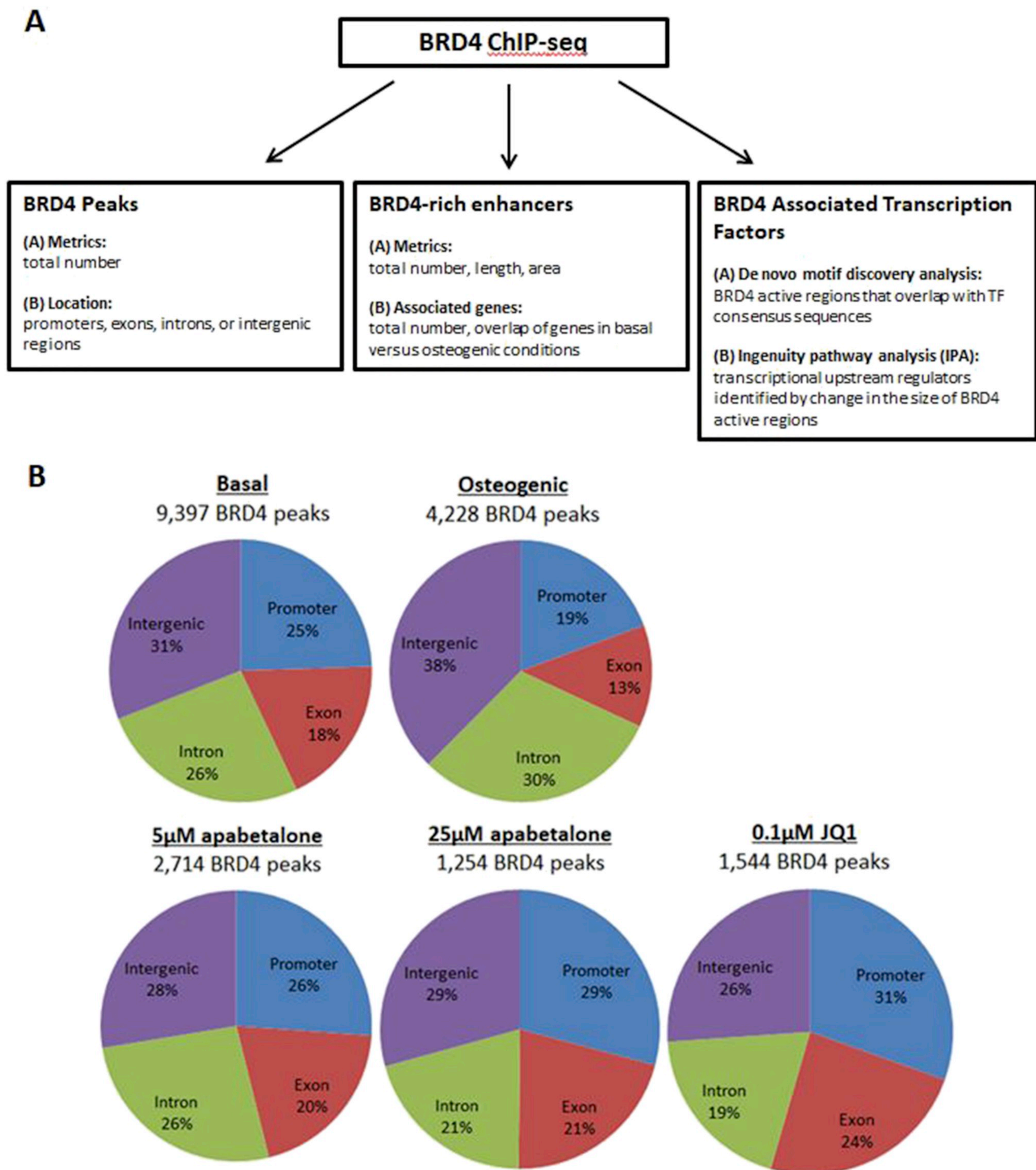

Fig. 4. BRD4 ChIP-seq analysis in VSMCs.
(A) Flow chart of BRD4 ChIP-seq analysis. (B) BRD4 peaks found in ChIP-seq are grouped according to their location in promoters (0–3 kb to transcription start site), exons, introns, or intergenic regions.

ALP in CKD patients with a history of CVD [13]. In addition to TNAP, which comprises > 90% of enzyme activity in the serum, the intestinal-type, placental-type and placental-like isozymes make up the total ALP in circulation [26]. In this study, we demonstrate that apabetalone suppresses TNAP expression in liver cells (Supplemental Figs. 1A–B), the major source of circulating TNAP [26], which is consistent with reduced serum ALP observed in patients receiving apabetalone [13,14]. The present study also shows that apabetalone or JQ1 attenuates TNAP gene expression, protein levels and enzyme activity in VSMCs during osteogenic transdifferentiation (Figs. 2, 3A-B, Supplemental Fig. 1C). Upregulation of TNAP is critical for mineralization [5,33]; therefore, the suppression of TNAP induction by apabetalone could potentially reduce VSMC calcification. Chemical inhibition of TNAP has been proposed as a therapeutic for VC [34,35], however, to our knowledge, this is the first clinical stage molecule that modifies TNAP production.

While clinical trials demonstrate apabetalone beneficially affects inflammation [15], plasma lipid profile [11], and may improve kidney function [13], data in this mechanistic study shows apabetalone also

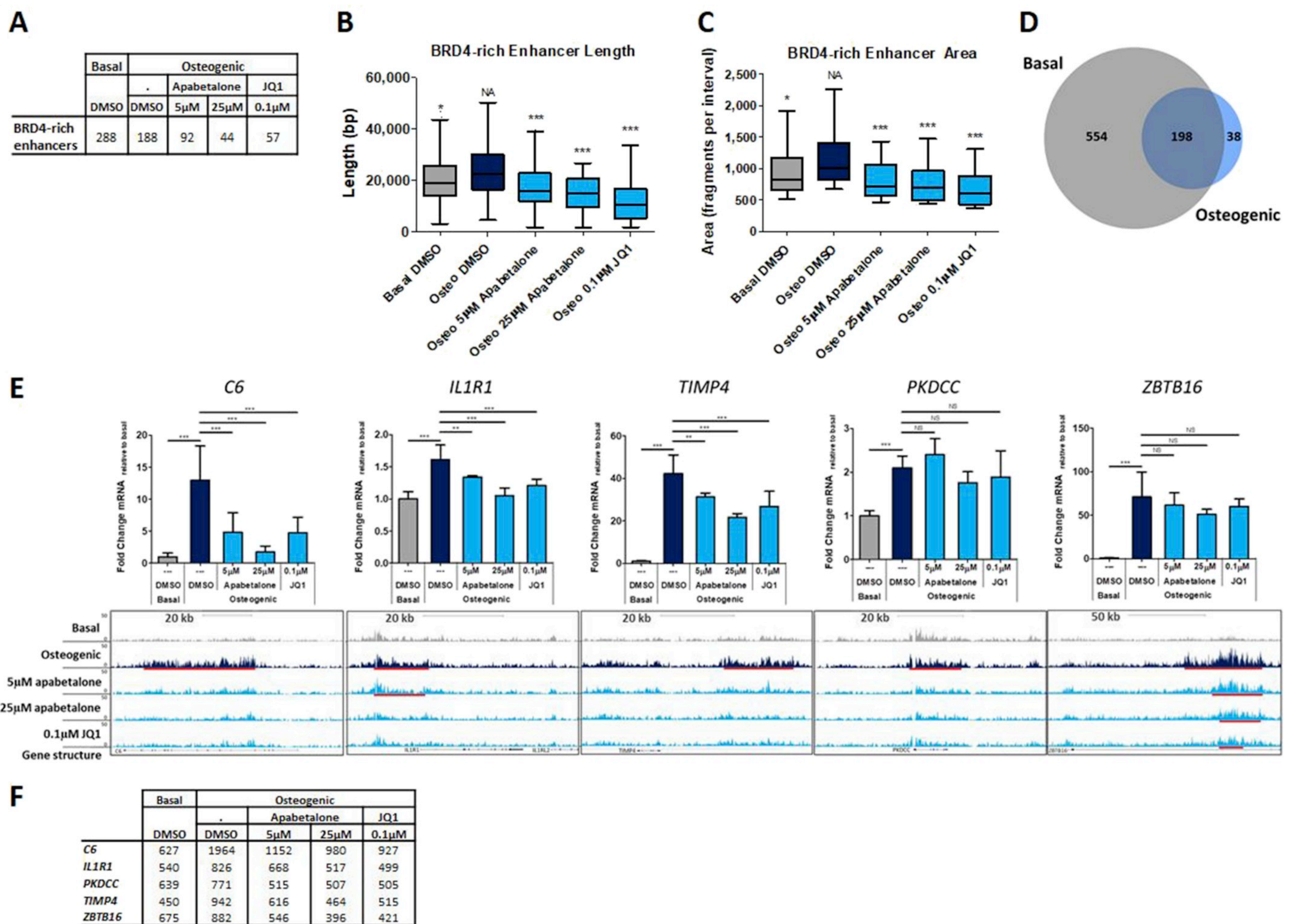

Fig. 5. BRD4-rich enhancers and associated genes.
(A) Number of BRD4-rich enhancers. (B–C) Box and whisker plots showing (B) length and (C) area of all BRD4-rich enhancers in each group. Osteo = osteogenic conditions. (D) VENN diagram: number of genes within 25,000 bp of BRD4-rich enhancers. (E) VSMCs were cultured in osteogenic conditions ± apabetalone or JQ1 for 12 days. Cells treated in parallel were analyzed for gene expression by real-time PCR (top; mean ± SD of 6 independent replicates/group) or by ChIP-seq (bottom) showing the UCSC genome browser view of BRD4 tracks in the region of the gene. Red lines indicate location of BRD4-rich enhancers. (F) Area of BRD4-rich enhancers in proximity to the indicated genes (fragments per interval). Statistical analysis was via one-way ANOVA followed by Dunnett's Multiple Comparison Test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, NS = not significant, NA = not applicable as it was the reference sample.

impacts calcification of VSMCs. BETi downregulated expression of genes with established roles in osteogenic reprogramming or markers of the transdifferentiation process: *RUNX2*, *ALPL*, *WNT5A* and *MGP* (Fig. 3A–B). The transcription factor RUNX2 is considered the master regulator of VSMC transdifferentiation [8]. Suppressing *RUNX2* gene induction is consistent with impeding calcification. Interestingly, the increase in *ALPL* expression in osteogenic conditions preceded *RUNX2* induction (Fig. 3A). A similar temporal arrangement was observed in uremic rats, where aortic regions with increased *ALPL* expression coincided with calcification but preceded *RUNX2* elevations [36]. In this model, *ALPL* induction appears to be dissociated from *RUNX2* upregulation. *WNT5A* is induced in calcifying VSMCs [37], and *MGP* is an established marker of osteogenic transdifferentiation of VSMCs [38]. In our study, attenuation of *WNT5A* and *MGP* gene induction by apabetalone indicated transdifferentiation was impeded by apabetalone. Collectively, osteogenic conditions induced genes associated with transdifferentiation of VSMCs, an effect hindered by BETi.

Transdifferentiation of VSMCs can be induced by stimuli other than

Table 1
TFs associated with BETi sensitive BRD4 assemblies: *de novo* motif discovery analysis combined with IPA upstream regulators of transcription.

TF/upstream regulator	5 μM Apabetalone		25 μM Apabetalone		0.1 μM JQ1		
	IPA *p*-value	TF motif e-value	IPA *p*-value	TF motif e-value	IPA *p*-value	TF motif e-value	Calcification references
SMAD4	< 0.05	2.5E-24	< 0.001	1.1E-44	< 0.01	3.0E-29	[53,54]
Smad2/3-Smad4	< 0.01	2.5E-24	< 0.01	1.1E-44	< 0.01	–	[55,56]
FOS	< 0.01	9.3E-25	< 0.01	–	< 0.001	2.0E-51	[50,57]
ATF3	< 0.05	5.9E-36	< 0.05	–	–	4.4E-126	[51,58]
TWIST2	< 0.05	1.3E-35	< 0.01	2.1E-61	< 0.05	2.8E-51	[59,60]
TBX2	< 0.01	8.9E-29	< 0.01	1.8E-24	–	5.8E-50	[61]
MEIS1	–	–	< 0.05	5.9E-26	–	2.2E-27	–

osteogenic culture conditions. We determined that BETi reduced the expression of mediators of VSMC transdifferentiation driven by leptin, periostin, NFATC1, TGF-β and non-canonical WNT5A-ROR2 signaling (Supplemental Fig. 2). Leptin and periostin are pro-calcific proteins synthesized by VSMCs [39,40]. Their expression was reduced in osteogenic *versus* basal conditions. However, further suppression occurred when apabetalone or JQ1 were present, suggesting BETi could be effective in conditions where leptin or periostin drive calcification. OxLDL, an inducer of VSMC calcification, is elevated 10-fold in patients with CKD [41]. OxLDL stimulates VSMC transdifferentiation through activation of NFATC1, a TF that binds the RUNX2 promoter to induce its transcription [42]. In this study, osteogenic conditions increased *NFATC1* gene expression by 30%. Apabetalone or JQ1 downregulated *NFATC1* mRNA, indicating potential for BETi to impede NFATC1 mediated mineralization of VSMCs. Both canonical (β-catenin-dependent) and non-canonical (β-catenin-independent) Wnt signaling programs control VSMC cell phenotype [43]. ROR2 is an orphan tyrosine kinase and a receptor for WNT5A involved in non-canonical WNT signaling cascade [44]. Apabetalone and JQ1 opposed induction of *WNT5A* and *ROR2*, indicating that BETi could suppress the pro-calcific programming induced by this pathway. Finally, TGF-β enhances osteogenic transdifferentiation and mineralization of VSMCs [45]. The expression of *ACVR2A*, a TGF-β receptor, was suppressed through BETi, potentially impairing TGF-β′s capacity to initiate VC. Further investigation will be required to verify that BETi can suppress calcification induced by stimuli other than osteogenic culture conditions.

BRD4 is involved in epigenetic rearrangements that alter the function of vascular cells. Das et al. showed that VSMC response to angiotensin II involves redistribution of BRD4 to assemble a new repertoire of enhancers to promote expression of genes leading to VSMC dysfunction and hypertension [46]. Similarly, BRD4 was redistributed in endothelial cells activated by tumor necrosis factor-α to drive inflammatory gene expression [47]. In both cases, JQ1 disrupted enhancer assembly and muted the transition to an activated state. Our ChIP-seq results revealed that genome wide redistribution of BRD4 accompanied osteogenic transdifferentiation. The number of BRD4 peaks was reduced from 9,397 in basal to 4,228 in osteogenic conditions (Fig. 4B). Interestingly, BRD4 was found more predominantly in gene promoters and exons in basal conditions, which shifted to introns and intergenic regions in osteogenic conditions (Fig. 4B). The shift may represent movement of BRD4 from proximal regulatory regions, including transcription start sites, to distal regulatory regions such as BRD4-rich enhancers. This redistribution of BRD4 may alter gene expression to induce cellular reprogramming and transdifferentiation. The BRD4-rich enhancers in osteogenic conditions were longer and had a larger area than those in basal conditions (Fig. 5B–C; $p < 0.05$). Large BRD4-rich enhancers are associated with high levels of gene expression [48], which promote expression of select genes to enable transdifferentiation. Apabetalone and JQ1 reduced the number, length and area of BRD4-rich enhancers (Fig. 5A–C), consistent with displacement of BRD4 from chromatin. BETi disruption of BRD4-rich enhancers may counter pathological cellular response, similar to previously reported results in vascular cells [46,47]. Of note, *RUNX2*, *ALPL*, *WNT5A* and *MGP* were not amongst the BRD4-rich enhancer associated genes (Supplemental File 1), and neither were genes identified as key components in pro-calcific pathways other than the osteogenic conditions used in this study (*LEP*, *POSTN*, *NFATC1*, *ACUR2A* and *ROR2*; Supplemental Fig. 2). Regulation by BRD4 may occur via a distal enhancer, or expression of these genes may depend on other factors regulated by BRD4. Alternately, the mechanism may be independent of BRD4, as apabetalone and JQ1 also inhibit BRD2 and BRD3 [28]. This is an area of ongoing investigation.

ChIP-seq identified 38 genes in proximity (within 25,000bp) of BRD4-rich enhancers in osteogenic, but not basal conditions (Fig. 5D and Supplemental Table 2). Eleven of these genes have connections to atherosclerosis or calcification in VSMCs, dental follicle cells, chondrocytes, osteoclasts, osteoblasts, mesenchymal stem cells or aortic cells (references in Supplemental Table 2). Their role in calcification may be maintained between cell types, but previously unrecognized in VSMCs. Expression of 5 genes with connections to calcification or atherosclerosis was measured (Fig. 5E: *C6*, *IL1R1*, *TIMP4*, *PKDCC* and *ZBTB16*). Expression of each increased in osteogenic conditions, consistent with the newly formed BRD4 enhancers in proximity. While BETi reduced the size of these BRD4-rich enhancers (Fig. 5E–F), a corresponding reduction in expression was observed for *C6*, *IL1R1* and *TIMP4*, but not for *PKDCC* or *ZBTB16*. Additional factors working independently of BRD4 likely dictate the transcription of *PKDCC* and *ZBTB16*.

BRD4 cooperates with TFs to regulate gene expression [49]. BRD4-TF complexes likely enable osteogenic transdifferentiation. Therefore, we strove to identify TFs that disproportionately associate with BETi sensitive BRD4 assemblies on chromatin in osteogenic conditions using *de novo* motif discovery analysis and IPA® upstream regulators of transcription. Seven TFs were identified by both methods, of which 6 have previously been linked to calcification (Table 1). Of note, proto-oncogene c-FOS (FOS) is involved in calcification of VSMCs induced by inflammatory cytokines [50]. FOS physically interacts with activating transcription factor 3 (ATF3) [51], which we also identified as a TF that may cooperate with BRD4 in transdifferentiating VSMCs (Table 1). Interestingly, a BRD4-ATF3 interaction was suggested by Tasdemir et al. through a similar TF motif analysis [52]. Disruption of the BRD4-TF associations through BETi could regulate expression of select genes that drive the transdifferentiation process.

Taken together, this study demonstrates that inhibitors of BET proteins reduce osteogenic transdifferentiation and calcium deposition in VSMCs, in part through suppression of *RUNX2* and *ALPL* gene induction. Apabetalone also downregulates expression of key factors that promote calcification via oxLDL, inflammation, leptin, periostin, or non-canonical WNT signaling. BRD4, a target of apabetalone, is redistributed on chromatin during transdifferentiation to alter gene expression and generate unique BRD4-rich enhancers associated with calcification. To our knowledge, this is the first report to implicate BET proteins in VSMC calcification, and to demonstrate a clinical stage compound that regulates TNAP production. While JQ1 was efficacious, its short half-life in vivo makes it unsuitable for clinical use [19]. Further assessment of apabetalone as a therapeutic for VC, alone or combined with agents targeting mineral imbalances, is warranted. Future studies will determine if apabetalone affects biomarkers or coronary artery calcium score [1] in patients at risk of VC and its complications. The impact of apabetalone on biomarkers, kidney function and CVD outcomes in patients with renal impairment is being evaluated in a subgroup of the phase 3 BETonMACE trial (ClinicalTrials.gov Identifier: NCT02586155).

Conflicts of interest

All authors are either employees or advisory board members of Resverlogix, which funded the study.

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Author contributions

DG and LMT conducted the research with the assistance of SW. DG, LMT, CDS and CH analyzed data and performed statistical analyses. DG, LMT, CDS, SW, SS, RJ, MS, JOJ, KKZ, NCWW and EK developed hypotheses and study designs. EK directed the research. DG drafted the manuscript and all authors performed critical review.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2018.11.002.

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