Retinoids repress human cardiovascular cell calcification with evidence for distinct selective retinoid modulator effects

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Abstract

Objective ---Retinoic acid (RA) is a ligand for nuclear receptors that modulate gene transcription and cell differentiation. Whether RA controls ectopic calcification in humans is unknown. We tested the hypothesis that RA regulates osteogenic differentiation of human arterial smooth muscle cells (SMC) and aortic valvular interstitial cells (VIC) that participate respectively in atherosclerosis and heart valve disease.

Approach and Results ---Human cardiovascular tissue contains immunoreactive retinoic acid receptor (RAR), a retinoid-activated nuclear receptor directing multiple transcriptional programs. RA stimulation suppressed primary human cardiovascular cell calcification while treatment with the RAR inhibitor AGN 193109 or *RARα* siRNA increased calcification. RA attenuated calcification in a coordinated manner – increasing levels of the calcification inhibitor matrix Gla protein while decreasing calcification-promoting tissue non-specific alkaline phosphatase activity. Given that nuclear receptor action varies as a function of distinct ligand structures, we compared calcification responses to cyclic retinoids and the acyclic retinoid, peretinoin. Peretinoin suppressed human cardiovascular cell calcification without inducing either secretion of apolipoprotein-CIII, which promotes atherogenesis, or reducing cytochrome P450 family 7 subfamily A member 1 expression, which occurred with cyclic retinoids all-trans retinoic acid, 9-cis retinoic acid, and 13-cis retinoic acid. Additionally, peretinoin did not suppress human femur osteoblast mineralization, whereas all-trans retinoic acid inhibited osteoblast mineralization.

Conclusions -—These results establish retinoid regulation of human cardiovascular calcification, provide new insight into mechanisms involved in these responses, and suggest selective retinoid modulators, like acyclic retinoids may allow for treating cardiovascular calcification without the adverse effects associated with cyclic retinoids.

Graphical Abstract
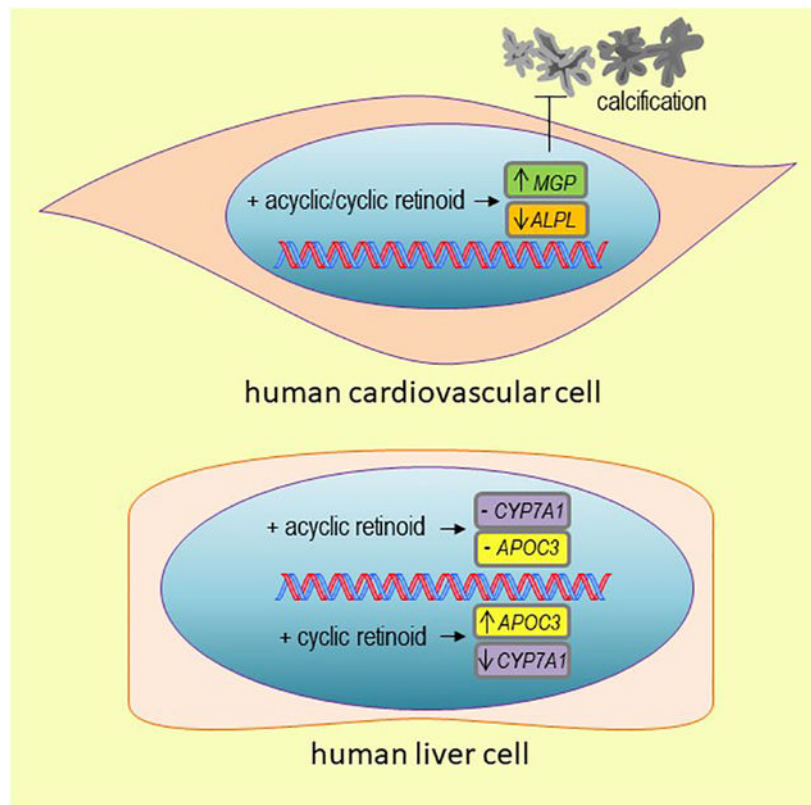
Keywords

retinoic acid; matrix Gla protein; cardiovascular calcification; apolipoprotein-CIII

Subject codes:

Cell Signaling/Signal Transduction; Vascular Disease; Valvular Heart Disease

Introduction

Cardiovascular calcification predicts risk for coronary heart disease events.[1] Arterial calcium deposition impairs vascular elasticity[2] while microcalcifications within the fibrous cap of atheromata may promote plaque rupture.[3,4] Clinical observations link cardiovascular calcification and bone metabolism although often in a divergent manner:[5] bone density and fractures associate inversely with the extent of vascular calcification and cardiovascular risk. [6,7] Vascular smooth muscle cells (SMC) and valvular interstitial cells (VIC) differentiate to osteoblast-like cells that express several osteogenic-related genes.[8,9,10] Mechanistic studies

support this cardiovascular calcification-bone osteogenesis connection, with overlapping pathways observed in cardiovascular and skeletal bone mineralization.[11] Retinoic acid (RA), a metabolite of vitamin A, is a mediator linking bone and cardiovascular mineralization through its potent, proximal regulation of transcriptional programs. Despite this, the role of retinoid signaling in cardiovascular calcification, particularly in humans remains poorly defined.

We previously reported deficiency of retinaldehyde dehydrogenase 1 increases bone formation in mice in a specific, restricted pattern.[12,13] Vitamin A metabolism produces retinaldehyde, which is further metabolized by retinaldehyde dehydrogenase 1 to generate the key retinoid, all-*trans*-retinoic acid (ATRA), which exerts its effect in large part by activating the nuclear receptor, retinoid activated receptor (RAR). While RA convincingly increases ectopic calcification in rodents and birds,[14,15,16] the role of RA in human cardiovascular cell calcification is unknown. Vitamin A supplementation can suppress inflammatory processes in human atherosclerotic patients;[17] however, beta carotene, which is converted into vitamin A does not impact cardiovascular events in women at high risk of cardiovascular disease.[18] Previous studies have demonstrated major cell type and species differences in RA-mediated gene expression.[16,19,20,21] Given these divergent reports, an understanding of the effects of RA on cardiovascular calcification in human cells and tissues is important. Here, we demonstrate a novel species difference in retinoid regulation of cardiovascular calcification, with retinoids inhibiting human cardiovascular cell calcification. Additionally, this study shows the cyclohexene hydrocarbon moiety present in cyclic retinoids mediates adverse retinoid responses, while acyclic retinoid inhibited cardiovascular cell calcification without adverse effects.

Materials and Methods

The authors declare that all supporting data are available within the article and its online supplementary files.

Cell culture

Human coronary artery SMC (PromoCell, Heidelberg, Germany) were expanded in Smooth Muscle Cell Growth Medium 2 (PromoCell) supplemented with epidermal growth factor (0.5ng/mL), insulin (5μg/mL) basic fibroblast growth factor- B (2ng/mL), and 5% fetal bovine serum, and cells were used between passages 3 and 8. SMC identity was confirmed by α-smooth muscle actin flow cytometry using a BDFACSAria™ II flow cytometer (BD Biosciences, Franklin Lakes, NJ). SMC were cultured up to 21 days in either control medium, CM: DMEM, 4.5g/L glucose, L-glutamine, 10% FBS, 1% penicillin/streptomycin; or osteogenic medium, OM: CM supplemented with 10nmol/L dexamethasone, 10mmol/L β–glycerol phosphate, and 100μmol/L L-ascorbic acid 2-phosphate. Cells were treated with combinations of the following reagents: 0.1% DMSO vehicle; ATRA (Sigma, St. Louis, MO); AGN 193109 (Sigma), 9-cis retinoic acid (Sigma), or peretinoin (MedChem Express LLC, Monmouth Junction, NJ). Human femur bone alkaline phosphatase positive osteoblasts (PromoCell) were expanded and cultured in an identical manner as with human SMC. Vimentin positive primary human VIC were obtained using isolated human aortic

valve tissue from patients undergoing valve replacement (Institutional Review Board protocol #2011P001703; written informed consent was obtained for human tissue donors), and cultured similarly to SMC, but with some changes to the calcification inducing media, pro-calcifying media, PM: CM supplemented with 2mmol/L NaH_2PO_4 (Sigma) and 50μg/mL L-ascorbic (Sigma). Medium was changed every three to four days. For knockdown experiments, siRNA (used at a final concentration 20 nmol/L) was added with each media change according to the Lipofectamine RNAiMAX (Thermo Fisher Scientific, Waltham, MA) protocol. The following validated siRNA (Thermo Fisher Scientific) were used and independently confirmed: $RAR\alpha$ siRNA (#s11801), $RAR\beta$ (#s11804), $RAR\gamma$ siRNA (#s11807), and negative control siRNA (#4390843). HepG2 cells (ATCC, Manassas, VA) were grown in EMEM with 1% penicillin/streptomycin and 10% FBS. For RA stimulation experiments, HepG2 were switched to EMEM with 1% penicillin/streptomycin, and without FBS but containing 1% bovine serum albumin at the time of RA stimulation and cultured for 24 hours.

Calcification analysis

Alizarin red staining was performed after two to three weeks in CM, OM, or PM. Cells were fixed in 4% paraformaldehyde and incubated in 2% Alizarin red solution at room temperature for one hour, and then rinsed three times with water. The stain was extracted by incubating cells in 100mmol/L cetylpyridinium chloride (Sigma) for 30 minutes with gentle shaking, and supernatant absorbance was read at 540nm. Von Kossa staining was performed by incubating fixed cells in 5% silver nitrate (American MasterTech Scientific, Lodi, CA) under ultraviolet light for 30 minutes, followed by rinsing in distilled water and incubating with 5% sodium thiosulfate (American MasterTech Scientific) for 2 minutes, and then rinsed in distilled water. OsteoSense 680 (PerkinElmer, Waltham, MA) staining was performed with a 1:100 dilution and overnight incubation at 37 degrees Celsius, followed by imaging on a Nikon A1 confocal microscope (Nikon Instruments, Melville, NY).

TNAP activity and extracellular vesicles

Cellular TNAP activity was determined by the Alkaline Phosphatase Activity Colorimetric Assay Kit (BioVision, Milpitas, CA) according to the manufacturer's protocol, and normalized by protein content, determined with the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Extracellular vesicle TNAP activity was assayed in vesicles in a similar manner after isolating vesicles by centrifuging cell culture media at 2,000 X g to remove any cell debris, and then centrifuging at 100,000G and four degrees Celsius for one hour to pellet vesicles. Vesicle diameter and particle number was determined by nanoparticle tracking analysis using a NanoSight LM10 (Malvern Instruments, Malvern, United Kingdom). Samples were diluted 1:10 in phosphate buffered saline prior to injection, and continuously injected via a syringe pump, with five nanoparticle tracking analysis videos collected for each minute. Camera gain was set at a constant value of 9, and the threshold value for vesicle detection was set to 2. Size and concentration from the data in each video was averaged to generate the distribution per sample.

RNA and protein analysis

Total RNA was extracted with TRIzol (Thermo Fisher Scientific), and cDNA was generated using the qScript cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, MD). Calcified and non-calcified human tissues were assessed by gross morphology and OsteoSense 680 staining. Quantitative PCR was performed using the following Taqman probes (Thermo Fisher Scientific): Hs01029144_m1 (*ALPL*); Hs00985000_g1 (*APOA1*); Hs00163644_m1 (*APOC3*); Hs00167982_m1 (*CYP7A1*); Hs01054040_m1 (*ENPP1*); Hs02758991_g1 (*GAPDH*); Hs00230853_m1 (*HNF4a*); Hs00969490_m1 (*MGP*); Hs05013609_mH (LPA); Hs01016719_m1 (*PPARGC1a*); Hs00751239_s1 (*MSX2*); Hs00940445_g1 (*RARa*);Hs00977140_m1 (*RARβ*); Hs01559234_m1 (*RARγ*); Hs00231692_m1 (*RUNX2*); Hs01001343_g1 (*SOX9*). Gene expressions were normalized to *GAPDH* using the Ct method. Secreted matrix Gla protein (MGP) was measured by ELISA (Lifespan Biosciences Inc, Seattle, WA, #LS-F4628) according to the manufacture's protocol. APO(a) protein was assessed in HepG2 cells by taking HepG2 treated for 24 hours and lysing in RIPA buffer (Thermo Fisher Scientific) containing 1:100 Halt protease and phosphatase inhibitor (Thermo Fisher Scientific). Cell lysates were run on PROTEAN TGX precast gels (Bio-Rad Laboratories, Hercules, CA) and blotted using APO(a)/Lp(a) antibody (Lee Biosolutions Inc., Maryland Heights, MO, #400–42, 1:1000) and β-actin antibody (Novus Biologicals, Centennial, CO, #NB600–501, 1:5,000). Human liver microsome fraction (Thermo Fisher Scientific, #HMMCPL) was used as an APO(a) positive blotting control.

Secreted apolipoprotein analysis

Conditioned media was collected from HepG2 cells 24 hours post-treatment. ELISA was used to measure lipoproteins according to manufacturer's protocols: APOA1 (Abcam #ab189576), APO(a) (Cell Biolabs Inc. #STA-359), APOB (Abcam #ab190806), APOC3 (Abcam #ab55984).

Immunohistochemistry

Carotid artery samples were obtained from patients undergoing endarterectomy (Brigham and Women's Hospital Institutional Review Board protocol #1999P001348) and aortic valve samples were obtained from patients undergoing valve replacement surgery (Institutional Review Board protocol #2011P001703; written informed consent was obtained for human tissue donors). Tissues were embedded in optimum cutting temperature compound, cut into slices with 7μm thickness, and cryosections were fixed in acetone. Following blocking in 4% serum, sections were incubated in RARα antibody (#MA1–810A, Thermo Fisher Scientific, 1:100) with two sections stained per donor. Negative control staining was performed by substituting the primary antibody with an IgG control antibody (#sc-32342, Santa Cruz Biotechnology, Dallas, TX, 1:100). For immunofluorescence, cryosections were incubated with primary antibody overnight (RARα, #MA1–810A, Thermo Fisher Scientific, 1:100; vimentin, #ab92547, Abcam, 1:200; α-smooth muscle actin, #ab5694, 1:200), with two sections stained per donor. Cryosections were then incubated with Alexa Fluor 488 and 594-labelled secondary (Fisher Scientific). 4',6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific) was used for nuclear staining. Imaging was performed with a confocal microscope A1 (Nikon Instruments). Cell culture immunofluorescence was performed by

growing cells on 0.1% gelatin coated Lab-Tek II chambered cover glass #1.5 borosilicate slides (Lab-Tek, Rochester, NY), fixing in 4% paraformaldehyde for 15 minutes, followed by permeabilization and blocking using 0.3% Triton X-100 and 5% serum for 30 minutes. Cells were incubated with primary antibody (MGP, #sc-81546, Santa Cruz Biotechnology, 1:200; SOX9, #82630S, Cell Signaling Technology, Danvers, MA, 1:400) overnight at 4 degrees Celsius, and incubated with Alexa Fluor 488-labelled secondary (Fisher Scientific). 4',6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific) was used for nuclear staining. Imaging was performed with a confocal microscope A1 (Nikon Instruments).

Statistical analysis

Analysis of variance (ANOVA) with Tukey's multiple comparison testing and Student's t-tests were performed using Prism 6 (GraphPad, La Jolla, CA). Data was analyzed for normality and equal variance to determine whether the applied parametric test was appropriate using Prism 6. Five donors were used for human tissue experiments. Three to nine donors were used at similar passage number for primary human cell culture experiments, and six independent experimental replicates were used for HepG2 cells.

Results

Retinoic acid attenuated human SMC calcification

As retinoic acid regulates SMC differentiation,[22] we hypothesized that ATRA the major form of RA in humans, might be a transcriptional mediator of human cardiovascular cell osteogenic differentiation and calcification. ATRA exerts its effects primarily through ligand activation of the retinoic acid receptor-α (RARα) in vascular SMC.[23] We examined RARα by immunohistochemistry in human carotid arteries and aortic valves, which stained positive for RARα (Figure 1A and 1B; online-only Data Supplement Figures I and II). Additionally, RARα localized in SMC (α-smooth muscle actin-positive) in human carotid artery and in VIC (vimentin-positive) in human aortic valve, assessed by immunofluorescence (Figure 1A and 1B; online-only Data Supplement Figures I and II). To assess effects of retinoic acid on cellular calcification, we used an established *in vitro* method with primary human coronary artery SMC cultured for up to three weeks in control media (CM) or osteogenic differentiation media (OM: CM containing β-glycerophosphate, ascorbic acid, and dexamethasone), and primary human VIC cultured for up to three weeks in pro-calcifying media (PM) containing inorganic phosphate and L-ascorbic acid.[24,25] *RARα* and *RARβ* mRNA levels where not altered in calcified human cardiovascular tissues and primary cells compared to controls (Figure 1C and 1D; online-only Data Supplement Figures I and II). *RARγ* mRNA levels were unchanged in calcified human cardiovascular tissues and SMC in OM, and slightly increased in VIC in PM (online-only Data Supplement Figures I and II). siRNA against *RARα* but not *RARβ* or *RARγ* induced earlier calcification in human SMC exposed to OM for two weeks (Figure 2A). OM increased SMC mineralized matrix as assessed by three different methods: Alizarin Red staining (Figure 2B and 2C), von Kossa staining (Figure 2D), and OsteoSense 680 near-infrared fluorescence[26] (Figure 2E). In contrast, the mineralization induction effect of OM was almost completely suppressed in the presence of ATRA in a concentration-dependent manner: 1 μmol/L (Figure 2B, 2D, and 2E), and to a slightly lesser extent with 0.1 μmol/L ATRA (Figure 2B). We also tested the RA

isomer 9-cis RA, which has reported distinct effects *in vitro* but debated as to its existence *in vivo*, to assess isomer specificity. In similar experiments, 9-cis retinoic acid also attenuated human SMC calcification (Figure 2C). While ATRA and 9-cis retinoic acid can both bind to RAR, only 9-cis retinoic acid also binds to the retinoid X receptor (RXR). To test whether RA effects on calcification were through RAR, we treated human SMC with a well-established, specific RAR inhibitor AGN 193109, which dose-dependently increased SMC mineralization (Figure 2B). Taken together these data support a selective RARα-mediated mechanism for ATRA-induced inhibition of human SMC calcification.

Retinoic acid suppressed OM-induced TNAP activity in human SMC

We previously reported a novel mechanism identifying tissue non-specific alkaline phosphatase (TNAP protein; *ALPL* gene) as a key enzyme mediating OM mineralization of SMC through extracellular vesicles (EV).[27] To assess whether ATRA inhibited SMC calcification via TNAP regulation, we measured *ALPL* mRNA levels and TNAP activity in SMC stimulated with ATRA. As expected, OM increased SMC *ALPL* mRNA levels (Figure 2F), and SMC cellular (Figure 2G) and EV TNAP (Figure 3A) activity; in contrast, ATRA suppressed OM-induced SMC *ALPL* mRNA levels (Figure 2F) and SMC cellular (Figure 2G) and EV (Figure 3A) TNAP activity, doing so without altering either EV abundance (Figure 3B) or size (Figure 3C). These data demonstrate ATRA suppressed TNAP-mediated calcification in human SMC and EV.

Retinoic acid attenuated an osteogenic transcriptional program in human SMC

To examine whether ATRA signaling modulates gene expression involved in differentiating SMC to an osteoblast-like cell phenotype with elevated TNAP, we investigated ATRA effects on osteogenic-related transcription factors. As ATRA mediates transcriptional effects through RAR, we first examined ATRA effects on *RARα* expression. Indeed, ATRA increased *RARα* mRNA levels in human SMC (Figure 4A). To assess whether ATRA changed the osteogenic transcriptional program of human coronary artery SMC, we measured mRNA levels of three transcription factors strongly implicated in cardiovascular calcification: runt related transcription factor 2 (*RUNX2*), Msh homeobox 2 (*MSX2*), and SRY-box 9 (*SOX9*).[28] OM increased human SMC *RUNX2* mRNA levels, which was attenuated in the presence of ATRA (Figure 4B). While none of the stimulation conditions altered *MSX2* expression in human SMC (Figure 4C), ATRA stimulation did increase *SOX9* mRNA levels and SOX9 protein nuclear localization, as visualized by confocal immunofluorescence (Figure 4D). Taken together, these data demonstrate that ATRA signaling inhibits an OM-induced osteogenic transcriptional program in human SMC.

Retinoic acid increased matrix Gla protein in human SMC

Given the overlap between bone, SMC, and ATRA responses, we next assessed whether ATRA altered the levels of matrix Gla protein (MGP), a calcification inhibitor[29] induced by RA in human osteoblastic cells.[19] Exposure to ATRA over two weeks strongly increased human SMC *MGP* mRNA levels, intracellular MGP protein visualized by confocal microscopy, and MGP protein secretion in human SMCs cultured in the presence of OM (Figure 4E). These data demonstrate that ATRA increased the abundance of the calcification inhibitor MGP in human SMC.

Retinoic acid increased MGP and suppressed human valvular interstitial cell calcification

As calcification in the aortic valve is of clinical relevance in addition to this process in arteries, we investigated ATRA responses in a second human cardiovascular cell type: primary human valvular interstitial cells (VIC). We used an established osteogenic differentiation protocol, with human VIC exposed to PM containing inorganic phosphate and L-ascorbic acid for three weeks.[25,30,31] PM was used for VIC studies given our prior report that PM generates more consistent calcification in VIC as compared to OM.[31] Unlike OM, PM contains inorganic phosphate and induces VIC calcification independent of TNAP activity.[31] This distinction allows assessment of the ATRA impact on mineralization through TNAP-independent calcification mechanisms like MGP. ATRA increased VIC *MGP* mRNA levels and MGP protein secretion in PM (Figure 5A). Human VIC formed a mineralized matrix after three-week PM treatment, visualized by Alizarin red staining, which was suppressed in the presence of ATRA (Figure 5B). In agreement with our SMC data, siRNA against *RARα* but not *RARβ* or *RARγ* promoted earlier induction of calcification in human VIC cultured for two weeks in PM (Figure 5C). These data demonstrate that in human VIC, ATRA regulates similar transcriptional programs as seen in human SMC, including induced expression and release of the calcification inhibitor MGP.

The acyclic retinoid, peretinoin does not unfavorably alter lipids as do classical retinoids

Therapeutic use of cyclic retinoids like ATRA in humans has been limited by their adverse effects, including increased triglyceride levels via retinoid induction of the lipoprotein lipase inhibitor apolipoprotein CIII (APOC3).[32] Increased APOC3 levels have been associated with coronary calcification[33] and adverse cardiovascular outcomes, as supported by recent strong human genetic evidence,[34] consistent with causality in these responses. Cyclic retinoids also decrease levels of cytochrome P450 family 7 subfamily A member 1 (CYP7A1), a major hepatic enzyme involved in cholesterol metabolism.[35] While ATRA is selective for RAR and 9-cis retinoic acid activates both RAR and RXR, naturally occurring retinoids can undergo distinct modifications and interconversion *in vivo*,[32] with distinct ligand structures known to exert divergent biologic effects.[36] Therefore, we tested cellular responses consistent with these potential adverse cellular effects in response to three natural retinoid isomers containing a cyclic cyclohexene hydrocarbon, namely ATRA, 9-cis retinoic acid, and 13-cis retinoic acid that is in clinical use, in comparison to the synthetic, clinically-tested acyclic retinoid, peretinoin, which lacks a cyclohexene hydrocarbon moiety (online-only Data Supplement Figure III). As expected, all three cyclic retinoids significantly elevated *APOC3* mRNA levels and secreted APOC3 protein in human liver HepG2 cells (Figure 6A). However, in direct contrast, the acyclic retinoid peretinoin did not alter *APOC3* mRNA or secreted APOC3 protein levels in HepG2 cells (Figure 6A). A similar pattern was seen in apolipoprotein A1 (APOA1) levels, with cyclic retinoids inducing *APOA1* mRNA and secreted APOA1 protein, while peretinoin had no such effect (Figure 6B). Neither cyclic nor acyclic retinoid treatment significantly affected secreted levels of apolipoprotein B (APOB) (online-only Data Supplement Figure IVA) and had no impact on the very low basal expression of apolipoprotein (a) (APO(a)) mRNA as well as APO(a) protein, assessed by Western blotting, or media secretion, measured by ELISA (online-only Data Supplement Figure IVB). Cyclic retinoids can repress *CYP7A1* mRNA expression,[35] which can alter metabolic responses, prompting us to assess cyclic versus acyclic retinoid regulation of

CYP7A1 transcription. Cyclic retinoid stimulation but not the acyclic retinoid peretinoin significantly reduced *CYP7A1* mRNA levels in HepG2 cells (Figure 6C). Expression of *APOC3* and *APOA1* are regulated by several nuclear receptors, including hepatocyte nuclear factor 4 alpha (HNF4α)[37,38] and retinoid X receptor (RXR).[32,38] *CYP7A1* is transcriptionally induced by HNF4α and the co-activator peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PPARGC1α).[39] We therefore investigated cyclic versus acyclic retinoid effects on these transcription factors. None of the retinoids tested altered *HNF4α* and *PPARGC1α* mRNA levels (Figure 6D). These data demonstrate that cyclic retinoid transcriptional regulation of *APOC3*, *APOA1*, and *CYP7A1* did not involve increasing *HNF4α* or decreasing *PPARGC1α* mRNA levels.

Acyclic retinoid, peretinoin inhibited human cardiovascular cell calcification without inhibiting human femur bone osteoblast mineralization

Since the acyclic retinoid peretinoin differed from cyclic retinoids by not exhibiting adverse effects on genes expected to alter triglyceride levels and other hepatic responses, we next tested if peretinoin retained the inhibition of human cardiovascular cell calcification seen with ATRA. Indeed, peretinoin significantly attenuated OM-induced human SMC calcification to a similar level as seen with ATRA stimulation (Figure 6E). Peretinoin also significantly reduced SMC *RUNX2* and *ALPL* mRNA levels, and increased *MPG* mRNA levels in SMC, but to a lesser extent than ATRA (online-only Data Supplement Figure VA). In agreement with its effect in SMC, peretinoin attenuated PM-induced human VIC calcification to a similar extent as ATRA (Figure 6F). Peretinoin also significantly increased VIC *MGP* mRNA levels, although to a lesser extent than ATRA (online-only Data Supplement Figure VIA). Peretinoin did not alter SMC or VIC *SOX9* mRNA levels (online-only Data Supplement Figure VA and VIA). We also assessed whether RA worked via ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENNP1) that can repress calcification,[2] but found no RA regulation of *ENPP1* mRNA expression in SMC or VIC (online-only Data Supplement Figures VB and VIB). Lastly, given evidence for differences in calcification depending on cell and bone contexts,[25] we tested the effects of ATRA versus peretinoin on human femur bone osteoblasts. ATRA reduced bone osteoblast mineralization in OM, which was not seen in response to peretinoin (Figure 6G). Furthermore, in agreement with our human SMC and VIC data, siRNA against *RARα* but not *RARβ* or *RARγ* induced earlier calcification in human femur osteoblasts in OM (Figure 6H).

Discussion

We report here the following novel findings: 1) ATRA inhibits human artery SMC and aortic VIC calcification via RARα and subsequent coordinated transcriptional regulation. 2) Likely involvement of the retinoid cyclohexene hydrocarbon in the adverse effects of ATRA and other cyclic retinoids on plasma lipids. Most studies investigating RA action in calcification have focused on its role in bone mineralization, with evidence for RA-mediated repression of bone mineralization through retinoid signaling.[12,13,40] Limited and often conflicting data exists regarding retinoic acid and cardiovascular calcification.[41] As bone formation and cardiovascular calcification share common biologically-mediated pathways,[42] we investigated the effect of retinoids on factors implicated in both processes. Among these

was the osteogenic transcription factor RUNX2 that induces vascular calcification.[43] Another transcription factor, SOX9, inhibits bone formation[44] and valvular calcification,[14] while taking part in providing feedback inhibition of RUNX2.[45,46] Our finding that ATRA decreased *RUNX2* and increased *SOX9* mRNA levels in human SMC indicates that ATRA drives a coordinated transcriptional program. This RA program suppressed osteogenic differentiation-mediated calcification via RAR activation, as further supported by findings with both chemical modulators of RAR activity and *RARa* siRNA, which accelerated calcification in primary human cells.

ATRA suppressed OM-induced cellular and EV TNAP activity in human SMC without altering EV abundance. We and others reported a role for EV[47] and EV TNAP[27] in mediating increased SMC calcification; these results place retinoid signaling upstream of this novel calcific mechanism, opening new ways in which to consider inputs into TNAP action in EV. To model calcification in human SMC and VIC, the use of two different conditions allowed for isolating potential contributory mechanisms. In OM, human SMC calcify readily through TNAP-mediated metabolism of β-glycerophosphate to generate inorganic phosphate. In human VIC, we used PM that contains inorganic phosphate to promote matrix calcification, thus bypassing any dependency on TNAP for inorganic phosphate formation.[31] In both experimental conditions, ATRA suppressed calcification. This result suggested that ATRA induced additional calcification inhibitory mechanisms beyond TNAP suppression. In human SMC and VIC, ATRA induced the transcription of MGP, a well-established calcification inhibitor. In human aortic VIC, knockdown of *MGP* increases inflammation-induced calcification whereas overexpression of *MGP* suppresses inflammatory driven calcific responses.[48] Post-translational modifications can alter MGP activity by shifting between carboxylated (active) and uncarboxylated MGP (inactive) forms. In a human proof-of-concept study, the slowing of aortic valve calcification seen with vitamin K supplementation correlated with decreased uncarboxylated MGP.[49] Thus, increasing MGP abundance and/or MGP activity has been proposed as a potential therapeutic strategy for reducing cardiovascular calcification.[50] Of note, MGP inhibition of SMC calcification may involve MGP binding to EV,[51] suggesting ways in which these mechanisms may be integrated, as also occurs through the action of ATRA. The data presented here raises the intriguing prospect that combining vitamin K and retinoid supplementation may be particularly efficacious in reducing cardiovascular calcification and warrants further exploration. Complicating assessment of MGP's role in human cardiovascular calcification is an unclear association between Keutel syndrome and coronary artery disease. Keutel syndrome patients lack functional MGP and have abnormal cartilage calcification.[52] While cardiovascular calcification is not a key disease feature, one case report observed coronary, hepatic, renal, meningeal and cerebral artery concentric calcification in a 31-year old male Keutel syndrome patient.[53] Additionally, a significant association between the MGP gene rs1800801 polymorphism and increased risk of vascular calcification and atherosclerotic disease in Caucasians has been reported in a meta-analysis.[54] Regardless, our data supports that RA inhibition of human cardiovascular cell calcification occurs via both MGP and MGP-independent mechanisms. As the data here establishes, further studies dissecting the contributions of these or other potential

mechanisms to retinoid-inhibited cardiovascular calcification are warranted and should consider species-specific differences in response.

Despite evidence for benefit, the clinical use of RA has been complicated. Based on demonstrated improvements, RA is in use for certain dermatologic[55] and oncologic conditions[56]. For cardiovascular disease, the RA data has been limited and often conflicting. [41] While some positive results have been seen in observational studies, supplementation with beta-carotene, which can be converted into the ATRA precursor vitamin A, did not reduce cardiovascular events.[18] However, beta-carotene, vitamin A and retinoid biology involves multiple distinct components and variables that could impact trial results. Along these lines, our present study demonstrates that species, cell type, and experimental differences may well underlie divergent reports regarding ATRA effects on cardiovascular cells. As shown, we determine definitive ATRA-mediated decreases in calcification in multiple human cell models, with coordinated modulation of distinct cellular targets and functional responses using various methodologies. We found ATRA either increased or had no effect on human cardiovascular cell *SOX9*. In contrast to these findings, ATRA has been reported to increase mouse heart valve calcification, which was attenuated by *Sox9* overexpression.[15] In mice, high 10-fold excess vitamin A intake reduced *Sox9* expression and promoted aortic valve calcification.[14] In avian mitral valve explants, ATRA also increased calcification, a response reversed with a RAR antagonist.[14] In contrast to these studies, ATRA decreased 1α,25-dyhydroxycholecalciferol-induced renal calcification in male mice while increasing carboxylated MGP but not *Mgp* expression,[57] results that contrast with the increased MGP expression and activity in human cells shown here. We previously reported species differences in OM-induced calcification between primary human and mouse SMC.[25] Differential regulation of several genes also occurs between mice and humans with the peroxisome proliferator-activated receptor-α, a nuclear receptor like RAR.[58] In human fibroblasts, chondrocytes, osteoblasts, osteosarcoma cell lines, and rat type II pneumocytes, ATRA increases *MGP* mRNA levels.[19,20] In contrast, in rat kidney cells, rat vascular SMC, and human breast cancer cells ATRA was found to decrease *MGP* mRNA levels.[16,21] RA signaling may act to suppress human cardiovascular cell calcification directly by binding to regulatory elements in the promoter regions of calcification-related genes or through more indirect mechanisms. The RAR/RXR heterodimeric complex binds to the human MGP promoter,[21] and the SOX9 promoter contains a RAR response element.[59] In mouse preadipocytes, RARγ but not RARα stimulates Sox9 expression;[59] whereas selective RARα activation stimulates SOX9 expression in human breast cancer cells.[60] RA-mediated regulation of RUNX2 is less clear, as it may likely occur indirectly. In mouse mesenchymal stem cell-like cells, RA stimulates the disassociation of CCAAT/enhancer binding protein β from the Runx2 promoter, which induces Runx2 expression.[61] In mouse MC-3T3 pre-osteoblastic cells, RA was reported to inhibit calcification in two separate studies[40, 62], but the reported effects on Runx2 mRNA diverged in these studies for unknown reasons. In one MC-3T3 cell line study, 0.4 μmol/L RA did not alter Runx2 mRNA but reduced RUNX2 protein.[40] In another report, 0.5 μmol/L RA increased Runx2 mRNA although RUNX2 protein levels were not reported.[62] Further investigation into what underlies differences in RA responses in rodent versus human cells may uncover additional mechanisms controlling calcification in humans, studies hopefully prompted by this report.

In humans, in general, cyclic retinoids like ATRA can increase triglyceride levels; postulated mechanisms include ATRA increases in expression of the lipoprotein lipase inhibitor APOC3.[32] Of particular relevance here, in patients with diabetes, elevated APOC3 was associated with higher coronary artery calcification.[33] Retinoids are also reported to decrease bone mineralization, with ATRA inhibiting bone osteoblast differentiation and mineral deposition.[12,13,40,63] These reports regarding adverse effects of ATRA highlight the potential importance of our finding that the acyclic retinoid peretinoin retains decreased calcification, but does not induce cellular responses associated with adverse ATRA effects. Although the structure of peretinoin is similar to ATRA, it lacks the cyclohexene hydrocarbon, which likely precludes this molecule from undergoing isomer conversion. Distinct molecular structures, including retinoid species, are known to differ in their ligand interaction with nuclear receptors, like RAR and RXR, thus altering nuclear receptor conformational states, accessory molecule recruitment or release and biologic responses, as is likely the case for peretinoin. There is a paucity of studies specifically examining RA signaling differences between cyclic and acyclic RA. Our study supports differences in overall magnitude of transcriptional response and induction of *SOX9* in OM between cyclic and acyclic RA, but further work is needed to identify additional differences, including in the specific activation of RARα/β/γ and/or RXR in human cells and tissues. The prospect of harnessing distinct molecular structure interaction with nuclear receptors to maintain clinical benefits while removing adverse effects has been pursued in other settings, including the estrogen receptor (selective estrogen receptor modulators)[64] and peroxisome proliferator activated receptors (selective PPAR modulators).[65] Whether acyclic retinoids might offer such options for retinoid receptors remains unknown but worthy of consideration given the data shown here.

Despite the impact calcification has on common, life-threatening clinical conditions like aortic valve disease and aortic stenosis, currently no agents have been approved for slowing or reversing cardiovascular calcification. Our findings provide new insight into retinoid regulation of human cardiovascular cell calcification and offer new prospects for developing therapies to address the unmet need of treating calcification and avoiding retinoid side effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Non-standard Abbreviations and Acronyms:

9-cis RA
9-cis retinoic acid

13-cis RA
13-cis retinoic acid

ATRA
all-*trans*-retinoic acid

APO(a)
apolipoprotein-(a)

APOA1
apolipoprotein-A1

APOB
apolipoprotein-B

APOC3
apolipoprotein-CIII

CYP7A1
cytochrome P450 family 7 subfamily A member 1

CM
control media

ENNP1
ectonucleotide pyrophosphatase/phosphodiesterase 1

EV
extracellular vesicles

HNF4α
hepatocyte nuclear factor 4 alpha

MGP
matrix Gla protein

MSX2
Msh homeobox 2

PGC-1α
peroxisome proliferator-activated receptor gamma coactivator 1-alpha

OM
osteogenic differentiation media

RA

retinoic acid

RAR

retinoic acid receptor

RUNX2

runt-related transcription factor 2

RXR

retinoid X receptor

SMC

vascular smooth muscle cells

SOX9

SRY-box 9

TNAP protein; *ALPL* **gene**

tissue non-specific alkaline phosphatase

VIC

aortic valvular interstitial cells

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Highlights

- Retinoic acid increased mRNA levels and protein secretion of matrix Gla protein, a calcification inhibitory molecule, in human coronary artery and aortic valve cells.

- In contrast to non-human models, retinoic acid suppressed calcification in human arterial smooth muscle cells and aortic valvular interstitial cells.

- Cyclohexene hydrocarbon moiety in cyclic retinoids likely mediates APOC3 induction, which can be avoided by using acyclic retinoids.
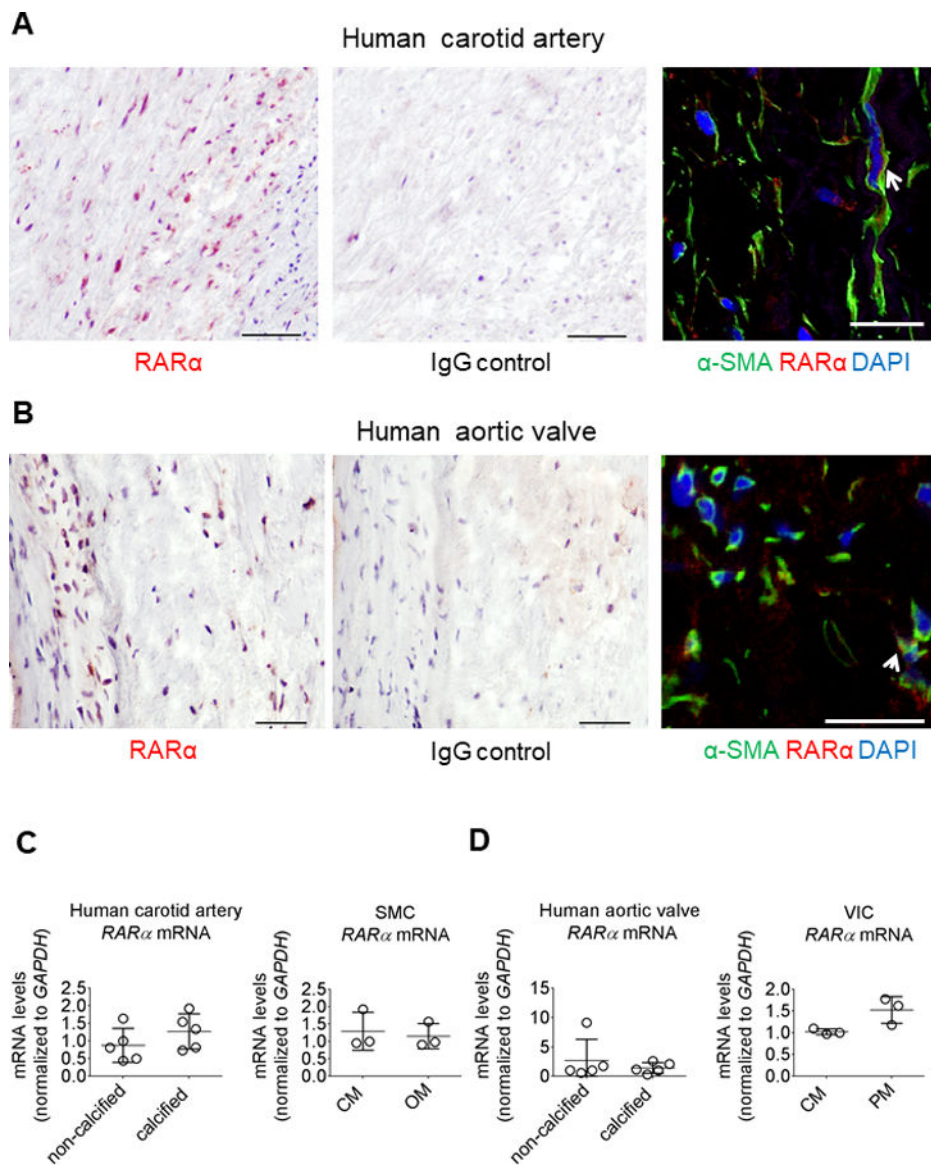
Figure 1.
Human cardiovascular tissue contained immunoreactive retinoic acid receptor. (A) Human carotid artery and (B) aortic valve RARα immunohistochemistry (red color); scale bars, 50 μm. IgG control antibody immunohistochemistry staining for matching donor adjacent sections used as negative controls. RARα (red color) immunofluorescence showing localization with α-smooth muscle actin (α-SMA) positive cells (green color) in human carotid artery (white arrow) and with vimentin-positive cells (green color) in human aortic valve (white arrow); scale bars, 20 μm. One of five carotid artery and aortic valve donors shown, with representative RARα immunohistochemistry shown for two additional donors in Figures I and II in the online-only Data Supplement. (C) Human carotid artery, SMC, (D) aortic valve, and VIC *RARα* mRNA levels in calcified and non-calcified tissues (n = 5 donors) and CM, OM or PM treated cells after two weeks in culture (n = 3 donors). Error bars indicate STDEV of mean. Data analyzed by Student's t-test.
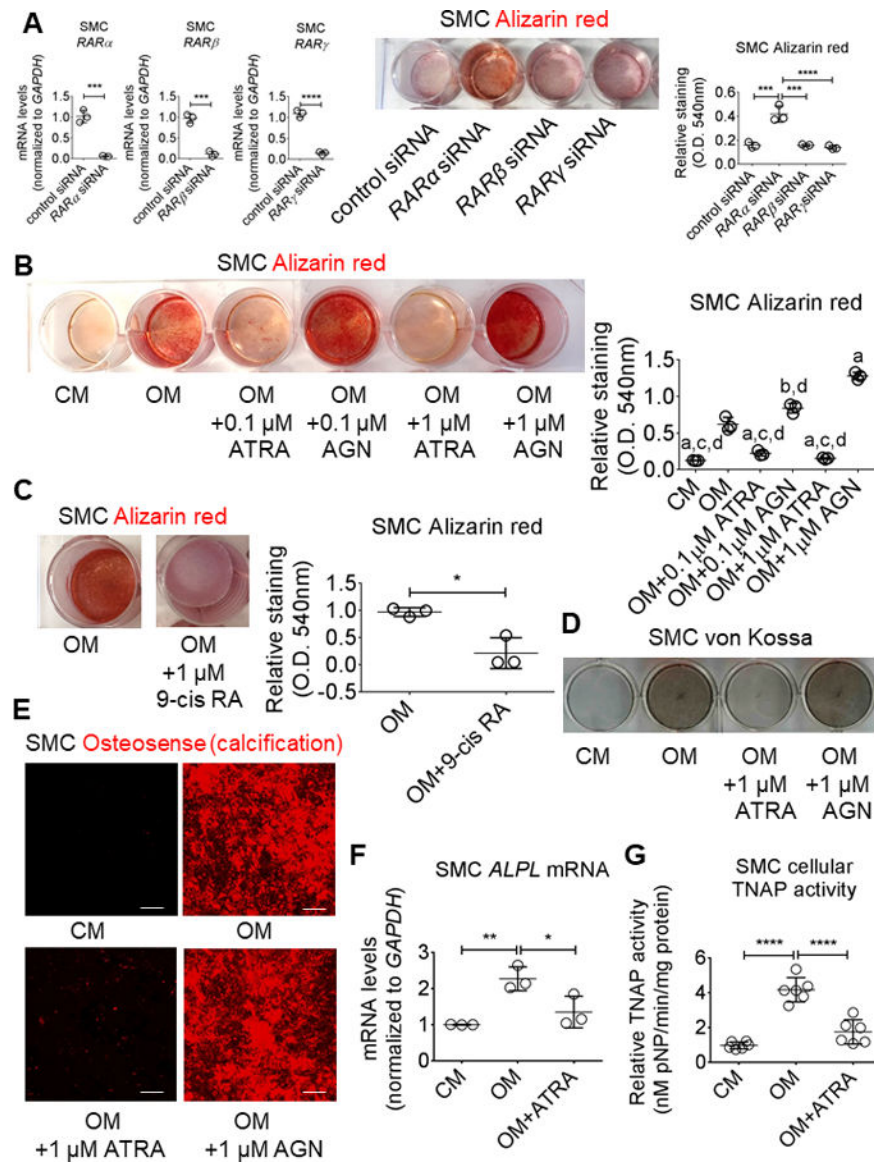
Figure 2.
Retinoic acid attenuated human SMC calcification. (A) *RAR* siRNA knockdown validation by mRNA level quantification, and Alizarin red stain and relative quantification for primary human SMC treated for two weeks in OM with negative control siRNA, *RARα* siRNA, *RARβ* siRNA, or *RARγ* siRNA. (B) Alizarin red stain and relative quantification for primary human artery SMC treated for three weeks in CM or OM with DMSO vehicle (0.1%), 0.1 μmol/L or 1 μmol/L ATRA, 0.1 μmol/L or 1 μmol/L AGN 193109 (AGN), or (C) 1 μmol/L 9-cis retinoic acid (9-cis RA). (D) Von Kossa and (E) OsteoSense 680 stain for SMC treated for three weeks; scale bars, 100 μm. (F) Cellular *ALPL* mRNA levels and corresponding TNAP enzyme activity (G) for SMC treated for two weeks. Error bars indicate STDEV of mean. [a]$P< 0.0001$ vs OM, [b]$P< 0.01$ vs OM, [c]$P< 0.0001$ vs OM + 0.1 μmol/L AGN, [d]$P< 0.0001$ vs OM + 1 μmol/L AGN, *$P< 0.05$, **$P< 0.01$, ***$P<0.001$,
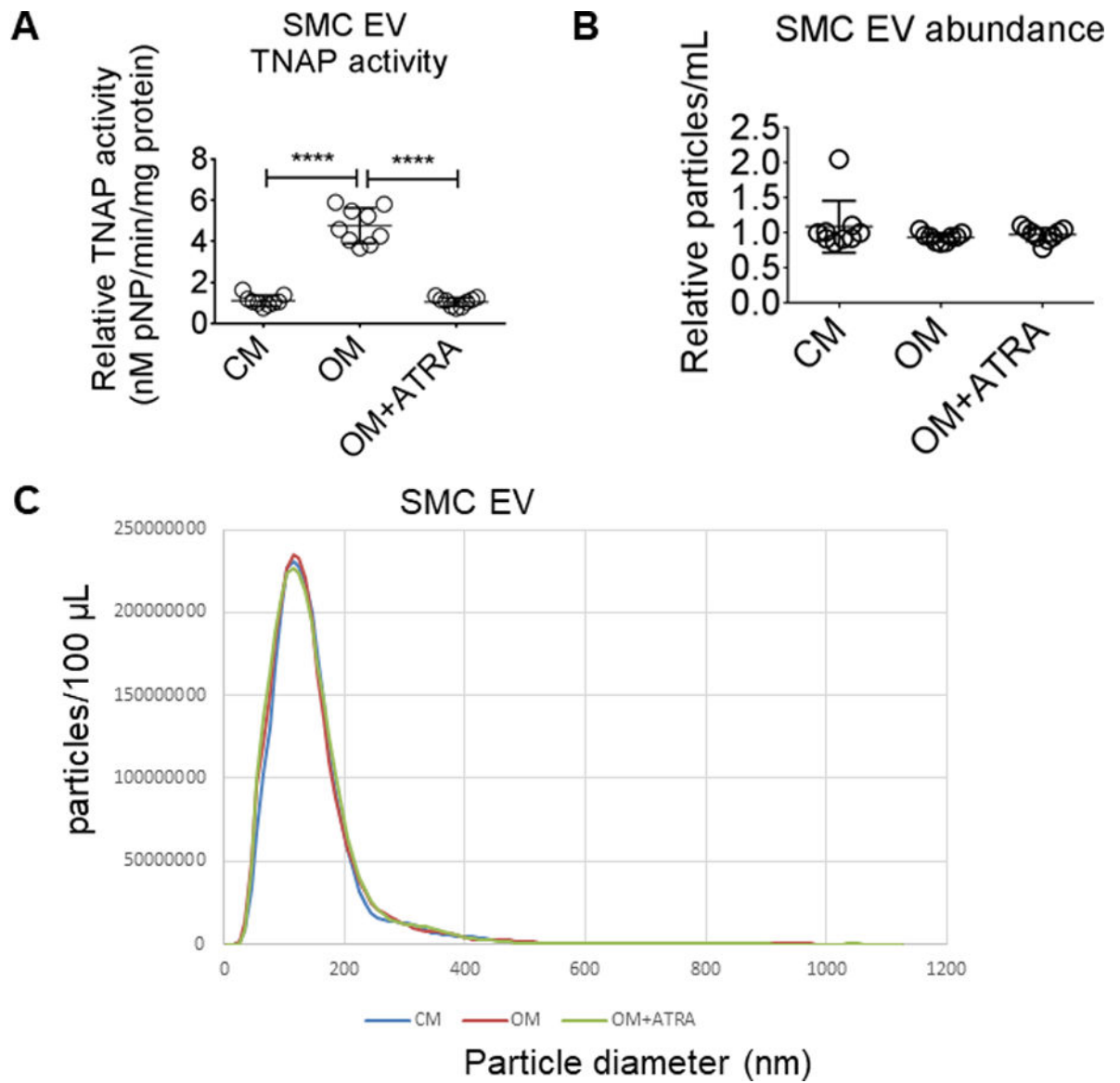
****P< 0.0001 analyzed by ANOVA or Student's t-test; n = 3 donors, except for TNAP activity in which n = 6 donors.
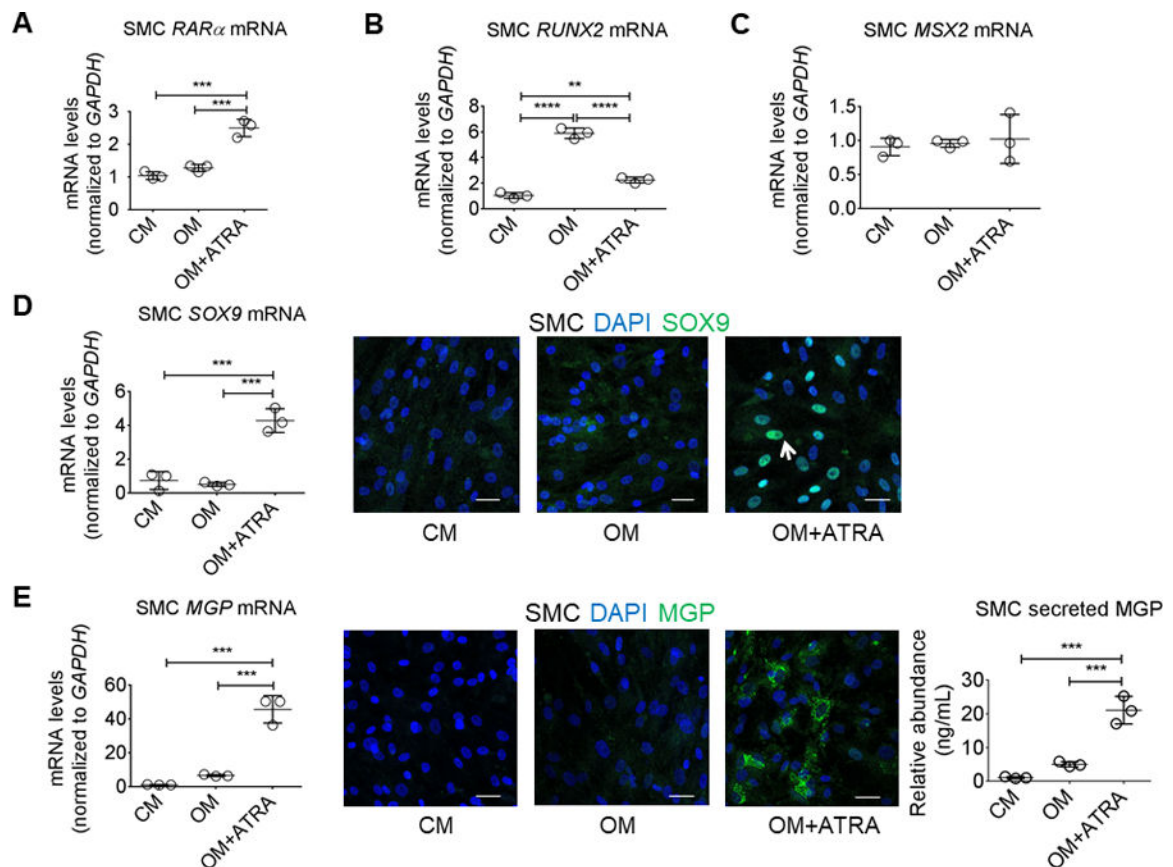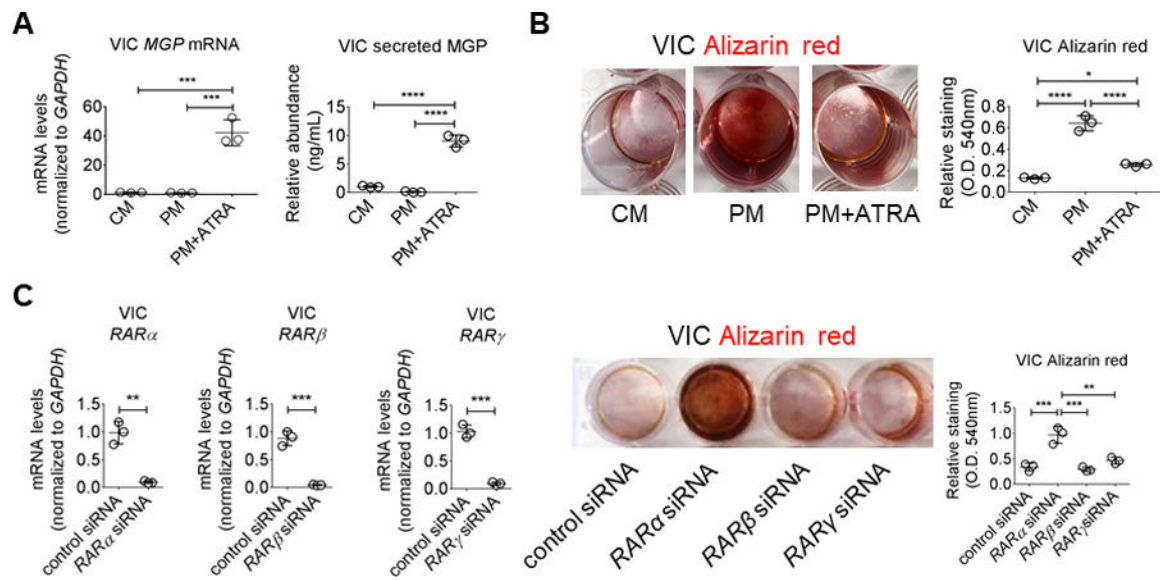
Figure 3.
Retinoic acid suppressed SMC EV TNAP activity. (A) EV TNAP activity from primary human coronary artery SMC treated for two weeks in CM or OM with DMSO vehicle (0.1%) or 1 μmol/L ATRA. (B) SMC EV abundance and (C) diameter from SMC treated for two weeks. Error bars indicate STDEV of mean. ****$P < 0.0001$ analyzed by ANOVA; n = 9 donors.
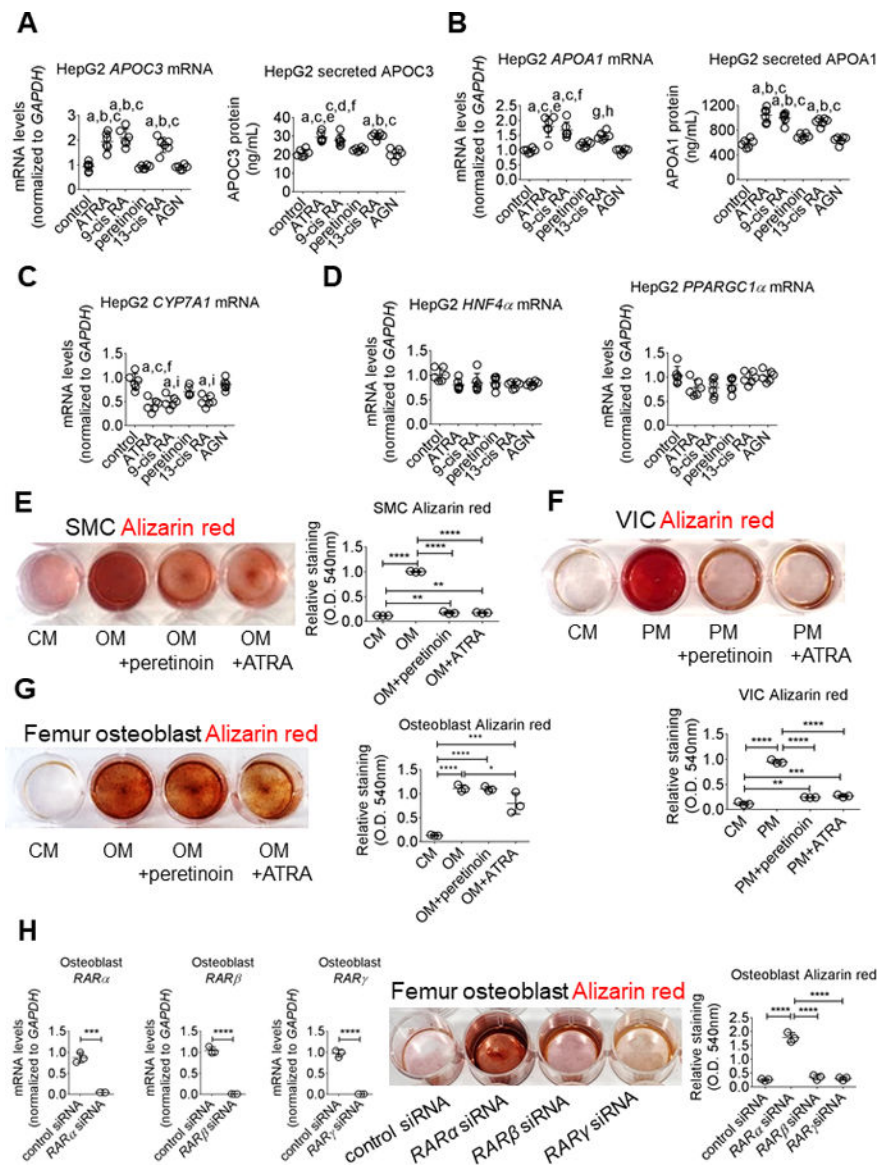
Figure 4.
Retinoic acid increased SOX9 and MGP in human SMC. (A) *RARa*, (B) *RUNX2*, and (C) *MSX2* mRNA levels from primary human coronary artery SMC treated for two weeks in CM or OM with DMSO vehicle (0.1%) or 1 µmol/L ATRA. (D) *SOX9* mRNA levels and representative SOX9 and 4',6-diamidino-2-phenylindole (DAPI) immunofluorescence from SMC treated for two weeks; white arrow depicts an example of SOX9 nuclear localization. (E) *MGP* mRNA levels, representative MGP and DAPI immunofluorescence, and secreted MGP protein abundance from SMC treated for two weeks. Error bars indicate STDEV of mean. **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ analyzed by ANOVA; n = 3 donors. Scale bars, 50 µm.

Figure 5.
Retinoic acid increased MGP in human VIC. (A) *MGP* mRNA levels and secreted protein from VIC treated for two weeks in CM or PM with DMSO vehicle (0.1%) or 1 μmol/L ATRA. (B) Alizarin red stain and relative quantification for VIC treated for three weeks. (C) *RAR* siRNA knockdown validation by mRNA level quantification, and Alizarin red stain and relative quantification for primary human VIC treated for two weeks in PM with negative control, *RARα*, *RARβ*, or *RARγ* siRNA. Error bars indicate STDEV of mean. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 analyzed by ANOVA or Student's t-test; n = 3 donors.

Figure 6.
Peretinoin inhibits human cardiovascular cell calcification without inducing liver cell APOC3 secretion. (A) *APOC3* and (B) *APOA1* mRNA levels and secreted protein in HepG2 treated for 24 hours with 0.1% DMSO vehicle (control), 1 μmol/L ATRA, 1 μmol/L 9-cis retinoic acid (9-cis RA), 1 μmol/L peretinoin, 13-cis retinoic acid (13-cis RA), or 1 μmol/L AGN 193109 (AGN). (C) *CYP7A1* and (D) *HNF4α*, *PPARGC1α* mRNA levels in HepG2 cells treated for 24 hours. Alizarin red stain and relative quantification for (E) primary human coronary artery SMC and (F) VIC treated for three weeks, and (G) primary human femur osteoblasts treated for one three weeks in CM, OM, or PM with DMSO vehicle (0.01%), 1 μmol/L peretinoin, or 1 μmol/L ATRA. (H) *RAR* siRNA knockdown validation by mRNA level quantification, and Alizarin red stain and relative quantification for primary human femur osteoblasts treated for two weeks in OM with negative control, *RARα*, *RARβ*, or *RARγ* siRNA. Error bars indicate STDEV of mean. [a]*P* < 0.0001 vs control, [b]*P* < 0.0001

vs peretinoin, ^{c}$P < 0.0001$ vs AGN, ^{d}$P < 0.001$ vs control, ^{e}$P < 0.001$ vs peretinoin, ^{f}$P < 0.01$ vs peretinoin, ^{g}$P < 0.01$ vs control, ^{h}$P < 0.01$ vs AGN, ^{i}$P < 0.001$ vs AGN, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$ analyzed by ANOVA or Student's t-test; n = 6 independent experiments for HepG2 and n = 3 donors for primary human VIC, SMC, and osteoblasts.