

**Role of endothelin signaling in osteochondrogenic differentiation and matrix calcification  
of vascular smooth muscle cells**

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**Abstract**

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Type II diabetes mellitus (T2DM) and high serum levels of endothelin-1 (ET1) are associated with accelerated ectopic calcification of blood vessels. Most often, this calcification occurs in the medial layer, a layer populated with vascular smooth muscle cells (SMCs). Additionally, T2DM-related hyperglycemia has been implicated in increased incidence and size of atherosclerotic plaques. We aimed to determine whether ET1 signaling through its type A receptor (ETA) is critical for matrix calcification of SMCs in culture conditions that mimic hyperglycemia. Furthermore, we aimed to determine whether this effect on calcification is associated with ET1-ETA interaction alone or its interplay with receptor for advanced glycation end-products (RAGE) signaling that has been implicated in diabetes-associated nephropathy and vascular calcification. Although glucose in hyperglycemic concentrations did not initiate calcification, high glucose in procalcific medium enhanced SMC calcification up to three-fold after seven days. Additionally, we found that adding ET1 to hyperphosphatemic-hyperglycemic cultures enhanced glucose accelerated SMC calcification by 21%, but ET1 had no effect on calcification under high-phosphate conditions alone. Delivery of ETA antagonists BQ-123 and atrasentan to

high phosphate, high glucose cultures, resulted in a 71% inhibition of SMC calcification. In addition to a substantial inhibition of calcification, SMCs treated with 1  $\mu$ M atrasentan had higher mRNA expression levels of myocardin (the SMC master transcription factor co-activator) and lower expression levels of Runx2 compared to SMCs treated with procalcific high glucose media alone, suggesting the preservation of SMC phenotype in their original fate decision. Moreover, blocking RAGE signaling with a neutralizing antibody reduced glucose-accelerated calcification of SMCs, and blocked ET1-enhanced calcification. These results show that ETA antagonism is an efficacious inhibitor of matrix calcification under diabetic conditions and imply interplay between the RAGE and ETA pathways.

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To Doris

## Table of contents

<b>Acknowledgements</b> .....	<b>i</b>
<b>Dedication</b> .....	<b>ii</b>
<b>Table of contents</b> .....	<b>iii</b>
<b>List of figures</b> .....	<b>vi</b>
<b>List of abbreviations</b> .....	<b>viii</b>
<b>Chapter 1: Background and significance</b> .....	<b>1</b>
<b>1.1. Diabetes and vascular calcification</b> .....	<b>2</b>
1.1.1. Is hyperglycemia critical for T2DM-associated vascular calcification?.....	3
<b>1.2. SMC reprogramming and vascular calcification in T2DM</b> .....	<b>4</b>
<b>1.3. T2DM, the receptor for advanced glycation end-products (RAGE), and osteochondrogenic differentiation of SMCs</b> .....	<b>5</b>
<b>1.4. The endothelin system</b> .....	<b>7</b>
1.4.1. Normal endothelin function in vasculature.....	7
1.4.2. Endothelin system dysfunction in vasculature.....	8
1.4.3. ETA signaling in vascular disease .....	8
1.4.4. ET1-ETA signaling in vascular calcification.....	9
<b>1.5. Research objectives</b> .....	<b>9</b>
1.5.1 Hypothesis.....	10
<b>Chapter 2: Materials and methods</b> .....	<b>11</b>
<b>2.1. Mouse models and genetic fate mapping</b> .....	<b>11</b>
<b>2.2. X-gal staining</b> .....	<b>12</b>
<b>2.3. Immunohistochemical staining</b> .....	<b>12</b>

<b>2.4. Quantification of aortic arch calcium content.....</b>	<b>13</b>
<b>2.5. Vascular smooth muscle cell culture .....</b>	<b>13</b>
2.5.1. SMC isolation .....	13
2.5.2. SMC subculture .....	13
2.5.3. <i>In vitro</i> model for study of hyperglycemia-related SMC calcification.....	14
<b>2.6. Reverse transcription-polymerase chain reaction (RT-PCR) and quantitative RT-PCR (qRT-PCR) .....</b>	<b>15</b>
<b>2.7. Western blotting.....</b>	<b>15</b>
<b>2.8. Creation of RAGE knockdown SMC line via small RNA interference .....</b>	<b>16</b>
<b>2.9. Endothelin-1 ELISA .....</b>	<b>17</b>
<b>2.10. Statistics .....</b>	<b>17</b>
<b>Chapter 3: Results and discussion.....</b>	<b>18</b>
<b>3.1. Aim 1: Determine whether ET1 signaling through ETA is important for osteochondrogenic differentiation and matrix calcification of vascular SMCs enhanced by high glucose conditions.....</b>	<b>18</b>
3.1.1. Rationale .....	18
3.1.2. Hypothesis.....	19
3.1.3. ET1 and ETA are found in osteochondrogenic areas of diabetic blood vessels.....	19
3.1.4. Exogenous ET1 enhances matrix calcification of SMCs in procalcific cultures only under high glucose conditions .....	20
3.1.5. Antagonism of ETA, but not ETB, substantially reduces osteochondrogenic differentiation and matrix calcification enhanced by high glucose .....	25
3.1.6. Conclusions.....	34



<b>3.2. Aim 2: Determine whether signaling through RAGE is required for ET1-ETA-associated SMC calcification.....</b>	<b>35</b>
3.2.1. Rationale .....	35
3.2.2. Hypothesis.....	35
3.2.3. Loss of RAGE inhibits SMC osteochondrogenic differentiation and calcification to a similar extent as ETA antagonism .....	35
3.2.4. RAGE is required for ET1-related calcification under high glucose conditions .....	41
3.2.5. RAGE is not required for ET1 production in procalcific SMC cultures .....	42
3.2.6. Conclusions.....	45
<b>Chapter 4: Future directions .....</b>	<b>48</b>
<b>4.1. Investigation of kinase pathways downstream of ET1-ETA and RAGE.....</b>	<b>48</b>
<b>4.2. Investigation of a possible Erk-Runx2-ctRAGE complex.....</b>	<b>49</b>
<b>4.3. Does ETA antagonism inhibit T2DM-related vascular calcification <i>in vivo</i>? .....</b>	<b>50</b>
<b>List of references .....</b>	<b>52</b>

## List of figures

Figure 1: ET1 and vascular calcification in T2DM. Serum ET1 (A) and aortic calcium content (B) from LDLr <sup>-/-</sup> mice fed with HFCD, HFD, or NC. Error bars are reported as SEM. ....	19
Figure 2: ET1-ETA signaling and osteochondrogenic differentiation of vascular SMCs in T2DM. Aortic arches of diabetic LDLr <sup>-/-</sup> mice were stained immunohistochemically (brown) for ET1 (A), ETA (B), and Runx2 (C). Blue X-gal stain designates cells originally derived from SMCs that were labeled through SM22a-Cre and R26R transgenes. ....	20
Figure 3: Representative results from an <i>in vitro</i> SMC matrix calcification assay. High glucose (25 mM) alone does not induce calcification, but addition of high glucose to SMCs induced with high phosphate (2.4 mM) enhances matrix calcification measured after 6 days. ....	21
Figure 4: Working model of osteochondrogenic differentiation and calcification of SMCs in response to glucose, phosphate, ET1, and ETR antagonism <i>in vitro</i> . ....	22
Figure 5: ET1 and SMC matrix calcification after 6 days in normal glucose conditions. ET1 had no significant effect on SMC matrix calcification induced by high phosphate (2.4 mM) under normal glucose (5.56 mM) conditions. ....	23
Figure 6: ET1 and SMC matrix calcification after 6 days in high glucose conditions. ET1 (500 nM) had a significant effect on SMC matrix calcification induced by high phosphate (2.4 mM) under high glucose (25 mM) conditions. ....	24
Figure 7: ETA antagonism and SMC matrix calcification after 6 days in high glucose conditions. Atrasentan (1 nM and 1 μM) significantly inhibited SMC calcification induced by high phosphate (2.4 mM) under high glucose (25 mM) conditions. ....	26
Figure 8: ETA antagonism and SMC matrix calcification after 6 days in normal glucose conditions. No dose of atrasentan (1 nM to 1 μM) significantly affected SMC calcification induced by high phosphate (2.4 mM) in normal glucose conditions (5.56 mM). ....	27
Figure 9: The effect of ETA and ETB antagonism on SMC matrix calcification after 6 days in high glucose conditions. ETA antagonist BQ-123 (1 μM) significantly inhibited SMC calcification, and ETB antagonist BQ-788 (1 μM) only slightly inhibited calcification induced by high phosphate (2.4 mM) under high glucose (25 mM) conditions. Dual antagonism resulted in the greatest inhibition of calcification. ....	29
Figure 10: The effect of ETA and ETB antagonism on SMC matrix calcification after 6 days in normal glucose conditions. No combination of ETR antagonists significantly affected SMC calcification induced by high phosphate (2.4 mM) under normal glucose (5.56 mM) conditions. ....	30
Figure 11: The effect of ETA antagonism on Runx2 protein expression. MC3T3 lysates served as a positive control for Runx2 (~75 kDa). Runx2 expression was upregulated in response to high glucose (25 mM) and high phosphate (2.4 mM), and expression was lower in groups treated with atrasentan (1 μM). ....	31
Figure 12: The effect of ETA antagonism on myocardin mRNA expression. Myocardin expression was downregulated in response to high phosphate (HP; 2.4 mM) and high glucose (HG; 25 mM), and expression was preserved in groups treated with atrasentan (1 μM). ....	32
Figure 13: The effect of ETA antagonism on SM22α mRNA expression. SM22α expression was downregulated in response to high phosphate (HP; 2.4 mM) and high glucose (HG; 25 mM), and expression was preserved in groups treated with atrasentan (1 μM). ....	33

Figure 14: RAGE expression levels in SMCs in procalcific conditions. RAGE (~45 kDa) is upregulated in response to procalcific conditions (2.4 mM Pi) with high glucose (25 mM). .....	36
Figure 15: RAGE and ETA in SMC calcification after 6 days under high glucose conditions. Both ETA antagonism (10 nM atrasentan) and neutralization of RAGE (2 µg/mL RAGE antibody) reduced SMC matrix calcification induced by high phosphate (2.4 mM) under high glucose (25 mM) conditions. ....	37
Figure 16: RAGE knockdown. Quantitative RT-PCR data show the shRNA knockdown of RAGE expression at the mRNA level (A), and western blot shows knockdown at the protein level (B). ....	39
Figure 17: RAGE and SMC matrix calcification after 6 days under high glucose conditions. Loss of RAGE (shRAGE) ablated the effect of high glucose (25 mM) on matrix calcification in procalcific conditions (2.4 mM Pi). Calcification (µg calcium/mg cellular protein) for shRAGE and mock lines were normalized to samples treated with 5.56 mM glucose (NG) and 2.4 mM phosphate (HP). ....	40
Figure 18: ET1-related matrix calcification of SMCs after 6 days with neutralized RAGE. Neutralization of RAGE (2 µg/mL RAGE ab) negated the aforementioned effect of ET1 (1 µM) on calcification induced by high phosphate (2.4 mM) in high glucose (25 mM) conditions. ....	41
Figure 19: RAGE-dependent ET1 secretion from SMCs under normal phosphate (NP; 1.0 mM) conditions, with normal (NG; 5.56 mM) or high (HG; 25 mM) glucose conditions. RAGE may be involved in, but not required for, the maintenance of ET1 levels in normal culture conditions. * = p<0.05 between mock and shRAGE groups. ....	43
Figure 20: RAGE-dependent ET1 secretion from SMCs under high phosphate (HP; 2.4 mM) conditions, with normal (NG; 5.56 mM) or high (HG; 25 mM) glucose conditions. RAGE is not required for ET1 secretion in procalcific conditions. * = p<0.05 between mock and shRAGE groups. ....	44
Figure 21: Effect of ETA antagonism on RAGE expression on SMCs. Atrasentan (1 µM) reduced RAGE expression in SMCs cultured in procalcific conditions (2.4 mM Pi) with high glucose (25 mM). ....	45
Figure 22: Possible pathway downstream of ETA and RAGE signaling, including an active Runx2 complex formed with ctRAGE and p-Erk1/2. ....	47

## List of abbreviations

<b>-/-</b>	Null
<b>ALP</b>	Alkaline Phosphatase
<b>ANOVA</b>	Analysis of Variance
<b>ApoE</b>	Apolipoprotein E
<b>bigET1</b>	Big Endothelin-1
<b>BMP-2</b>	Bone Morphogenetic Protein 2
<b>BSA</b>	Bovine Serum Albumin
<b>cDNA</b>	Complementary DNA
<b>Col I</b>	Collagen Type I
<b>ctRAGE</b>	Cytoplasmic Receptor for Advanced Glycation End-products
<b>DMEM</b>	Dulbecco's Modified Eagle's Serum
<b>DMSO</b>	Dimethyl Sulfoxide
<b>ECE1</b>	Endothelin Converting Enzyme 1
<b>ET</b>	Endothelin
<b>ETA</b>	Endothelin Receptor Type A
<b>ETB</b>	Endothelin Receptor Type B
<b>ETR</b>	Endothelin Receptor
<b>ET1</b>	Endothelin-1
<b>ELISA</b>	Enzyme-linked Immunosorbent Assay
<b>Erk1/2</b>	Extracellular Signal Regulated Kinase 1/2
<b>FBS</b>	Fetal Bovine Serum
<b>GAPDH</b>	Glyceraldehyde 3-phosphate dehydrogenase
<b>HFCF</b>	High Fat/Cholesterol Diet
<b>HFD</b>	High Fat Diet
<b>HG</b>	High Glucose (25 mM)
<b>HOMA-IR</b>	Homeostatic Model Assessment for Insulin Resistance
<b>HP</b>	High Inorganic Phosphate (2.4 mM)
<b>HRP</b>	Horseshoe Peroxidase
<b>IgG</b>	Immunoglobulin G
<b>LDLr</b>	Low Density Lipoprotein Receptor
<b>MAPK</b>	Mitogen-Activated Protein Kinase
<b>MEK</b>	Mitogen-Activated Protein Kinase Kinase
<b>MGP</b>	Matrix Gla Protein
<b>Msx2</b>	Msh Homeobox 2
<b>NC</b>	Normal Chow
<b>NFκB</b>	Nuclear Factor Kappa-light-chain-enhancer of Activated B Cells
<b>NG</b>	Normal Glucose
<b>NO</b>	Nitrous Oxide
<b>NP</b>	Normal Inorganic Phosphate (1.0 mM)
<b>NT</b>	Non-transduced
<b>OPN</b>	Osteopontin
<b>OSE2</b>	Osteoblast-specific Element 2

<b>PAGE</b>	Polyacrylamide Gel Electrophoresis
<b>PBS</b>	Phosphate Buffered Saline
<b>p-Erk1/2</b>	Phosphorylated Extracellular Signal Regulated Kinase 1/2
<b>Pi</b>	Inorganic Phosphate
<b>PI3K</b>	Phosphoinositide 3-kinase
<b>PLC</b>	Phospholipase C
<b>PMSF</b>	Phenylmethylsulfonyl Fluoride
<b>preproET1</b>	Prepro Endothelin-1
<b>PVDF</b>	Polyvinylidene Fluoride
<b>qRT-PCR</b>	Quantitative Reverse Transcription Polymerase Chain Reaction
<b>RAGE</b>	Receptor for Advanced Glycation End-products
<b>RNAi</b>	RNA interference
<b>Runx2</b>	Runt-related Transcription Factor 2 (Cbfa1)
<b>SDS</b>	Sodium Dodecyl Sulfate
<b>shRAGE</b>	Small Hairpin RNA Interference of Receptor for Advanced Glycation End-products
<b>shRNA</b>	Small Hairpin RNA
<b>SM</b>	Smooth Muscle
<b>SMC</b>	Smooth Muscle Cell
<b>SMMHC</b>	Smooth Muscle Myosin Heavy Chain
<b>sRAGE</b>	Secretory Receptor for Advanced Glycation End-products
<b>T2DM</b>	Type II Diabetes Mellitus
<b>TBST</b>	Tris-Buffered Saline and Tween 20
<b>TNF<math>\alpha</math></b>	Tumor Necrosis Factor Alpha
<b>VEGF</b>	Vascular Endothelial Growth Factor
<b>vWF</b>	von Willebrand Factor

## **Chapter 1: Background and significance**

Vascular calcification, the ectopic deposition of calcium-phosphate salts in blood vessels, greatly affects the aging and diabetic populations. Although often asymptomatic at early stages, the occurrence of vascular calcification is associated with a heightened risk for cardiovascular morbidity and mortality.<sup>1</sup> It is also a significant predictor of future coronary heart disease events, stroke, and lower-limb amputation.<sup>1,2</sup> Vascular calcification in animal models has been detected at the early stages with a near infrared probe technique,<sup>3</sup> but in the clinical setting, vascular calcification can only be detected when there is an obvious amount of calcium-phosphate salts deposited in blood vessels by X-ray and computed tomography and/or by the resulting hemodynamic change from increased stiffness of blood vessels.<sup>4,5</sup> The clinical effects of a lack of early detection method are worsened by a limited understanding of disease initiation and progression. Even though there are animal studies that demonstrate slowed progression of vascular

calcification in uremic mice treated with either phosphate binders,<sup>6</sup> vitamin D receptor activators,<sup>7</sup> bisphosphonates,<sup>8</sup> or BMP-7,<sup>9</sup> there are no pharmacological therapies proven to improve clinical outcomes for patients with vascular calcification.

### **1.1. Diabetes and vascular calcification**

Diabetes mellitus affects an estimated 25.8 million people and has been the sixth or seventh leading cause of death in the US since 2002.<sup>10</sup> Type II diabetes mellitus (T2DM) is the most common form of this disease, accounting for over 90 percent of cases.<sup>10</sup> Patients with T2DM showed a heightened risk for vascular calcification compared to those without.<sup>11</sup> T2DM also facilitates progression of calcification, and there is a significantly increased rate of new incidence compared to those without.<sup>11</sup> The long-term complications of T2DM in blood vessels include increased fibro-cartilaginous matrices and deposition of calcium-phosphate salts in both intimal and medial layers.<sup>12-15</sup>

Compared to diabetics without vascular calcification, diabetics with vascular calcification have a 1.5-fold increase in mortality, a 1.6-fold increase in incidence of coronary artery disease, a 2.4-fold increase in proteinuria, a 1.7-fold increase in retinopathy, and a 5.5-fold increase in amputation.<sup>16</sup> Additionally, vascular calcification increases arterial stiffness and decreases compliance, which impairs cardiac function and is correlated with increased coronary ischemic syndromes, such as myocardial infarction.<sup>17,18</sup> No effective clinical treatment to reduce vascular calcification has been found, but infliximab, a monoclonal antibody against tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), was shown to reduce vascular calcification in low density lipoprotein receptor null (LDLr $^{-/-}$ ) mice.<sup>19</sup> Despite our improved understanding of vascular calcification from recent epidemiological and histopathological studies, it is unknown whether T2DM has a causal effect on the early pathogenesis of vascular calcification. It is also unclear what regulatory molecules

and pathways underlying the T2DM-associated vascular calcification are critical for disease initiation and/or progression.

### **1.1.1. Is hyperglycemia critical for T2DM-associated vascular calcification?**

Hyperglycemia is a notable symptom of T2DM, but its role in vascular calcification is not clearly defined. In a study of 3,054 patients, impaired fasting glucose (5.6–6.9 mM) was found to be independently associated with high coronary artery calcification (measured using multidetector computed tomography).<sup>20</sup> Hyperglycemia is associated with superoxide formation,<sup>21</sup> impaired neovascularization and vascular endothelial growth factor (VEGF) production, and accelerated atherosclerosis in apolipoprotein-E null (apoE<sup>-/-</sup>) mice.<sup>22</sup> Dietary cholesterol is known to promote osteoblastic differentiation of SMCs in culture; concordantly, when fed with a fat-enriched, “Western” diet, LDLr<sup>-/-</sup> mice developed atherosclerotic lesions and ectopic calcium-phosphate salt deposition in arteries and cardiac valves.<sup>23,24</sup> To substantiate this, in an experiment with the LDLr<sup>-/-</sup> mice similar to those described above, mice fed with a high fat diet (HFD) with high cholesterol (HFCD) were shown to develop an intermediate increase in serum glucose and insulin levels.<sup>25</sup> Even though high cholesterol was required as an initiator of vascular calcification in LDLr<sup>-/-</sup> mice, the resulting severity of insulin resistance and hyperglycemia was indicative of an increased amount of calcium deposition in blood vessels and a higher incidence of osteochondrogenic differentiation of vascular wall cells.<sup>25</sup> High dietary cholesterol tripled the incidence of cartilaginous metaplasia and increased aortic arch calcification 13-fold in diabetic mice.<sup>25</sup> Insulin has been shown to enhance cholesterol transport into arteriolar smooth muscle cells (SMCs) and increase endogenous lipid synthesis by these cells, increasing the formation of and decreasing the regression of lipid plaques.<sup>26</sup> Additionally, cartilaginous metaplasia and calcification of blood vessels occurred earlier in mice that developed T2DM (as determined by



obesity, hyperglycemia, and hyperinsulinemia), with features often seen in human T2DM cardiovascular disease.<sup>25</sup> Thus, hypercholesterolemia likely initiated calcification, and insulin resistance and hyperglycemia likely exacerbated development of vascular calcification in T2DM.

## **1.2. SMC reprogramming and vascular calcification in T2DM**

Vascular calcification was once considered a passive, degenerative process involving tissue necrosis, but many studies have now shown that vascular calcification involves active, cell-mediated deposition of matrix mineral that resembles bone formation and remodeling.<sup>13,27,28</sup> Calcium deposits appear in conjunction with osteochondrogenic gene expression patterns, such as upregulated Runx2 and Sox9.<sup>13,23,27-29</sup>

Lineage tracing studies have highlighted the cell sources that give rise to osteochondrogenic precursor and chondrocyte-like cells in calcifying arteries. SM22 $\alpha$ -Cre recombinase and Rosa26-LacZ reporter alleles permanently mark cells derived from SMCs with  $\beta$ -galactosidase,<sup>30</sup> and these cells have been found to express osteochondrogenic (Runx2) and/or chondrocytic (Sox9, type II collagen) markers and lose expression of SMC lineage proteins (SM-MHC, SM22 $\alpha$ , and SM  $\alpha$ -actin). A genetic fate mapping study using this technique showed that approximately 90% of the Runx2-positive cells in atherosclerotic vessels of LDLr<sup>-/-</sup> mice fed with HFCD for 28 weeks were of SMC origin,<sup>31</sup> indicating that these cells are crucial mediators of vascular calcification.

Moreover, osteochondrogenic differentiation and matrix calcification were reproduced *in vitro* using cells isolated from normal vasculature.<sup>32</sup> Culture of SMCs under procalcific conditions *in vitro* resulted in a downregulation of the SMC marker proteins and a concurrent upregulation of osteochondrogenic markers.<sup>32,33</sup> Extracellular signal-regulated kinase (Erk1/2), a mitogen-activated protein kinase (MAPK) that mediates osteoblastic differentiation in skeletal bone through

the stimulation of Runx2 transcriptional activity, was activated in procalcific conditions.<sup>33</sup> Blocking Erk1/2 phosphorylation with a specific MEK inhibitor (U-0126) preserved SMC phenotype markers, and blocked upregulated expression of osteochondrogenic marker, Runx2, in procalcific conditions.<sup>34</sup> Runx2 activation via phosphorylation facilitated by phosphorylated Erk1/2 (p-Erk1/2) is important for calcification of osteoblasts,<sup>35</sup> and this pathway may be important to osteochondrogenic differentiation of SMCs as well.

The same genetic fate mapping strategy described above was applied to a T2DM mouse model to determine whether osteochondrogenic differentiation of SMCs is a common initiator of vascular calcification, occurring also in diabetes. SM22 $\alpha$ -Cre and R26R-LacZ transgenes were introduced into LDLr<sup>-/-</sup> mice prior to induction of T2DM and vascular calcification with HFCD. Co-localization of  $\beta$ -galactosidase activity with the loss of SMC lineage proteins in diabetic blood vessels, along with the expression of bone and cartilage marker proteins within a single cell, identified a lineage reprogramming of vascular SMCs toward osteochondrogenic fate.<sup>25</sup> These results show that SMCs are a major source of osteochondrogenic precursor- and chondrocyte-like cells in calcifying arterial media and identify these cells as crucial mediators of vascular calcification in the T2DM setting.<sup>36</sup>

### **1.3. T2DM, the receptor for advanced glycation end-products (RAGE), and osteochondrogenic differentiation of SMCs**

Hyperglycemia appears early in the pathogenesis of T2DM, and it has been hypothesized that sustained high glucose levels explain some partial effects T2DM-related cardiovascular complications.<sup>37</sup> High serum levels of advanced glycation end-products (AGEs), non-enzymatically glycated forms of proteins and lipids due mainly to sustained hyperglycemia, are associated with the progression of atherosclerosis in diabetic patients and correlated with the

occurrence of vascular calcification.<sup>38,39</sup> RAGE is a member of the immunoglobulin G (IgG) superfamily and is a pattern recognition receptor of AGEs and calgranulins,<sup>40-43</sup> and activation of the RAGE has been implicated in a wide variety of diabetic complications, including atherosclerosis.<sup>44</sup>

RAGE and its ligands were co-localized in calcified lesions,<sup>45-47</sup> but how RAGE signals osteochondrogenic differentiation of SMCs is presently unclear. Diabetic apoE<sup>-/-</sup> mice deficient in RAGE developed smaller atherosclerotic lesions than mice expressing RAGE, most likely through the reduction of inflammatory mediators and leukocyte recruitment, decreased nuclear factor  $\kappa$ -light-chain-enhancer of activated B cells (NF $\kappa$ B) activity and oxidative stress, and improved endothelial function.<sup>48</sup> Additionally, atherosclerotic plaque size and calcified plaque area were larger in transgenic ApoE<sup>-/-</sup> mice expressing S100A12, a RAGE ligand. This change was accompanied by an increase in Runx2, BMP-2, and osteocalcin expression in the aorta.<sup>49</sup> Aortic vascular SMCs overexpressing RAGE demonstrated reduced myocardin-dependent gene expression and induced Notch/Msh homeobox 2 (Msx2) signaling.<sup>50</sup> Furthermore, SMCs incubated with AGEs exhibited enhanced calcium accumulation in time- and dose-dependent manners.<sup>51</sup> Interestingly, sequence analysis of the docking sites among p-Erk1/2 substrates identified a conserved amino acid sequence within the putative binding sites of the cytoplasmic domain of RAGE (ctRAGE).<sup>52</sup> Using an antibody that recognizes ctRAGE, differences were observed in RAGE localization within calcifying lesions of diabetic blood vessels, likely dependent upon the stage of disease progression. In the early stages, RAGE was primarily located in the nuclei of vascular SMCs, co-localizing with p-Erk1/2 and osteochondrogenic transcription factor, Runx2. In the later stages, RAGE occurred primarily on the membrane of SMCs positive for chondrogenic transcription factor, Sox9, and types II and X collagen, markers of proliferating

and hypertrophic chondrocytes.<sup>25</sup> Thus, RAGE signaling likely plays a role in the development of vascular calcification in the diabetic setting at multiple levels.

#### **1.4. The endothelin system**

There are three types of endothelins (ETs), which are endogenously produced and highly regulated 21-amino-acid peptides involved in the regulation of vascular tone.<sup>53,54</sup> Endothelin-1 (ET1) is the most highly expressed member of the endothelin family, but both ET1 and ET3 are produced in the vasculature.<sup>55</sup> ETs transduce biochemical signals through two G protein-coupled receptors, type A (ETA) and type B (ETB).<sup>56</sup> ETA is the most highly expressed endothelin receptor (ETR) on the membrane of SMCs,<sup>55,57</sup> and the affinity of ET1 to ETA is approximately 100 times higher than that of ET3.<sup>58</sup> Although ET1 was originally identified as a potent vasoconstrictor produced by vascular endothelial cells,<sup>54</sup> it has now been found in multiple cell types and its signaling through ETRs has been implicated as a cause of the micro- and macro-vascular complications in patients with T2DM and atherosclerosis.<sup>59,60</sup>

##### **1.4.1. Normal endothelin function in vasculature**

ET1 production and clearance are highly regulated. ET1 is produced predominantly by vascular wall cells, such as endothelial cells and SMCs.<sup>55</sup> Biochemical and mechanical stressors, such as NFκB and shear stress, trigger transcription of ET1 mRNA.<sup>61,62</sup> ET1 mRNA is translated to preproET1, a precursor that may be cleaved by a furin-like protease to give a biologically inactive intermediate form, bigET1. The rate-limiting step is the cleavage of bigET1 by Endothelin Converting Enzyme-1 (ECE1) at the cell surface, producing the functional ET1 peptide. Binding of ET1 to ETA on SMCs results in calcium ion influx and contraction through phospholipase C (PLC) signaling. Binding of ET1 to ETB on endothelial cells induces ET1 reuptake (clearance)

and nitric oxide (NO) synthesis through phosphoinositide 3-kinase (PI3K) signaling for vasorelaxation through guanylyl cyclase.<sup>55,58,60,61,63</sup> ET1 initiates vasoconstriction within five minutes of being introduced to the blood stream, but the systemic half-life of ET1 is approximately 7 hours.<sup>59</sup>

#### **1.4.2. Endothelin system dysfunction in vasculature**

Recently, ET1 was found to be associated with a variety of micro- and macro-vascular complications associated with hyperglycemia and insulin resistance.<sup>64,65</sup> Increased serum levels of ET1 were correlated with endothelial dysfunction,<sup>66</sup> atherosclerosis,<sup>65,67</sup> diabetic nephropathy,<sup>68</sup> and calcific cardiac valve disease.<sup>69</sup> ET1 overexpression in apoE<sup>-/-</sup> mice exacerbates HFD-induced atherosclerosis, suggesting a role for ET1 in lipid biosynthesis in the vascular wall and the accelerated progression of atherosclerosis.<sup>70</sup>

#### **1.4.3. ETA signaling in vascular disease**

With the development of selective ETR antagonists, small molecule drugs that bind with a high affinity to a specific ETR subtype without conducting the signals associated with ET binding, ETA was found to play an important role in the progression of the diseases listed above. For example, BQ123 inhibited endothelial dysfunction in patients with T2DM.<sup>71</sup> Sildenafil reduced oxidative stress and insulin resistance,<sup>72,73</sup> and bosentan reduced macrophage infiltration associated with atherosclerosis.<sup>74,75</sup> Additionally, sitaxsentan led to reduced atherosclerotic lesion size and arterial stiffness.<sup>76</sup> Atrasentan improved coronary blood flow in patients with early atherosclerosis,<sup>77</sup> and avosentan improved creatinine clearance and attenuated both glomerulosclerosis and mesangial matrix accumulation in ApoE<sup>-/-</sup> mice with diabetic nephropathy.<sup>78</sup> However, blocking ETB with various selective antagonists resulted in salt and

water retention and slowed ET1 clearance and NO production.<sup>58,79</sup> These findings implicate ETA signaling in the pathogenesis of various vascular diseases.

#### **1.4.4. ET1-ETA signaling in vascular calcification**

Application of endothelin receptor antagonists was shown to reduce pulse wave velocity, a measure of arterial stiffness, in patients with chronic kidney disease.<sup>76</sup> Additionally, darusentan, an ETA antagonist, regressed medial arterial calcification in rats treated with warfarin and vitamin K, most likely through activation of membrane-bound carbonic anhydrase.<sup>80</sup> However, the direct effects of ET1-ETA signaling on SMC osteochondrogenic differentiation and diabetes-associated matrix calcification were not investigated. Furthermore, ET1-ETA signaling has been shown to activate Erk1/2 and induce the production of procalcific reactive oxygen species in vascular SMCs.<sup>81-83</sup> Although ET1 and ETA signaling have been shown to be important for normal bone formation and pathways associated with osteochondrogenic differentiation,<sup>84,85</sup> it is unknown whether ET1-ETA signaling plays a role in osteochondrogenic differentiation and calcification of SMCs, specifically in a diabetic setting.

#### **1.5. Research objectives**

The overall goal of this study is to determine whether ET1 is an important molecule that signals early pathogenesis of vascular calcification under diabetic conditions. Vascular calcification is an active, highly controlled process, and previous studies have highlighted a role of SMCs as a major cell source that gives rise to osteochondrogenic precursors and chondrocytes in calcifying blood vessels in a variety of disease models. Importantly, these cells showed increased susceptibility to osteochondrogenic differentiation and calcification upon exposure to hyperglycemia. Additionally, this phenotypic change was associated with increased expression of ET1, its receptor

ETA, and RAGE. Because both ET1-ETA and RAGE signals are propagated in similar conditions, it is likely that the pathways are interconnected. In an attempt to clarify the roles these molecules play in diabetic vascular calcification, we sought (1) to determine whether ET1 signaling through ETA is important for matrix calcification of vascular SMCs associated with high glucose conditions and (2) to determine whether signaling through RAGE is required for ET1-ETA-associated SMC calcification.

### **1.5.1 Hypothesis**

We hypothesize that ET1-ETA signaling interacts with the RAGE signaling pathway to prolong activation of Erk1/2 and Runx2, thus enhancing osteochondrogenic differentiation and matrix calcification under diabetic conditions.

## Chapter 2: Materials and methods

### 2.1. Mouse models and genetic fate mapping

LDLr<sup>-/-</sup> mice were purchased from the Jackson Laboratory (002207). The mice were bred onto the SM22 $\alpha$ -Cre recombinase (SM22 $\alpha$ -Cre, gift from Dr. Herz, UT) and Rosa26 Cre reporter (R26R-LacZ, gift from Dr. Soriano, FHCRC) transgenic background to produce SM22 $\alpha$ -Cre<sup>+ / 0</sup>:R26R-LacZ<sup>+ / 0</sup>:LDLr<sup>-/-</sup> mice. As characterized in a previous study,<sup>34</sup> SM22 $\alpha$ -Cre and R26R-LacZ transgenic alleles allow Cre recombination to occur in SM22 $\alpha$ -positive cells early in embryogenesis and onward, resulting in a permanent mark of these cells with active  $\beta$ -galactosidase. Therefore, SMCs that have lost their marker proteins in calcifying lesions are still identifiable by X-gal staining, which converts any intracellular  $\beta$ -galactosidase activity into a blue precipitate. At 10–12 weeks old ( $\geq 20$ g), baseline body weight and fasted blood glucose levels of SM22 $\alpha$ -Cre<sup>+ / 0</sup>:R26R-LacZ<sup>+ / 0</sup>:LDLr<sup>-/-</sup> mice were recorded. The mice were randomly assigned



into three groups, fed with previously described diabetogenic/procalcific diet (HFDC; 57.9% kcal fat, 26.9% kcal carbohydrates, 0.2% kcal cholesterol), along with diabetogenic diet (HFD; 58.7% kcal fat, 26.7 kcal carbohydrates, 0.03% kcal cholesterol) and normal chow (NC) dietary controls.<sup>25</sup> Progression of diabetes was monitored by body weight every two weeks and fasted blood glucose every four weeks under diets. At 18–24 weeks, mice were sacrificed with 50–180 mg/kg pentobarbital intraperitoneally followed by exsanguination via cardiac puncture for blood collection. Aortic arches were collected for histological analysis and quantification of calcium content (10–13 per group). Sera were collected for ET1 quantification through a sandwich enzyme-linked immunosorbent assay (ELISA) (R&D, DET100). A total of 48 SM22 $\alpha$ -Cre+/0:R26R+/0:LDLr $^{-/-}$  mice were examined. All animals were maintained in a specific pathogen-free environment and genotypes were determined as previously described.<sup>31,34</sup> All protocols are in compliance with the National Institutes of Health Guideline for the Care and Use of Laboratory Animals and have been approved by the Institutional Animal Care and Use Committee, University of Washington.

## **2.2. X-gal staining**

Tissue dissected from SM22 $\alpha$ -Cre+/0:R26R-LacZ+/0:LDLr $^{-/-}$  mice were stained using a  $\beta$ -galactosidase stain kit (Special Media) as described previously.<sup>34</sup>

## **2.3. Immunohistochemical staining**

X-gal-stained SM22 $\alpha$ -Cre+/0:R26R-LacZ+/0:LDLr $^{-/-}$  tissues were post-fixed with Methyl Carnoy's fixative prior to processing and embedding in paraffin. Five-micrometer sections were collected for immunohistochemical staining using rabbit polyclonal antibodies for ET1 (ab117757,

Abcam) and ETA (ab85163, Abcam) and a rat polyclonal antibody for Runx2/Cbfa1 (MAB2006, R&D Systems).

## **2.4. Quantification of aortic arch calcium content**

Around 6 mm of aortic arch from each LDLr<sup>-/-</sup> mouse was collected and lyophilized to a constant weight. Calcium was extracted from the lyophilized tissue with 0.6 N HCl and quantified colorimetrically using the o-Cresolphthalein complexone kit (Teco Diagnostics, C503-480). Values were normalized to the dry weight of the specimens.<sup>34</sup>

## **2.5. Vascular smooth muscle cell culture**

### **2.5.1. SMC isolation**

SMCs were isolated from the medial layer of aortas of wild-type C57BL/6 mice. Four to five week old mice were euthanized, and their aortas were retrieved. Whole aortas were rinsed and digested with collagenase and elastase, and the adventitial and endothelial layers were removed. Aortic medial layers were again digested in the enzyme solution and plated for primary culture. Purity was determined via staining for smooth muscle cell markers SMMHC, SM22 $\alpha$ , and SM $\alpha$ -actin. The absence of endothelial cells was determined via a negative staining result for von Willebrand Factor (vWF).<sup>86</sup>

### **2.5.2. SMC subculture**

To mimic physiological glucose levels, aortic SMCs were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 5.56 mM glucose supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotic-antimycotic. Medium was changed every 2-3 days until confluence, at which point the cells were detached from the flask with 0.05% trypsin and

progressively cultured into new flasks. For long-term storage, cells were cryopreserved in a 90% FBS and 10% dimethyl sulfoxide (DMSO) solution first at -70°C in an isopropanol-filled container, followed by storage in a liquid nitrogen dewar. Passage 6-10 SMCs were used for experiments.

### **2.5.3. *In vitro* model for study of hyperglycemia-related SMC calcification**

The calcification model was optimized to limit passive, spontaneous deposition of calcium phosphate crystals on the collagen-coated surface and cell death-related calcification, and to maximize difference between calcification observed in cultures treated with high phosphate alone and cultures treated with both high phosphate (concentrations above 1.0 mM) and high glucose (concentrations above 100 mg/dL, or 5.56 mM). Culture plates coated with Col I were used to provide the SMCs with a matrix similar to what they encounter *in vivo* and improve the survival of the SMCs when induced to calcify with various stressors. Concentration ranges of inorganic phosphate, glucose and FBS were tested against SMC density and collection time to give the optimal protocol described above. Thus, for calcification experiments, SMCs were seeded onto type I collagen (Col I)-coated 12-well plates (BD Biosciences) at a density of 18000 cells per well and induced to calcify with 2.4 mM inorganic phosphate (Pi) 24 hours after seeding. Role of high glucose at levels of hyperglycemia (25 mM, HG) in SMC calcification was determined with and without 2.4 mM phosphate (HP). Media was refreshed every other day until collection. Cell cultures were rinsed with phosphate buffered saline (PBS) and decalcified with 0.6 N HCl at 4°C for 24 hours. Calcium released from the cultures was quantified colorimetrically using the o-Cresolphthalein complexone kit (Teco Diagnostics, C503-480). Calcium content was normalized to cellular protein of the cultures and expressed as µg calcium/mg cellular protein.

## **2.6. Reverse transcription-polymerase chain reaction (RT-PCR) and quantitative RT-PCR (qRT-PCR)**

Total RNA was extracted from cell cultures using RNeasy Mini kit (Qiagen), and the contaminating genomic DNA was degraded on column by RNase-free DNase I (Qiagen). One  $\mu$ g RNA was used to synthesize first-strand complementary DNA (cDNA) using Omniscript reverse transcriptase (Qiagen) at 37°C for one hour. The resulting cDNA was amplified through PCR to determine expression of myocardin (forward primer: 5'-TCACTGTGTGGAGTCCTCAGGTC-3', reverse primer: 5'-TGGCATCGGCTGGCATTTC-3') and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (forward primer: 5'-ACCACAGTCCATGCCATCAC-3', reverse primer: 5'-TCCACCACCCTGTTGCTGTA -3'). Myocardin expression level was normalized to GAPDH. The cDNA was also used in qRT-PCR to quantify expression of SM22 $\alpha$  (Life Technologies, Mm00441661\_g1) and RAGE (forward primer: 5'-AGGGAAGGAGGTCAAGTCCAAC-3', reverse primer: 5'-TATTAGGGACACTGGCTGTGAGTTC-3', probe: 5'-FAM-CGAGTCTACCAGATTC-MGB-3') using qRT-PCR. To control loading, 18S ribosomal RNA levels of each sample was determined using TaqMan Ribosomal RNA Control Reagents (Life Technologies, 4308329). Gene expression levels were normalized to 18S ribosomal RNA and expressed as ratios.

## **2.7. Western blotting**

Whole cell lysates were prepared from SMC cultures using 0.1 mM Tris-HCl buffer, pH 6.8, supplemented with 2% sodium dodecyl sulfate (SDS), 2  $\mu$ g/mL aprotinin, 2  $\mu$ g/mL leupeptin, 2  $\mu$ g/mL pepstatin, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Protein content of the lysates was measured by the Micro BCA assay (Pierce). Equal amounts of whole cell lysates (10-40  $\mu$ g) were heated at 95°C for 5 minutes with Laemmli sample loading buffer and allowed to cool to

room temperature before being loaded into a 1.5 mm polyacrylamide gel, which consisted of a 5% acrylamide stacking layer and a 10% acrylamide resolving layer. Proteins were separated by SDS-polyacrylamide gel electrophoresis followed by a transfer to polyvinylidene fluoride (PVDF) membranes (Perkin Elmer). Membranes were blocked with a mixture of 5-10% milk and 1% BSA in Tris-buffered saline containing 0.05% tween-20 (TBST) and probed with polyclonal rabbit anti-RAGE (Abcam, ab3611), monoclonal mouse anti-Runx2 (MBL, D130-3), and/or polyclonal rabbit anti- $\beta$ -tubulin (Abcam, ab6046). An appropriate HRP-conjugated secondary antibody and Western Blot Chemiluminescence detection reagents (Perkin Elmer) were used to collect the signal.  $\beta$ -tubulin blotting of the same membrane was used to monitor sample loading. Densitometry analysis (ImageJ) was used to determine relative expression levels, and protein expression levels were expressed as ratios to  $\beta$ -tubulin.

## **2.8. Creation of RAGE knockdown SMC line via small RNA interference**

Nineteen nucleotide target sequence (5'-GAACTGAAGCTTGGAAGGT-3') was selected from the encoding region of mouse RAGE mRNA and synthesized by MWG Biotech Inc. The annealed double stranded oligonucleotide was inserted into Hind III and Bgl II sites of pSUPER retroviral vector. Positive clones were selected with ampicillin, and clones containing 64-bp insertions were sequenced to confirm the sequence identity. The recombinant sh-RAGE-pSUPER construct was then used to transiently transfect phoenix ecotropic packaging cells (the Nolan laboratory, Stanford University, CA) via calcium-phosphate precipitation. At 48-hour post-transfection, virus-containing culture media was collected and used to infect mouse vascular SMCs at passage 2 to establish RAGE knockdown (shRAGE) stable cell line. Mock transduced SMCs were used as control cells. Successfully transduced cells were selected with 2.5  $\mu$ g/mL puromycin, and the knockdown efficiency of RAGE was assessed through qRT-PCR and Western blot analysis.

## **2.9. Endothelin-1 ELISA**

SMCs were seeded onto collagen-coated plates and induced with procalcific medium under normal or high glucose conditions. Serum-free media with 0.1% BSA were conditioned for 24 hours prior to collection and stored at -20°C until use. Cellular protein was collected into 0.2 N NaOH and quantified by the microBCA assay. A sandwich ELISA (R&D, DET100) was used to determine ET1 concentrations in conditioned media. ET1 amounts were normalized to cellular protein of respective cultures and expressed as pg ET1/mg cellular protein.

## **2.10. Statistics**

For each data set, a normality test ( $p > 0.05$ ) and an equal variance test ( $p > 0.05$ ) were performed to identify normally distributed data. If normally distributed, a t-test (comparison of 2 groups) or ANOVA (comparison of multiple groups) with appropriate post-hoc test (Fisher's, Tukey, Bonferroni, etc.) were applied. If not normally distributed, nonparametric analyses of variance were performed using Kruskal-Wallis test followed by Bonferroni correction. Pearson correlation coefficient was used to determine associations between variables. Unless otherwise reported, error bars on graphs represent one standard deviation. A p-value less than 0.05 was considered statistically significant.

## **Chapter 3: Results and discussion**

### **3.1. Aim 1: Determine whether ET1 signaling through ETA is important for osteochondrogenic differentiation and matrix calcification of vascular SMCs enhanced by high glucose conditions**

#### **3.1.1. Rationale**

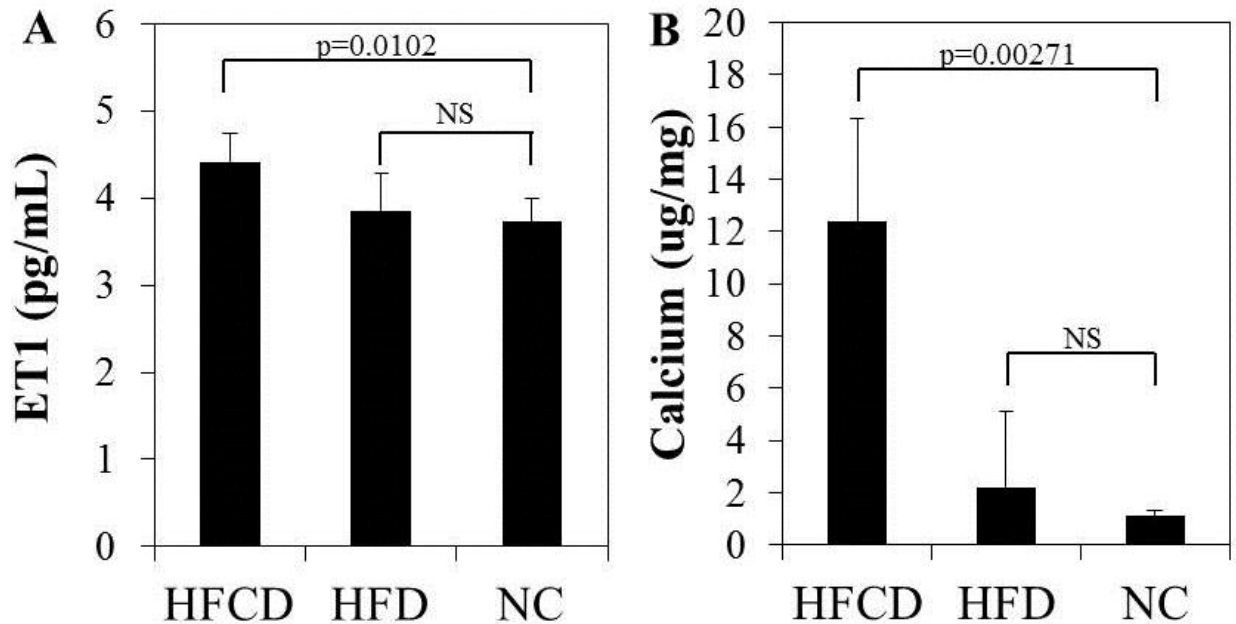
ET1, originally identified as a potent vasoconstrictor produced by vascular endothelial cells to regulate vascular tone, has now been implicated as a cause of the micro- and macro-vascular complications in patients with T2DM and atherosclerosis.<sup>65,67</sup> ET1-ETA signaling has been linked to calcification,<sup>80,84</sup> but the role of this signaling in osteochondrogenic differentiation and matrix calcification of SMCs in T2DM is unknown.

### 3.1.2. Hypothesis

We hypothesize that ET1 signaling through ETA is critical for glucose-enhanced osteochondrogenic differentiation and matrix calcification of SMCs. Thus, blocking ETA biological activity with a receptor-specific antagonist will preserve SMC phenotype and reduce glucose-associated SMC calcification.

### 3.1.3. ET1 and ETA are found in osteochondrogenic areas of diabetic blood vessels

To study whether ET1 and its receptors are involved in vascular calcification associated with T2DM, we determined serum ET1 and the immunoreactivity of ET1 and its receptors in calcifying blood vessels of LDLr<sup>-/-</sup> mice with T2DM. As shown in **Figure 1A**, ET1 was significantly higher in serum of diabetic mice that also developed vascular calcification (**Figure 1B**, HFDC).

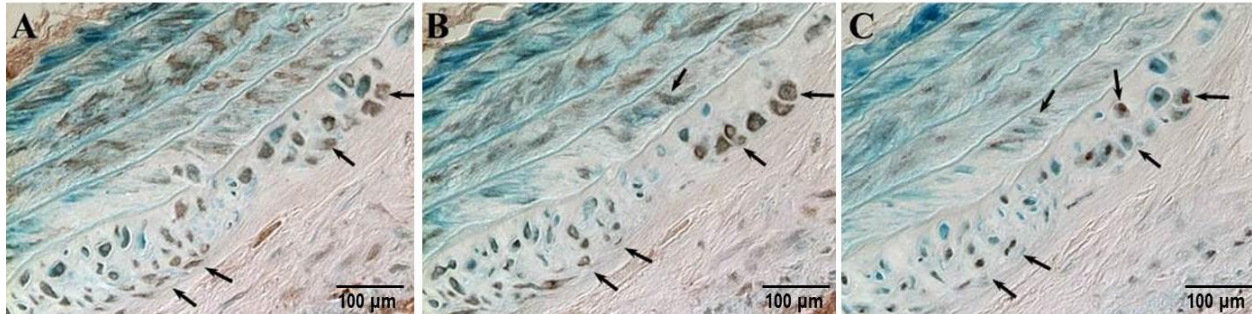


**Figure 1:** ET1 and vascular calcification in T2DM. Serum ET1 (A) and aortic calcium content (B) from LDLr<sup>-/-</sup> mice fed with HFCD, HFD, or NC. Error bars are reported as SEM.

Concordantly, in diabetic blood vessels, ET1 (**Figure 2A**, brown) was found in cells originally derived from SMCs (stained blue by X-gal), co-localizing with its type A receptor (**Figure 2B**).



Interestingly, the ET1-ETA co-localization corresponded with SMCs undergoing osteochondrogenic differentiation (**Figure 2C**, positive for Runx2, arrows).

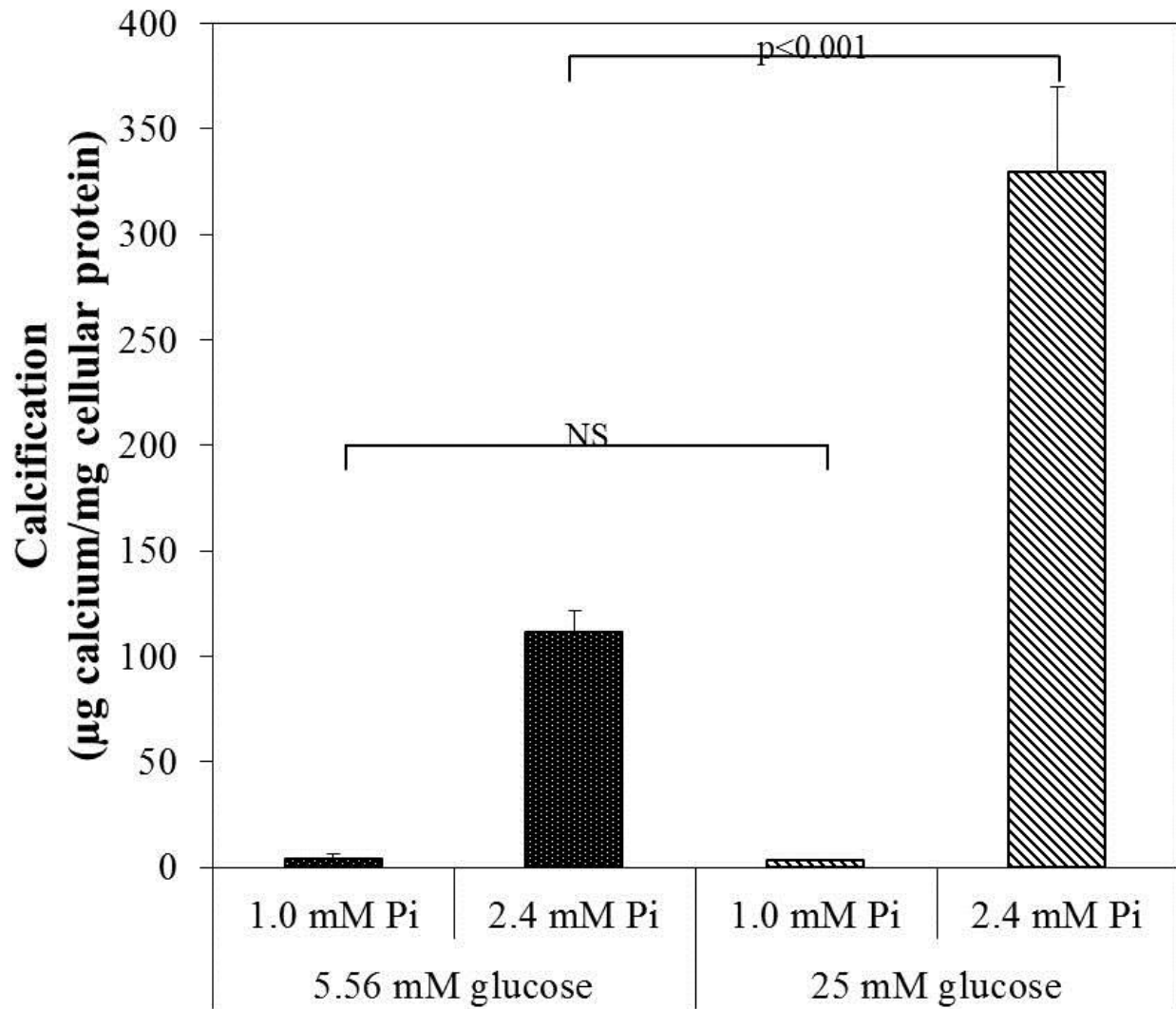


**Figure 2:** ET1-ETA signaling and osteochondrogenic differentiation of vascular SMCs in T2DM. Aortic arches of diabetic LDLr<sup>-/-</sup> mice were stained immunohistochemically (brown) for ET1 (A), ETA (B), and Runx2 (C). Blue X-gal stain designates cells originally derived from SMCs that were labeled through SM22a-Cre and R26R transgenes.

These results suggest a possible role of ET1-ETA signaling in SMC lineage reprogramming towards osteochondrogenic fate.

### 3.1.4. Exogenous ET1 enhances matrix calcification of SMCs in procalcific cultures only under high glucose conditions

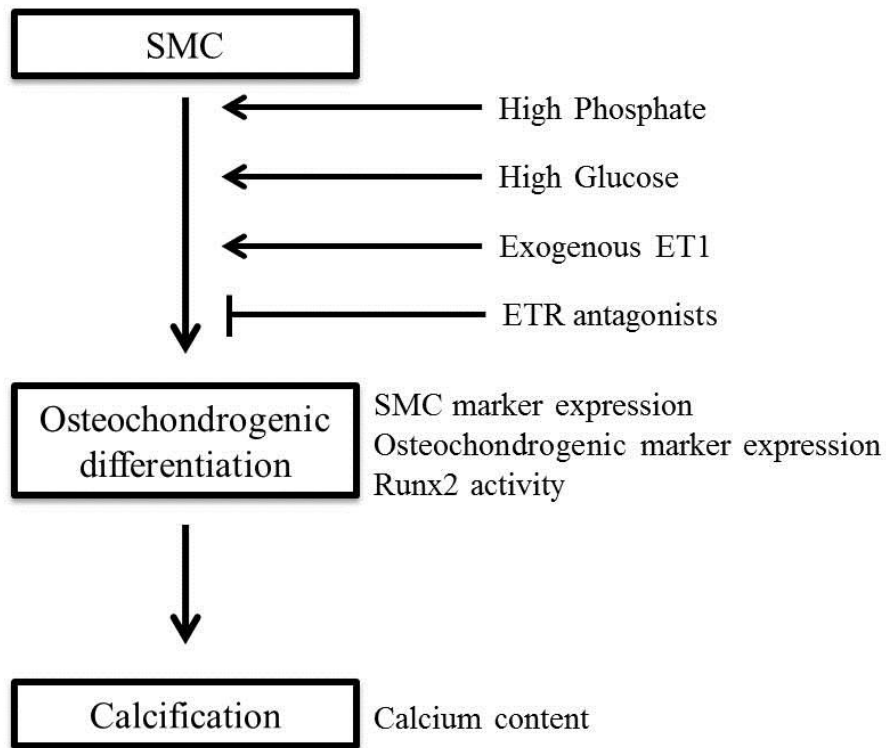
Our recent genetic fate mapping studies have identified vascular SMCs as major sources that give rise to osteochondrogenic precursors and chondrocytes observed in calcifying blood vessels of diabetic LDLr<sup>-/-</sup> mice, and hyperglycemia was likely to serve as an accelerator of the process.<sup>25</sup> To study whether and how hyperglycemia plays a role in the accelerated osteochondrogenic differentiation and calcification of vascular SMCs, we developed an *in vitro* model of SMC matrix calcification that mimics mouse serum glucose levels at normal (5.56 mM) and hyperglycemic (25 mM) ranges. As shown in **Figure 3**, we found that high glucose conditions alone did not induce SMC calcification, whereas addition of high glucose to procalcific medium containing inorganic phosphate at levels of hyperphosphatemia (2.4 mM) enhanced SMC calcification by up to 3-fold compared to cultures treated with high phosphate in normal glucose conditions.



**Figure 3:** Representative results from an *in vitro* SMC matrix calcification assay. High glucose (25 mM) alone does not induce calcification, but addition of high glucose to SMCs induced with high phosphate (2.4 mM) enhances matrix calcification measured after 6 days.

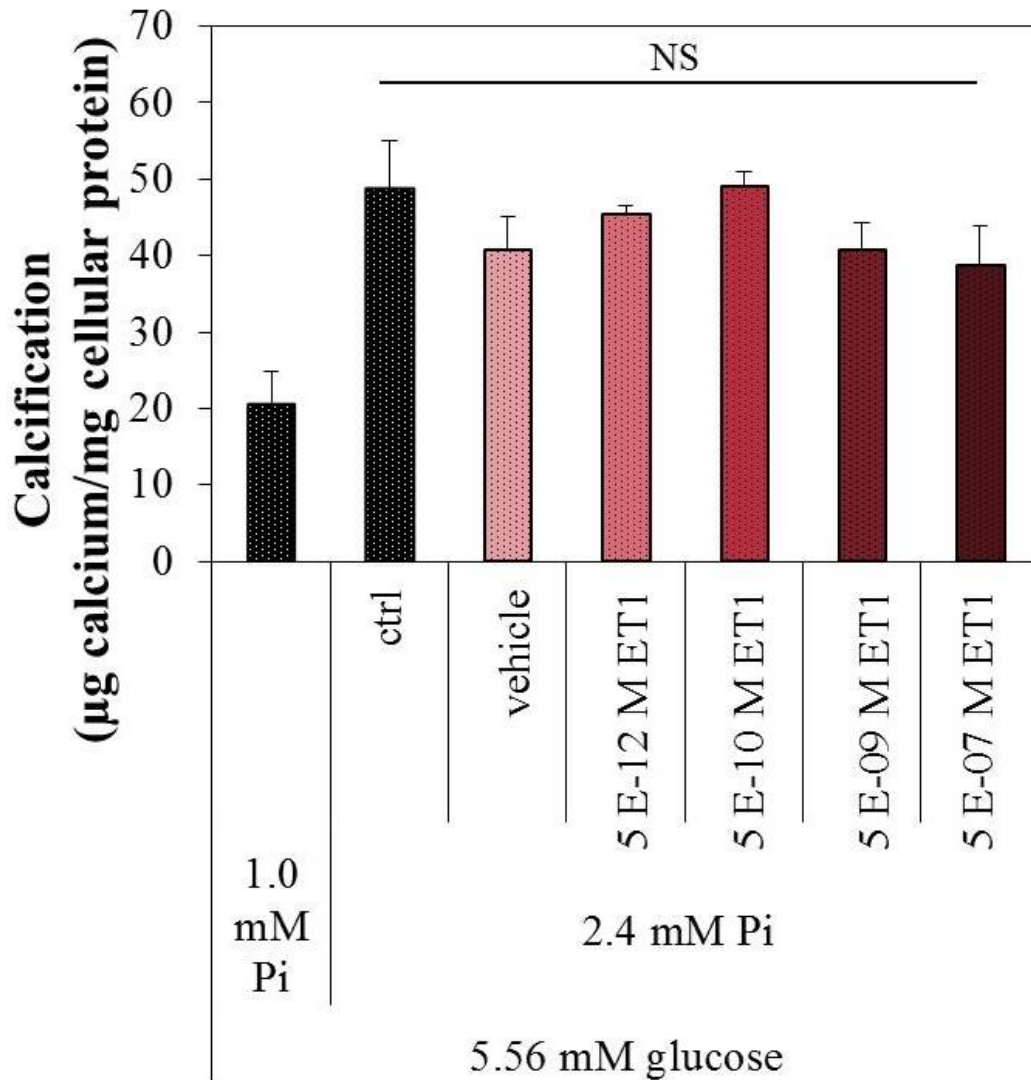
Our study with LDLr<sup>-/-</sup> mice fed with HFCD suggested that matrix calcification required an initiator. It is likely that high cholesterol induced aortic arch calcification, and hyperglycemia enhanced and accelerated it *in vivo*. In the *in vitro* system, phosphate at levels of hyperphosphatemia was required to stimulate matrix calcification, and a high concentration of glucose mimicking hyperglycemia exacerbated the extent of calcium deposition. Through controls without cells seeded on Col I-coated plates, it was found that treatment with phosphate and/or

glucose did not result in deposition of calcium, ruling out any passive mechanism of calcification in this system (data not shown). Additionally, protein levels were approximately equal across all treatments, implying that all treatment groups had approximately equal viability. This model was utilized in a series of experiments to determine the effects of ET1-ETA signaling on osteochondrogenic differentiation and matrix calcification of SMCs (**Figure 4**).



**Figure 4:** Working model of osteochondrogenic differentiation and calcification of SMCs in response to glucose, phosphate, ET1, and ETR antagonism *in vitro*.

To see the effect of ET1 on SMC matrix calcification, exogenous ET1 peptide was added to procalcific SMC cultures in both normal and high glucose conditions. As shown in **Figure 5**, addition of up to 500 nM ET1 to normal glucose cultures did not significantly alter matrix calcification of these cells.

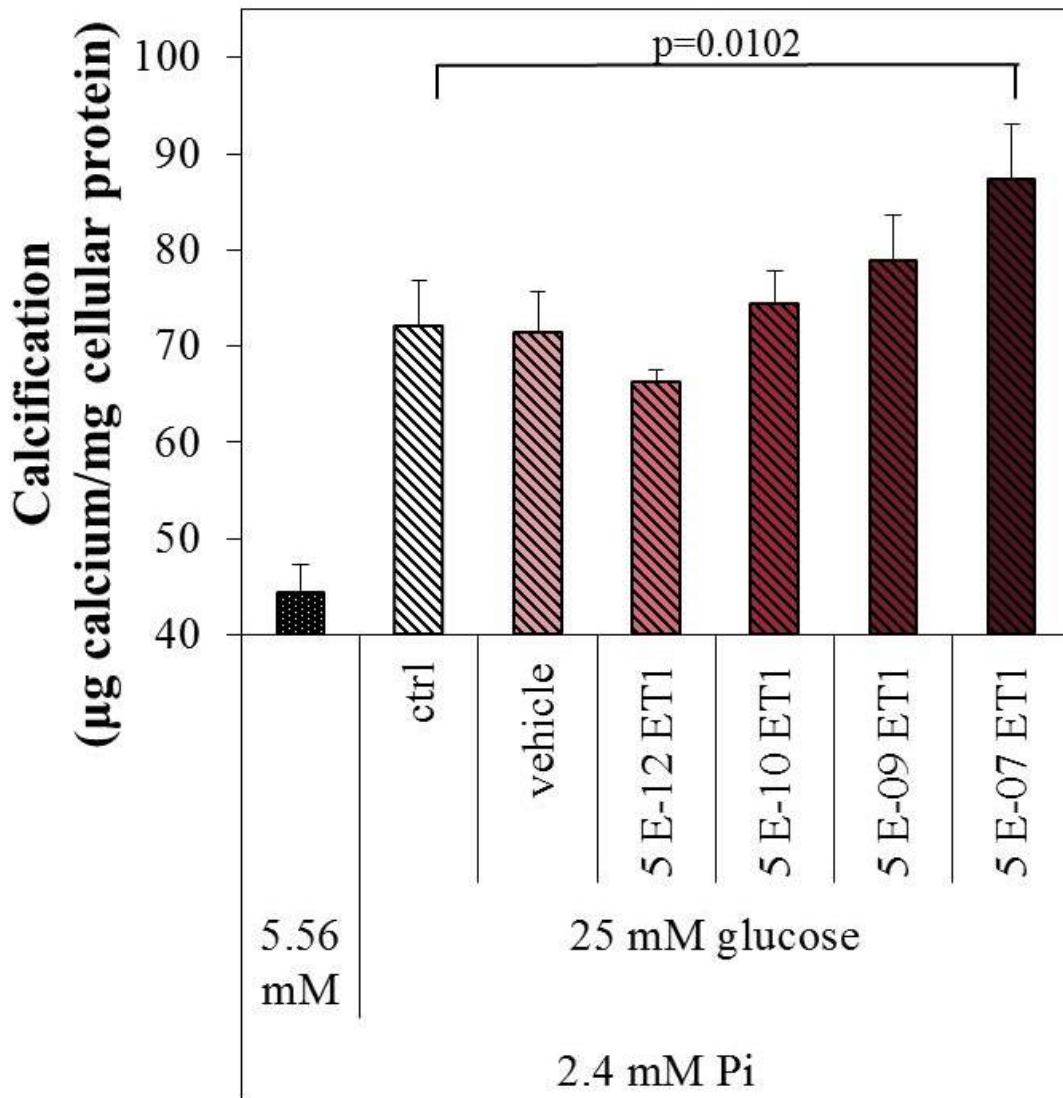


**Figure 5:** ET1 and SMC matrix calcification after 6 days in normal glucose conditions. ET1 had no significant effect on SMC matrix calcification induced by high phosphate (2.4 mM) under normal glucose (5.56 mM) conditions.

The normal physiological concentration of ET1 in mouse serum is approximately 100 pM, and this experiment tested initial doses above and below that average. However, clearance of the peptide was not monitored, so the effective dose is unknown. Additionally, the ET system is paracrine in nature, and it is likely that the local concentration of ET1 between endothelial cells and SMCs are effectively much higher than the systemic concentration *in vivo*. Thus, even though only small changes in serum ET1 levels have been noted in patients with atherosclerosis,<sup>67</sup> using

concentrations of ET1 multiple orders of magnitude above normal serum concentrations is likely clinically significant.

Interestingly, when ET1 was added to high glucose cultures, a 21% increase in total calcification was achieved when SMCs were exposed to 500 nM exogenous ET1 (**Figure 6**).



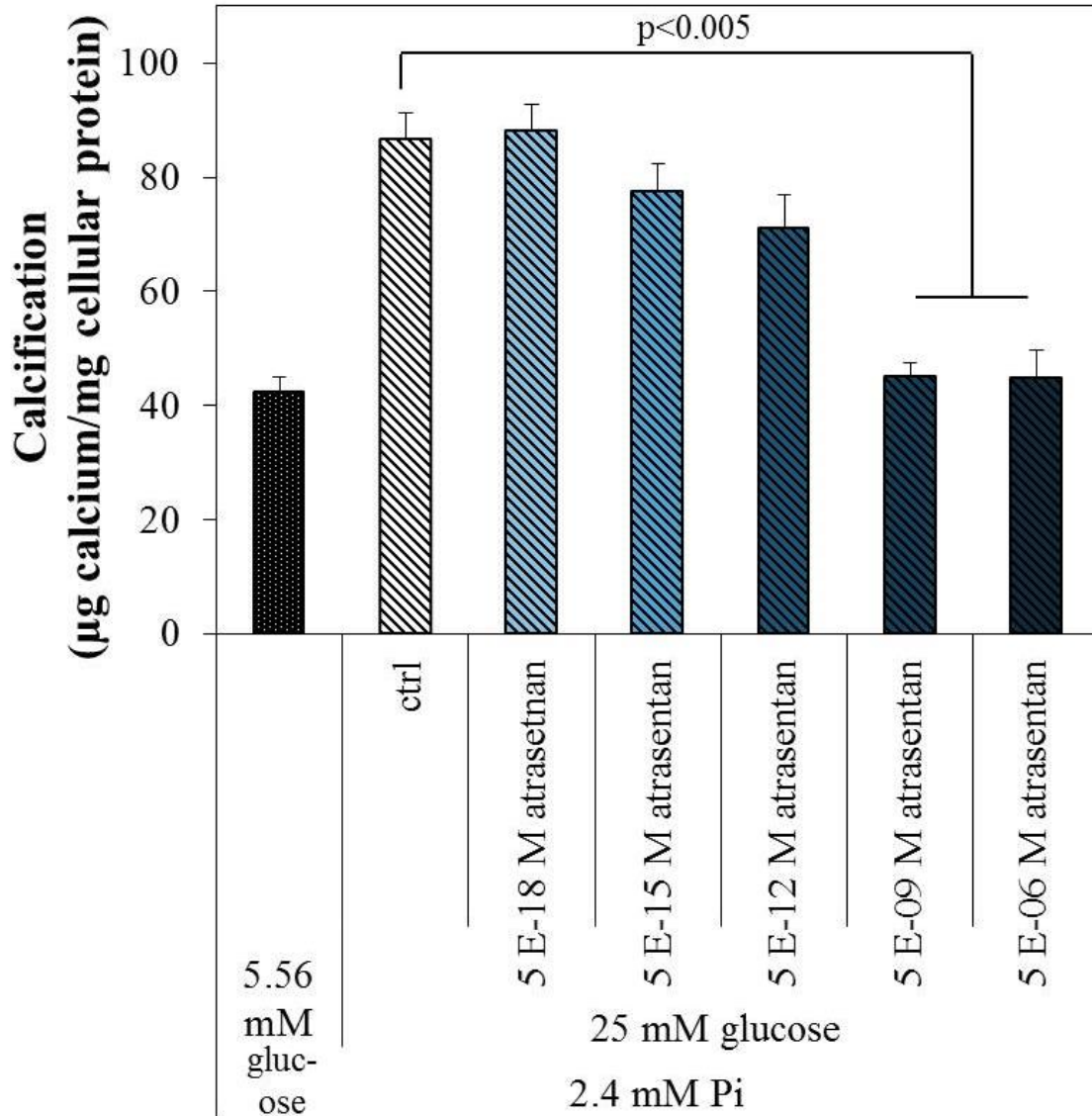
**Figure 6:** ET1 and SMC matrix calcification after 6 days in high glucose conditions. ET1 (500 nM) had a significant effect on SMC matrix calcification induced by high phosphate (2.4 mM) under high glucose (25 mM) conditions.

Thus, ET1 does have an effect on matrix calcification of SMCs, but the apparent glucose-dependent effect has yet to be explained. To investigate the signaling of ET1 and clarify a possible

glucose-dependent effect of ET1 on matrix calcification of SMCs, we first investigated signal propagation through its receptors.

### **3.1.5. Antagonism of ETA, but not ETB, substantially reduces osteochondrogenic differentiation and matrix calcification enhanced by high glucose**

ET1 signals through two G protein-coupled receptors, ETA and ETB, located on cell membrane of vascular SMCs.<sup>56</sup> To determine whether a particular receptor subtype was responsible for the ET1-enhanced SMC calcification, we utilized subtype-specific antagonists to block the signals mediated through their respective receptor. As shown in **Figure 7**, we found that antagonism of the ETA with atrasentan significantly reduced glucose-accelerated SMC calcification in a dose-dependent manner.

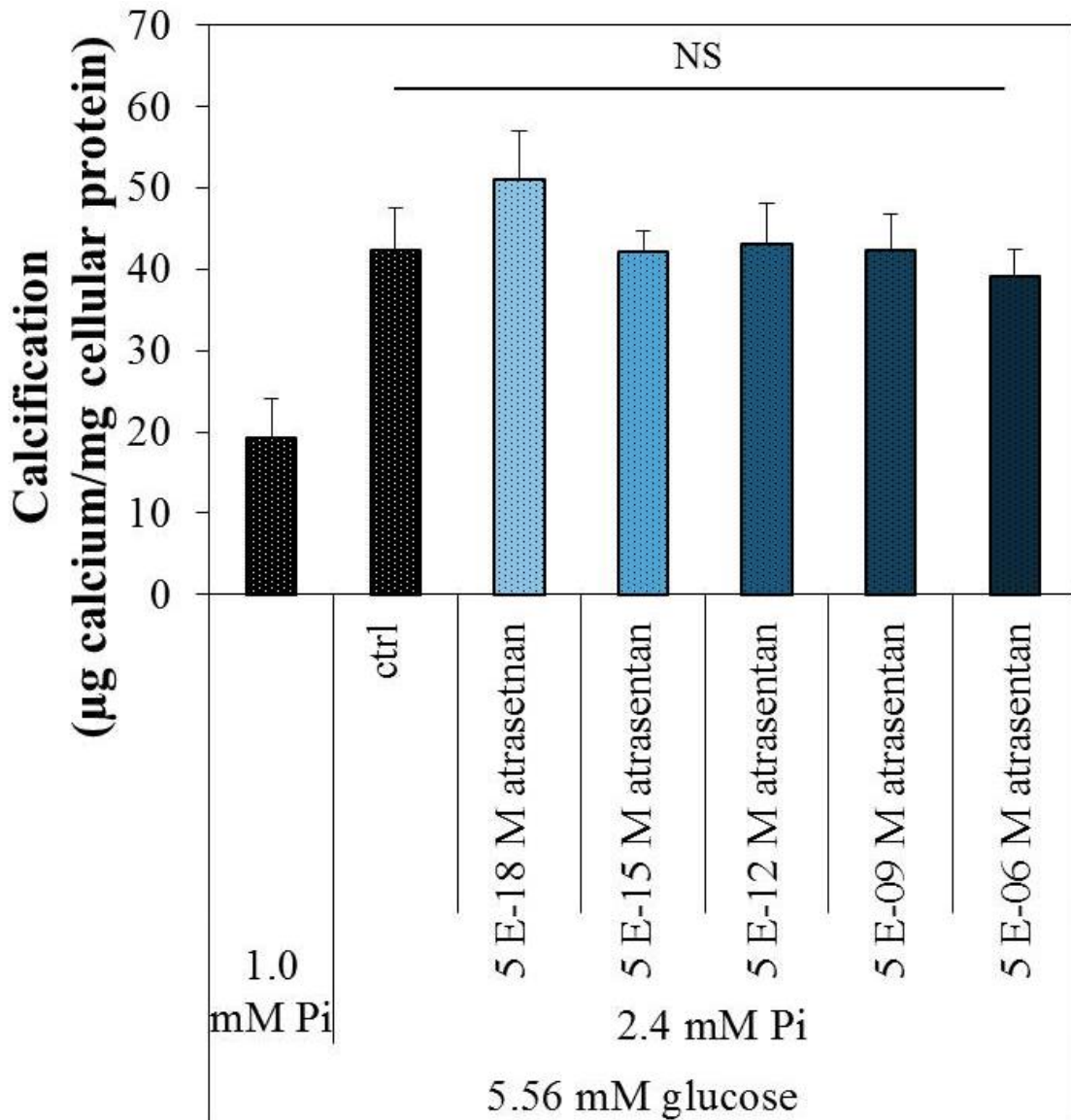


**Figure 7:** ETA antagonism and SMC matrix calcification after 6 days in high glucose conditions. Atrasentan (1 nM and 1 µM) significantly inhibited SMC calcification induced by high phosphate (2.4 mM) under high glucose (25 mM) conditions.

Up to 71% of total matrix calcification, i.e., nearly 100% of glucose-enhanced calcification was inhibited by atrasentan at a dose range of 1 nM to 1 µM. To see whether this inhibitory effect is truly glucose-dependent, higher doses of atrasentan in high glucose conditions, as well as a similar dose range in normal glucose conditions, were applied to the SMC calcification model. Higher doses of atrasentan (up to 500 µM) did not significantly inhibit calcification beyond that enhanced by high glucose (data not shown).



Additionally, atrasentan only slightly inhibited calcification in SMCs exposed to procalcific medium alone (**Figure 8**), and this result was not statistically significant. This further suggests a likelihood of the requirement of high glucose concentrations for ETA-mediated SMC calcification.

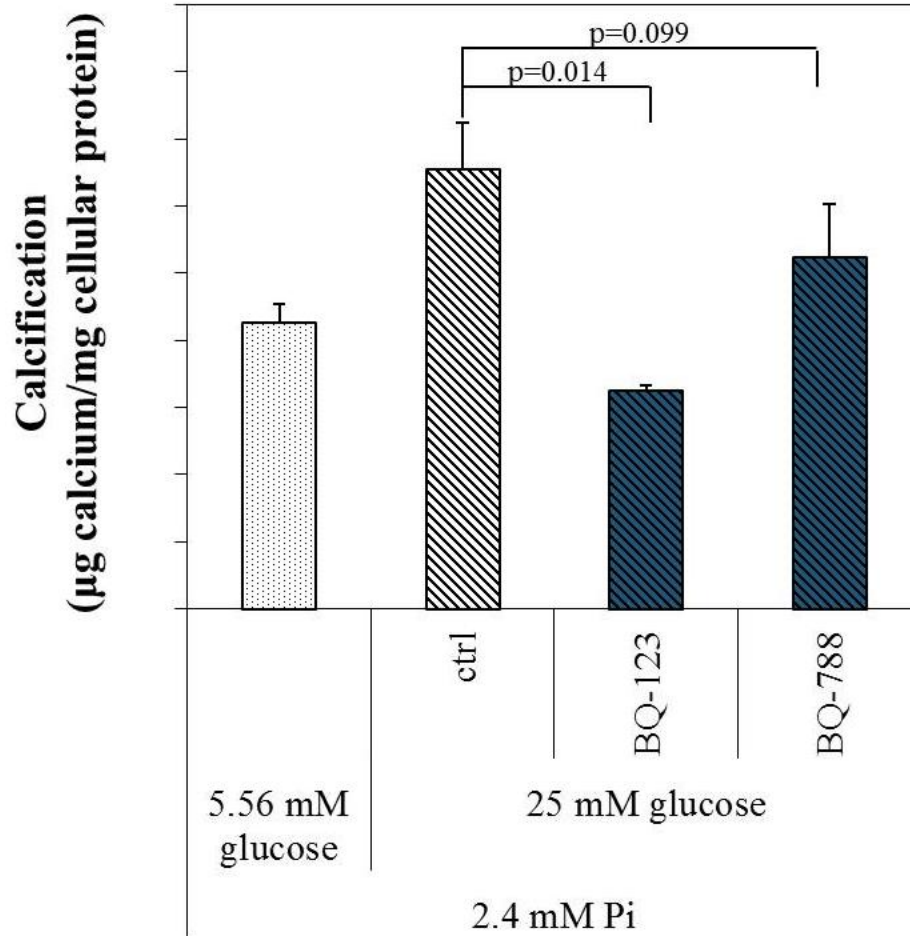


**Figure 8:** ETA antagonism and SMC matrix calcification after 6 days in normal glucose conditions. No dose of atrasentan (1 aM to 1 µM) significantly affected SMC calcification induced by high phosphate (2.4 mM) in normal glucose conditions (5.56 mM).



This result suggests that pathogenic effects of signaling through ETA are dependent upon high glucose conditions. High glucose may have resulted in a greater amount ETA on the cell membrane to transduce pathogenic ET1 signaling, but it is likely that related signaling pathways are triggered in high glucose conditions to enhance ET1-ETA signals.

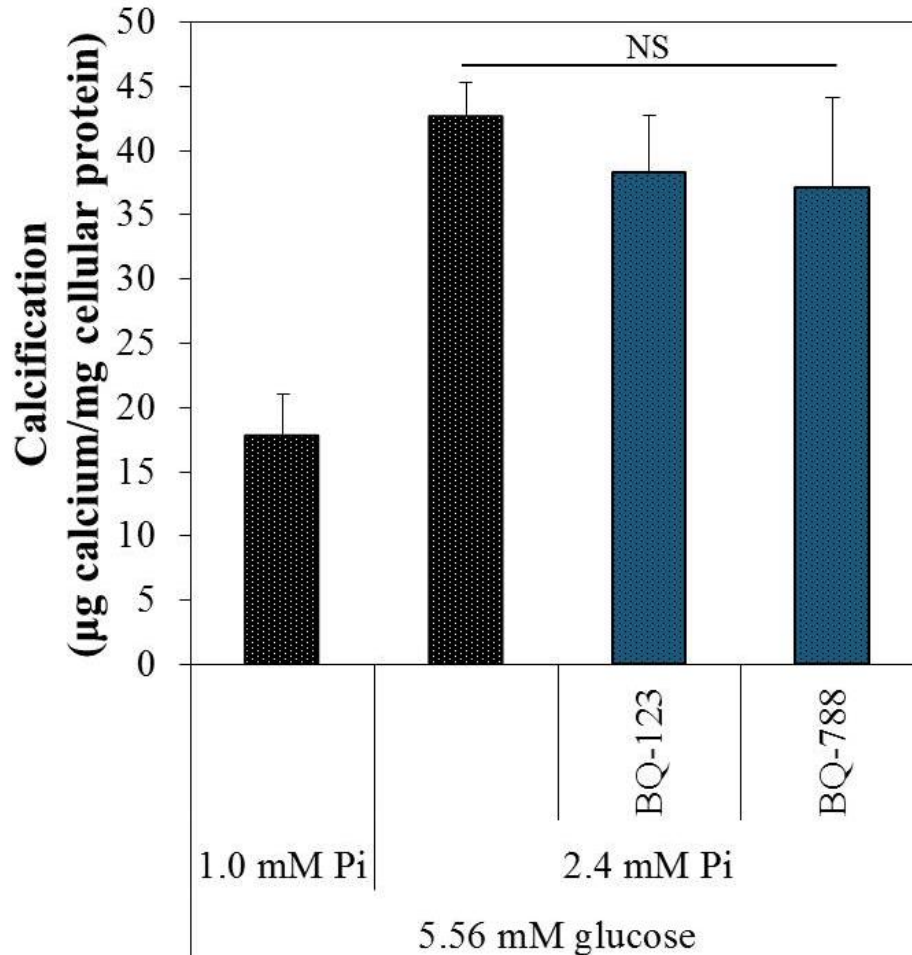
To compare the effects of ETA and ETB signaling pathways on the pathogenic effects of ET1 described in **3.1.4.**, we blocked signaling through each of the two receptors expressed on SMCs, ETA and ETB, with their respective antagonists. BQ-123, an ETA antagonist, and BQ-788, an ETB antagonist, have equal  $IC_{50}$  values and affinities for their respective receptors. As shown in **Figure 9**, 1  $\mu$ M BQ-123 efficiently reduced SMC calcification by 49%, whereas application of BQ-788 at the same dosage resulted in no significant reduction in glucose-enhanced SMC calcification.



**Figure 9:** The effect of ETA and ETB antagonism on SMC matrix calcification after 6 days in high glucose conditions. ETA antagonist BQ-123 (1 µM) significantly inhibited SMC calcification, and ETB antagonist BQ-788 (1 µM) only slightly inhibited calcification induced by high phosphate (2.4 mM) under high glucose (25 mM) conditions. Dual antagonism resulted in the greatest inhibition of calcification.

These results demonstrate that signaling through ETA is essential for SMC calcification exacerbated by high glucose conditions *in vitro*. The relative effectiveness of ETA and ETB antagonism is most likely due to dissimilar signaling pathways following the two receptors.

As seen with atrasentan, these antagonists had little inhibitory effect, if any, on matrix calcification of SMCs cultured in procalcific medium in normal glucose conditions (**Figure 10**).

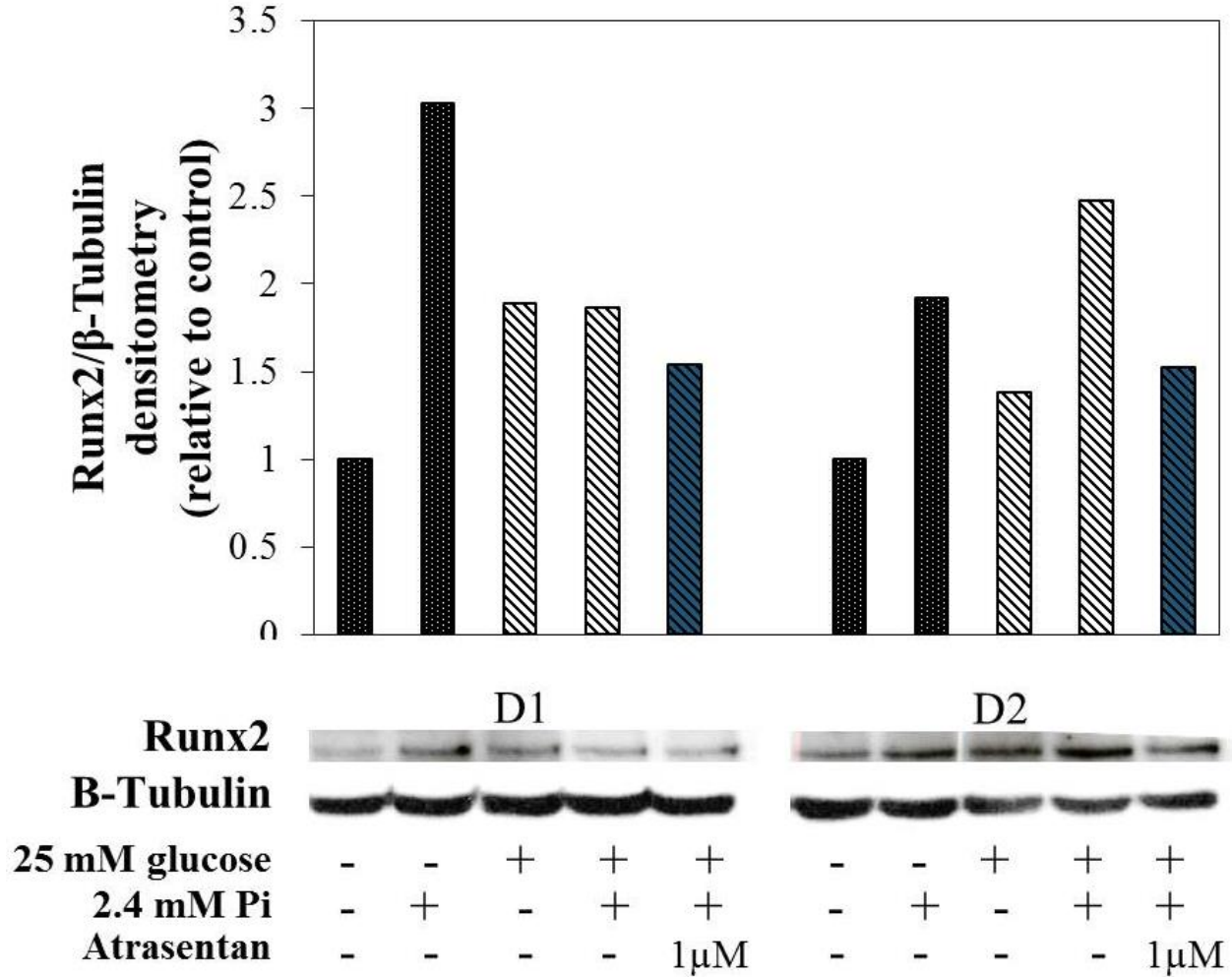


**Figure 10:** The effect of ETA and ETB antagonism on SMC matrix calcification after 6 days in normal glucose conditions. No combination of ETR antagonists significantly affected SMC calcification induced by high phosphate (2.4 mM) under normal glucose (5.56 mM) conditions.

Although ETR antagonism had a slight effect on SMC matrix calcification in normal glucose conditions, this effect was not significant. This result recapitulates the idea that pathogenic effects of signaling through ETRs may be dependent on high glucose conditions.

Further investigation into ETA signaling showed that ETA antagonism protects SMCs from lineage reprogramming towards osteochondrogenic fate. As shown in **Figure 11**, Runx2, a transcription factor critical for bone and cartilage formation,<sup>87,88</sup> was transiently increased at earlier time points, provoked by procalcific medium at day 1 and enhanced by high glucose concentrations at day 2, a result that recapitulates the expression pattern of Runx2 in calcifying blood vessels.<sup>33,34</sup>

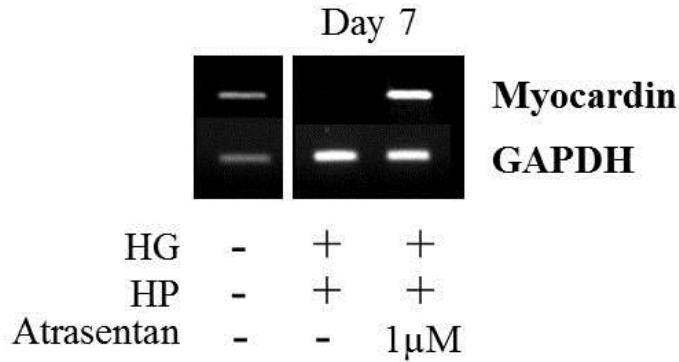
Treating SMCs exposed to high glucose in procalcific medium with atrasentan (1  $\mu$ M) resulted in a substantial reduction of Runx2 protein at later time points.



**Figure 11:** The effect of ETA antagonism on Runx2 protein expression. MC3T3 lysates served as a positive control for Runx2 (~75 kDa). Runx2 expression was upregulated in response to high glucose (25 mM) and high phosphate (2.4 mM), and expression was lower in groups treated with atrasentan (1  $\mu$ M).

This result suggests that signaling through ETA in procalcific, hyperglycemic conditions plays a role in upregulation of Runx2 expression, and reaffirms the role of ETA signaling in matrix calcification in high glucose conditions.

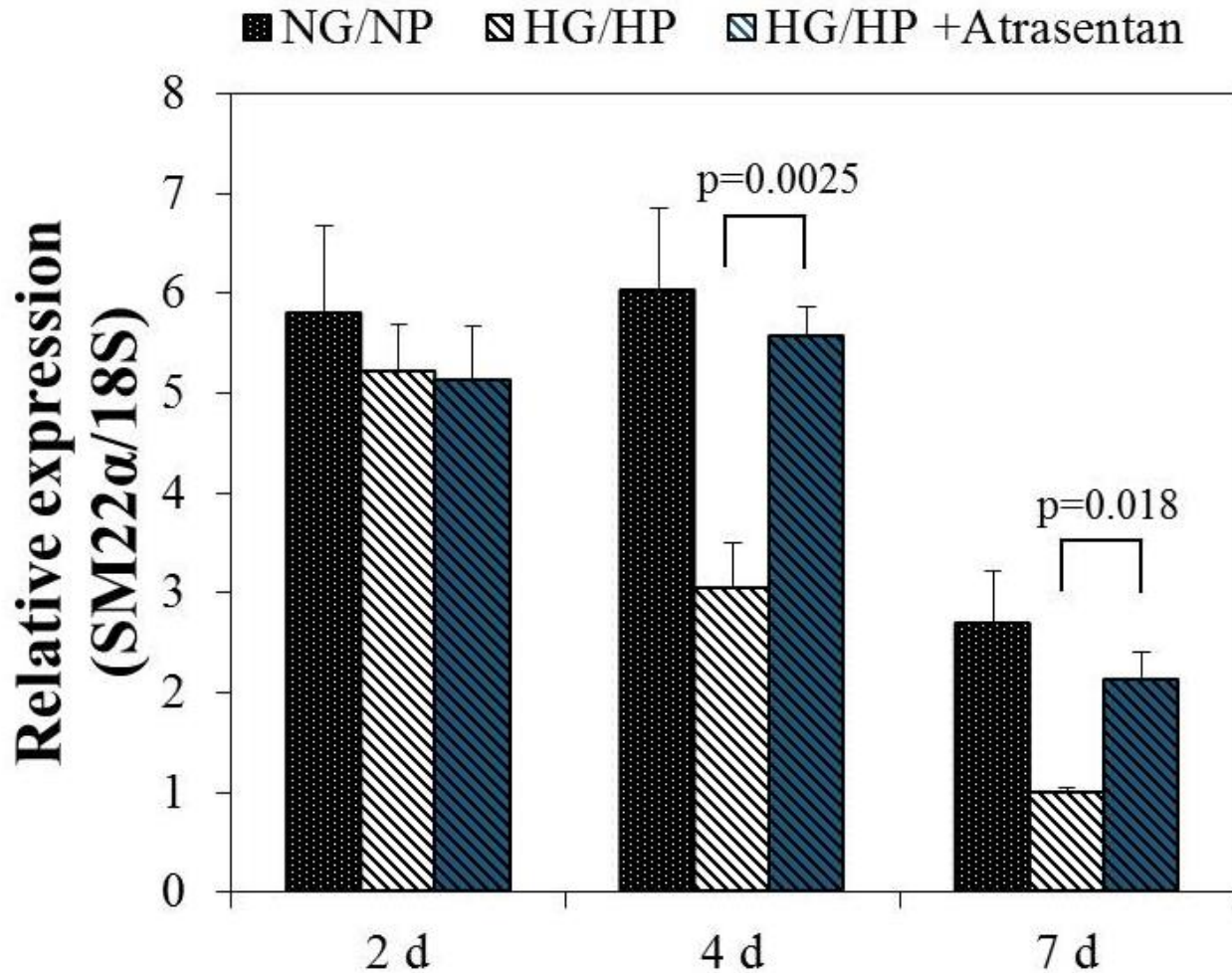
Likewise, atrasentan (1  $\mu$ M) rescued SMCs from losing their marker proteins in procalcific conditions with high glucose, as determined by mRNA expression of myocardin, a master transcription co-activator required for SMC development (**Figure 12**).



**Figure 12:** The effect of ETA antagonism on myocardin mRNA expression. Myocardin expression was downregulated in response to high phosphate (HP; 2.4 mM) and high glucose (HG; 25 mM), and expression was preserved in groups treated with atrasentan (1  $\mu$ M).

Myocardin expression is slightly downregulated in procalcific cultures at day 7, and addition of high glucose to these cultures further decreased myocardin mRNA expression. However, ETA antagonism preserved myocardin expression.

To recapitulate this idea of SMC marker gene preservation, qRT-PCR was used to quantify the expression of a downstream target gene of myocardin, SM22 $\alpha$  (**Figure 13**).



**Figure 13:** The effect of ETA antagonism on SM22 $\alpha$  mRNA expression. SM22 $\alpha$  expression was downregulated in response to high phosphate (HP; 2.4 mM) and high glucose (HG; 25 mM), and expression was preserved in groups treated with atrasentan (1  $\mu$ M).

Again, procalcific conditions with high glucose reduced SM22 $\alpha$  expression, and ETA antagonism restored SM22 $\alpha$  mRNA expression to levels observed in normal culture conditions.

These results indicate that ETA antagonism prohibits SMC phenotypic switch toward an osteochondrogenic state under procalcific high glucose conditions, but the mechanisms by which this occurs are currently unclear. It is possible that ETA antagonism inhibits activation of kinase pathways that activate Runx2 transcriptional activity, such as Erk1/2. ETA antagonism may also partially block activation of other cell-stress-related pathways, such as NF $\kappa$ B, that trigger osteochondrogenic marker gene expression.

### **3.1.6. Conclusions**

In this aim, we investigated the role of ET1-ETA signaling in osteochondrogenic differentiation and matrix calcification of SMCs under procalcific, hyperglycemic conditions. Our preliminary findings in calcifying vascular SMCs under high glucose conditions suggest that lineage reprogramming of SMCs toward an osteochondrogenic state is the initial step of vascular calcification. Hyperglycemia is likely critical in accelerating this process, thereby advancing matrix calcification of blood vessels. Our studies identified ET1 signaling through ETA as a mediator of osteochondrogenic differentiation and calcification of SMCs under high glucose concentrations in procalcific conditions. However, the signaling pathways that link ET1-ETA to osteochondrogenic marker gene expression and the requirement for high glucose levels to observe the effects of ET1-ETA on SMC matrix calcification have yet to be explained.

Unfortunately, due to the lack of a reasonable method to detect differences in ETA signaling activity, we were unable to determine whether ETA signaling levels varied across our experimental groups, particularly in procalcific conditions with high glucose. RT-PCR revealed no differences in ETA expression at the mRNA level, and attempts to determine ETA at protein level through western blotting analysis were hindered by the lack of a specific antibody. High ETA expression levels and/or enhanced signaling activity in high glucose conditions could provide clarity for the glucose-dependence of ET1-enhanced calcification and calcification inhibited by ETA antagonism. To investigate other possible explanations for the apparent requirement of high glucose for ET1- and ETA-related effects on SMC calcification, we looked to a known hyperglycemia-associated pathway: RAGE signaling.

## **3.2. Aim 2: Determine whether signaling through RAGE is required for ET1-ETA-associated SMC calcification**

### **3.2.1. Rationale**

We observed a glucose-dependent effect of ET1-ETA signaling on osteochondrogenic differentiation and matrix calcification of SMCs *in vitro*. RAGE is a member of the IgG superfamily and is a pattern recognition receptor for AGEs and calgranulins.<sup>42,43</sup> Activation of RAGE has been implicated in a wide variety of diabetic complications, including vascular calcification.<sup>50,51</sup> Because RAGE and ET1-ETA signals are involved in T2DM-related vascular calcification, it is possible that these pathways are interconnected in hyperglycemia-enhanced osteochondrogenic differentiation and matrix calcification of SMCs.

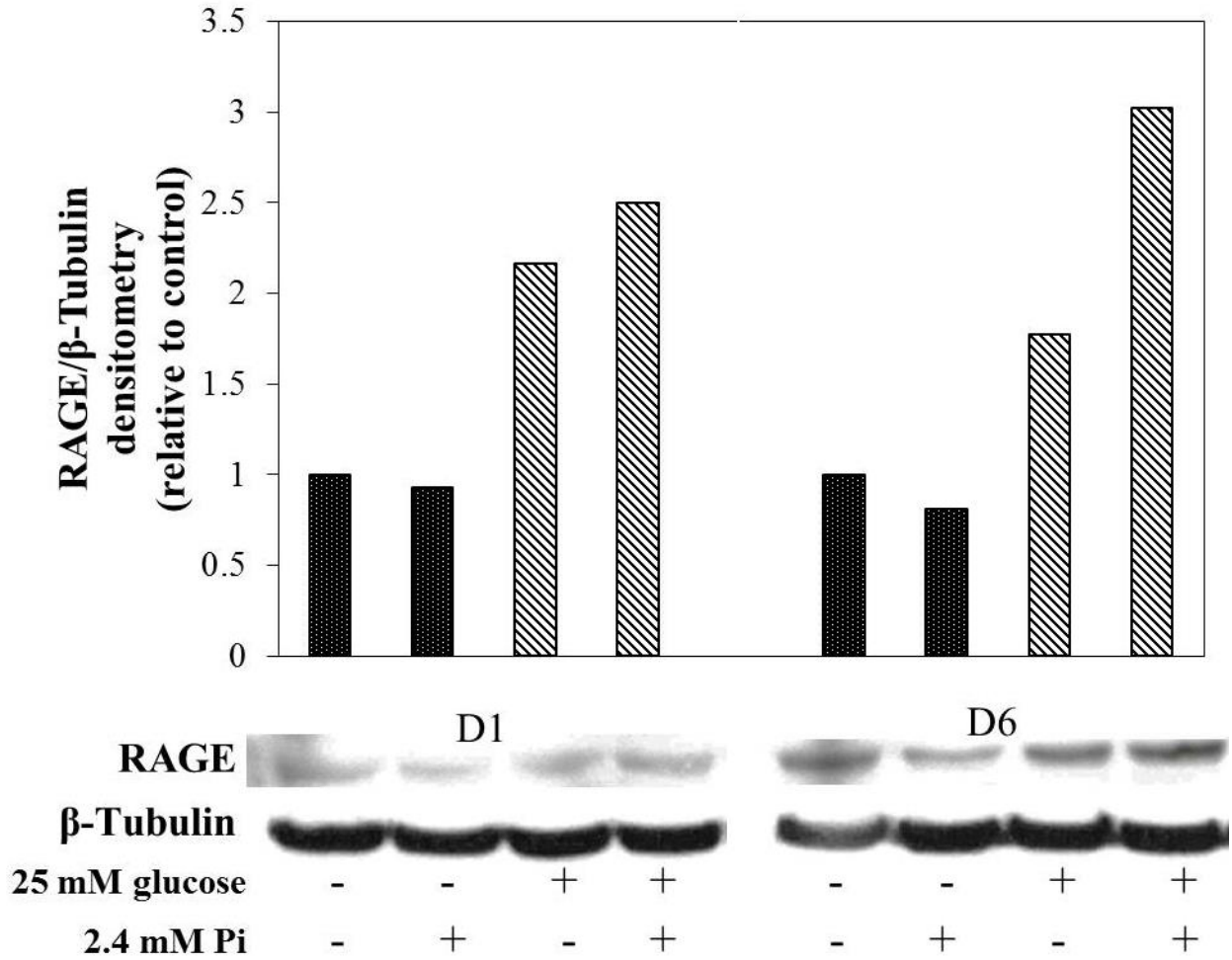
### **3.2.2. Hypothesis**

We hypothesize that signaling through RAGE is intertwined with ET1-ETA signaling via mutual downstream activation of Runx2 in high glucose conditions.

### **3.2.3. Loss of RAGE inhibits SMC osteochondrogenic differentiation and calcification to a similar extent as ETA antagonism**

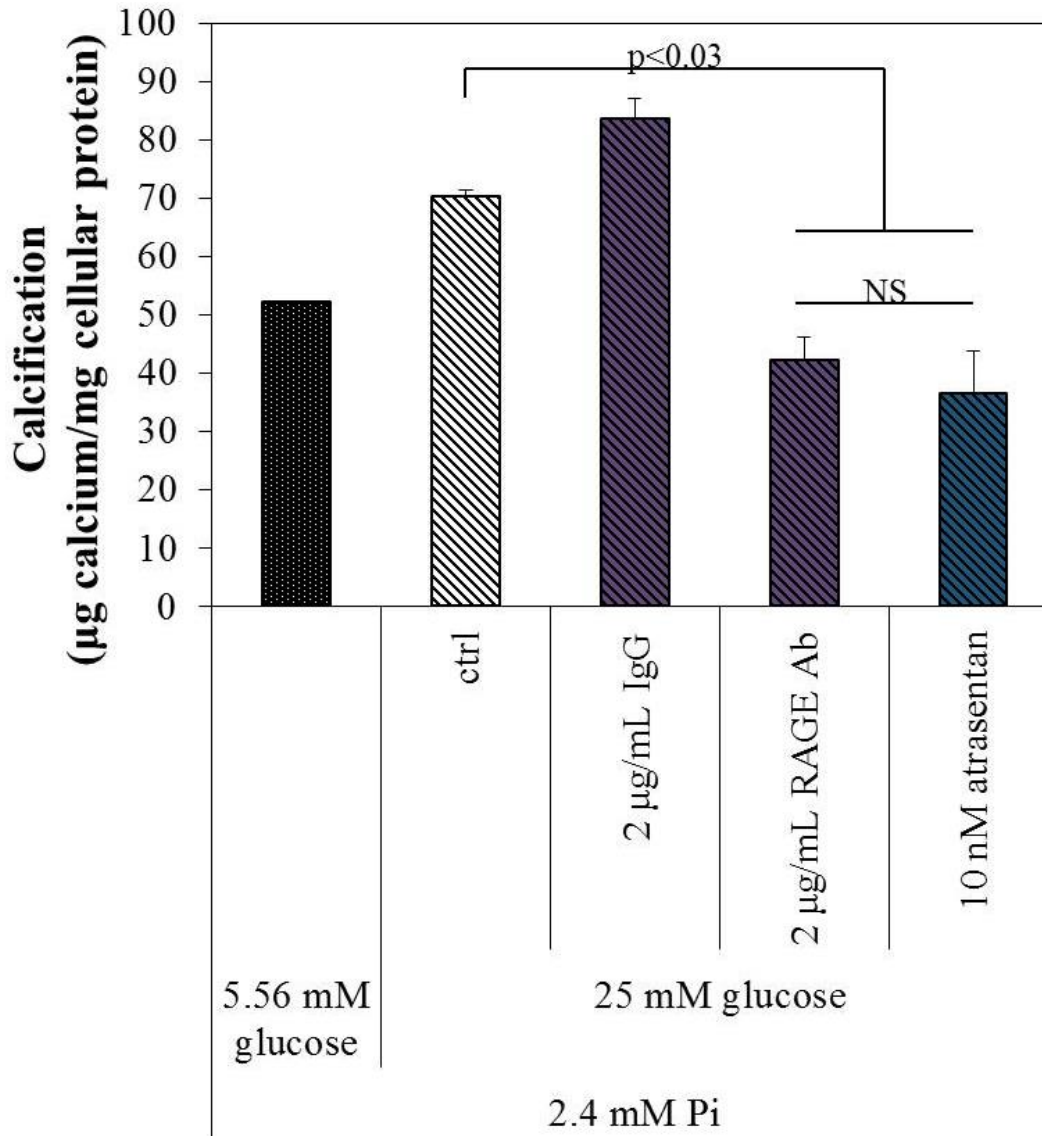
RAGE was found in SMCs within cartilaginous areas of calcifying blood vessels, co-localizing with osteochondrogenic and chondrocytic transcription factors, Runx2 and Sox9.<sup>25</sup> RAGE expression has been shown to be activated by high levels of AGEs and oxidative stress,<sup>45</sup> both of which have been shown to play a role in SMC calcification.<sup>51,89</sup> We sought to determine whether RAGE is required for SMC osteochondrogenic differentiation and calcification associated with high glucose conditions in our system. We found that RAGE was upregulated at days 1 and 6 in SMCs treated with procalcific conditions and high glucose concentrations (**Figure 14**).





**Figure 14:** RAGE expression levels in SMCs in procalcific conditions. RAGE (~45 kDa) is upregulated in response to procalcific conditions (2.4 mM Pi) with high glucose (25 mM).

To determine whether RAGE affects the calcification seen in procalcific conditions with high glucose, biological activity of RAGE was blocked with a neutralizing antibody. Interestingly, inactivation of the RAGE ameliorated matrix calcification enhanced by high glucose conditions to a similar extent as ETA antagonism with atrasentan (**Figure 15**).



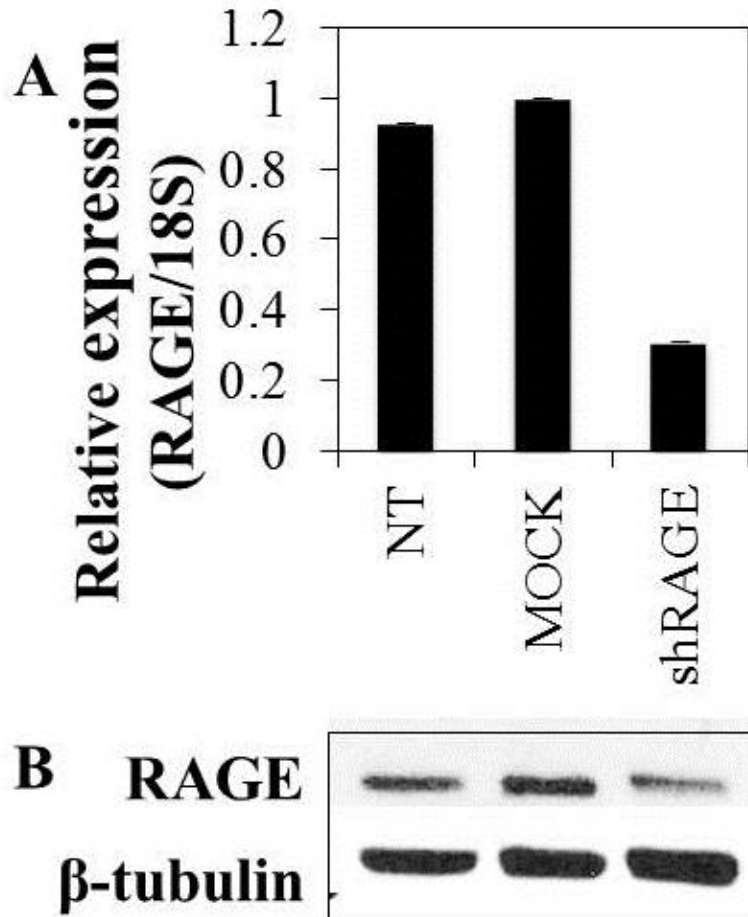
**Figure 15:** RAGE and ETA in SMC calcification after 6 days under high glucose conditions. Both ETA antagonism (10 nM atrasentan) and neutralization of RAGE (2 µg/mL RAGE antibody) reduced SMC matrix calcification induced by high phosphate (2.4 mM) under high glucose (25 mM) conditions.

Because independently blocking each pathway nearly blocked all glucose-associated calcification, it is likely that both pathways are required for matrix calcification in high glucose conditions.

To further study whether loss of RAGE expression is sufficient to inhibit SMC calcification associated with high glucose conditions, we knocked down RAGE expression through application of small hairpin RNA (shRNA). Introduction of synthetic 21- to 22-nucleotide double-stranded

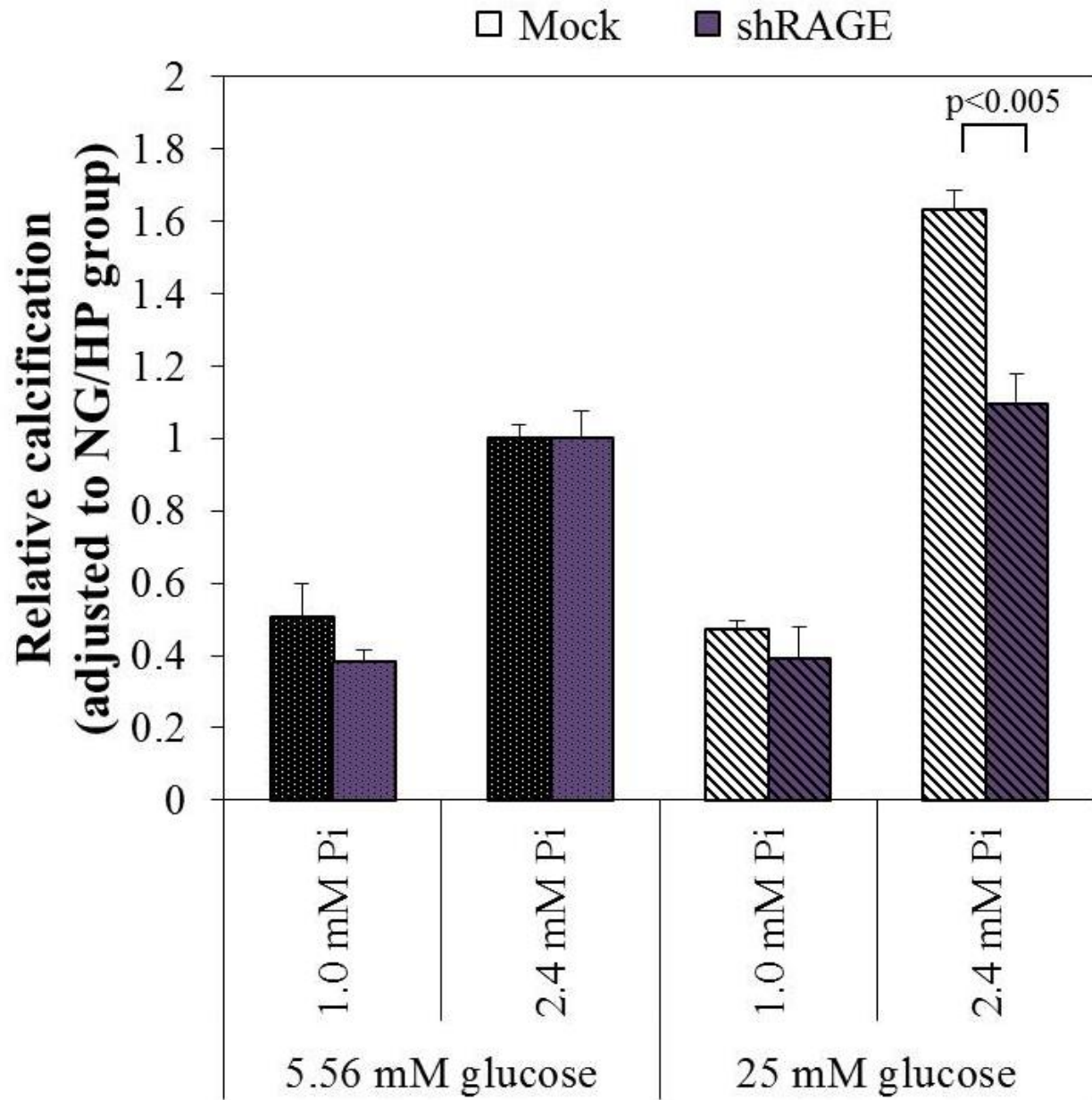
RNA has proven to be a powerful tool to suppress specific gene expression through a process known as RNA interference (RNAi). To overcome the limitation of transient interference, polymerase-III H1-RNA gene promoter-driven transcription of specific shRNAs via the pSUPER RNAi system was utilized to maintain RNAi, allowing for the analysis of loss-of-function phenotypes that develop over longer periods of time. An ecotropic Phoenix helper-free retrovirus producer line, a cell line that is easily transfected through a calcium phosphate-mediated method, was used to package the virus that was used to stably transduce SMCs with a construct that produced RAGE-specific shRNA.

RAGE mRNA levels were quantified with qRT-PCR, and protein levels were measured with western blot. RAGE mRNA level in the knockdown SMCs (shRAGE) was reduced by 70% compared to non-transduced (NT) and mock transduced SMCs (**Figure 16A**), and a 50% loss of RAGE protein was found in the shRAGE cells (**Figure 16B**).



**Figure 16:** RAGE knockdown. Quantitative RT-PCR data show the shRNA knockdown of RAGE expression at the mRNA level (A), and western blot shows knockdown at the protein level (B).

Finally, we determined whether knockdown of the RAGE gene expression would alter the susceptibility of SMCs to matrix calcification. RAGE knockdown and mock SMC lines were cultured in procalcific medium under normal or high glucose conditions. As shown in **Figure 17**, knockdown of RAGE expression had no effect on matrix calcification of SMCs treated with procalcific medium with normal glucose. However, similar to ETA antagonism, RAGE knockdown resulted in a complete inhibition of matrix calcification enhanced by high glucose in these cells.

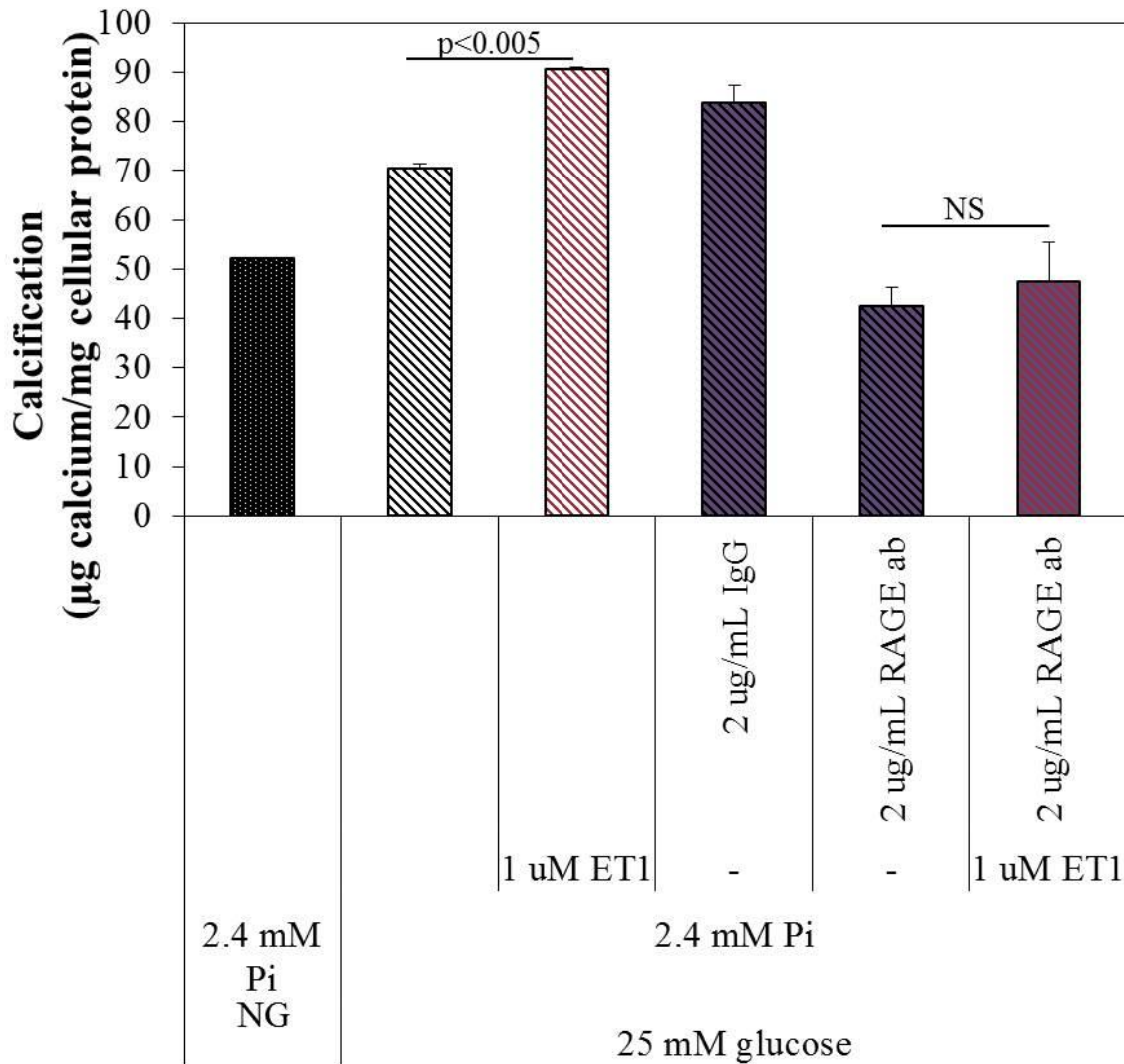


**Figure 17:** RAGE and SMC matrix calcification after 6 days under high glucose conditions. Loss of RAGE (shRAGE) ablated the effect of high glucose (25 mM) on matrix calcification in procalcific conditions (2.4 mM Pi). Calcification ( $\mu\text{g}$  calcium/mg cellular protein) for shRAGE and mock lines were normalized to samples treated with 5.56 mM glucose (NG) and 2.4 mM phosphate (HP).

The inhibition of glucose-associated calcification in shRAGE cells reaffirms the loss of RAGE signaling, and solidified these cell lines as an effective tool for detecting RAGE-related signaling in this model of SMC calcification.

### 3.2.4. RAGE is required for ET1-related calcification under high glucose conditions

In Aim 1, we showed that adding exogenous ET1 to high glucose cultures enhanced calcification. To determine whether this effect is dependent upon RAGE signaling, we added ET1 in cultures along with a RAGE neutralizing antibody and quantified its effect on ET1-induced calcification. We found that blocking RAGE signaling ameliorated not only SMC calcification enhanced by high glucose but also calcification enhanced by exogenous ET1 (**Figure 18**).

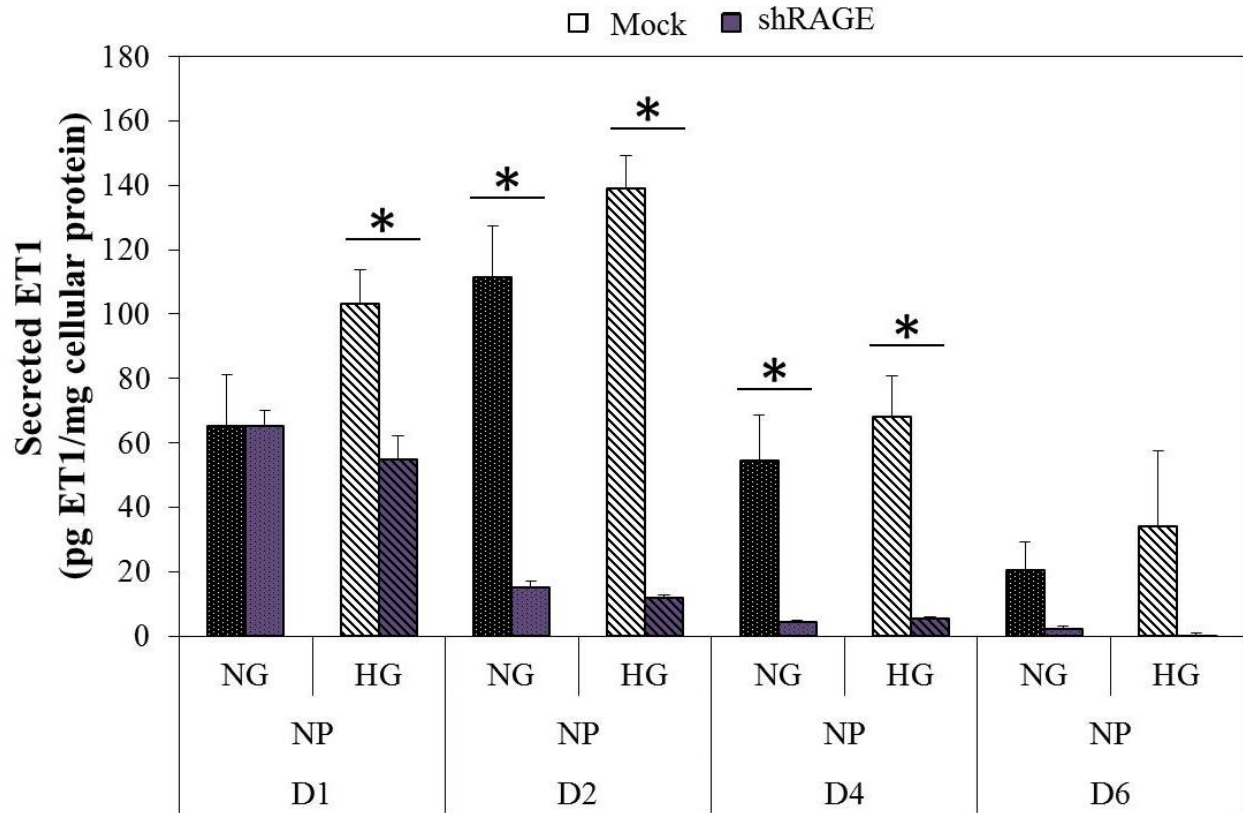


**Figure 18:** ET1-related matrix calcification of SMCs after 6 days with neutralized RAGE. Neutralization of RAGE (2 µg/mL RAGE ab) negated the aforementioned effect of ET1 (1 µM) on calcification induced by high phosphate (2.4 mM) in high glucose (25 mM) conditions.

These results indicate that RAGE is not only essential to high glucose-enhanced calcification but also ET1-mediated SMC calcification in high glucose conditions. Because RAGE expression is upregulated in procalcific cultures under high glucose conditions, it is likely that RAGE signaling is linked to the glucose-dependent nature of ET1-ETA-associated matrix calcification. It is possible that RAGE signaling affects ET1-ETA, or vice versa, and/or both pathways act on a mutual downstream component linked to calcification.

### **3.2.5. RAGE is not required for ET1 production in procalcific SMC cultures**

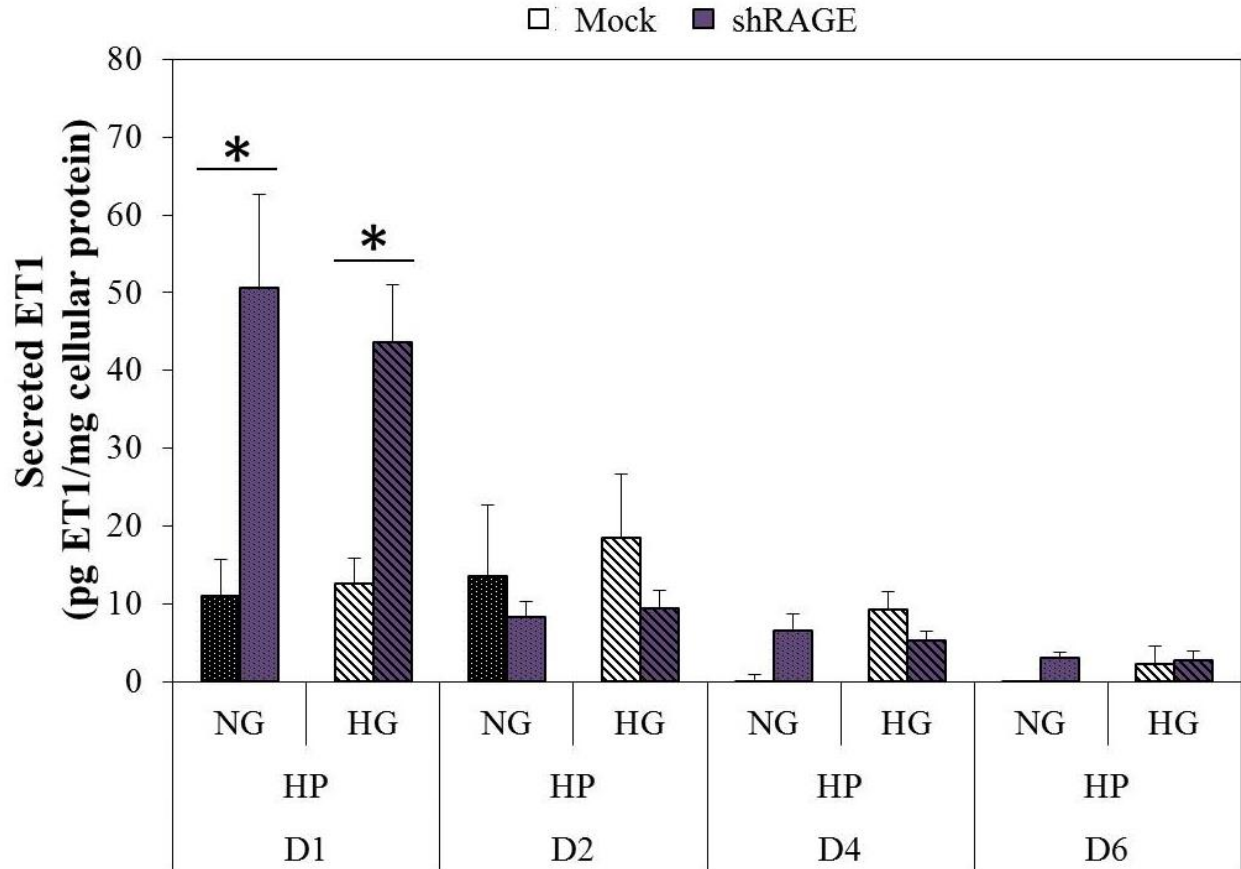
Our findings showed a requirement of ETA and RAGE signaling for SMC osteochondrogenic differentiation and calcification enhanced by high glucose conditions. To determine whether ET1 is downstream of RAGE, secreted upon RAGE activation through high glucose, we determined ET1 concentration in culture media of shRAGE and mock SMCs treated with normal or high glucose, and with or without high phosphate. As shown in **Figure 19**, under normal phosphate conditions, wild type SMCs secreted more ET1 over a 24 hour period than RAGE knockdown cells. ET1 was secreted from shRAGE cells, albeit at lower levels.



**Figure 19:** RAGE-dependent ET1 secretion from SMCs under normal phosphate (NP; 1.0 mM) conditions, with normal (NG; 5.56 mM) or high (HG; 25 mM) glucose conditions. RAGE may be involved in, but not required for, the maintenance of ET1 levels in normal culture conditions. \* =  $p < 0.05$  between mock and shRAGE groups.

Interestingly, in cultures treated with high phosphate, RAGE knockdown SMCs secreted more ET1 than their wild type counterparts cells during the first day of induction, suggesting that RAGE is not required for ET1 production under procalcific conditions (**Figure 20**).





**Figure 20:** RAGE-dependent ET1 secretion from SMCs under high phosphate (HP; 2.4 mM) conditions, with normal (NG; 5.56 mM) or high (HG; 25 mM) glucose conditions. RAGE is not required for ET1 secretion in procalcific conditions. \* =  $p < 0.05$  between mock and shRAGE groups.

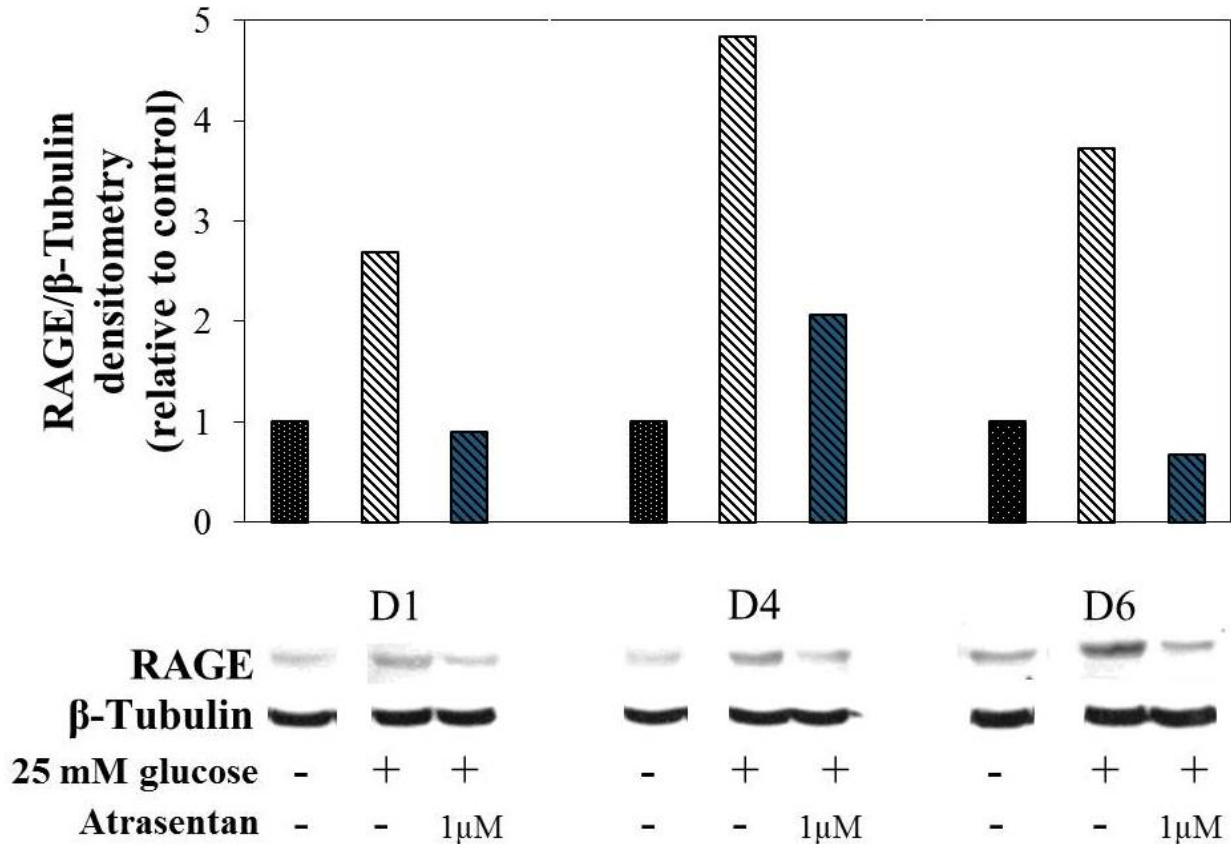
Because ET1 secretion occurred in shRAGE SMCs, RAGE is not required for ET1 secretion.

Although ET1 regulation may be affected by RAGE signaling in normal conditions, these results imply that ET1 expression is not downstream of RAGE in procalcific conditions.

### 3.2.6. ETA signaling affects RAGE expression

Our findings showed a requirement of ETA and RAGE signaling for SMC osteochondrogenic differentiation and calcification enhanced by high glucose conditions. Additionally, RAGE does not have a significant effect on ET1 secretion under procalcific, hyperglycemic conditions. To determine whether ETA signaling affects RAGE expression, RAGE expression levels were

analyzed in SMCs treated with atrasentan (1  $\mu$ M) in procalcific, hyperglycemic conditions (**Figure 21**).



**Figure 21:** Effect of ETA antagonism on RAGE expression on SMCs. Atrasentan (1  $\mu$ M) reduced RAGE expression in SMCs cultured in procalcific conditions (2.4 mM Pi) with high glucose (25 mM).

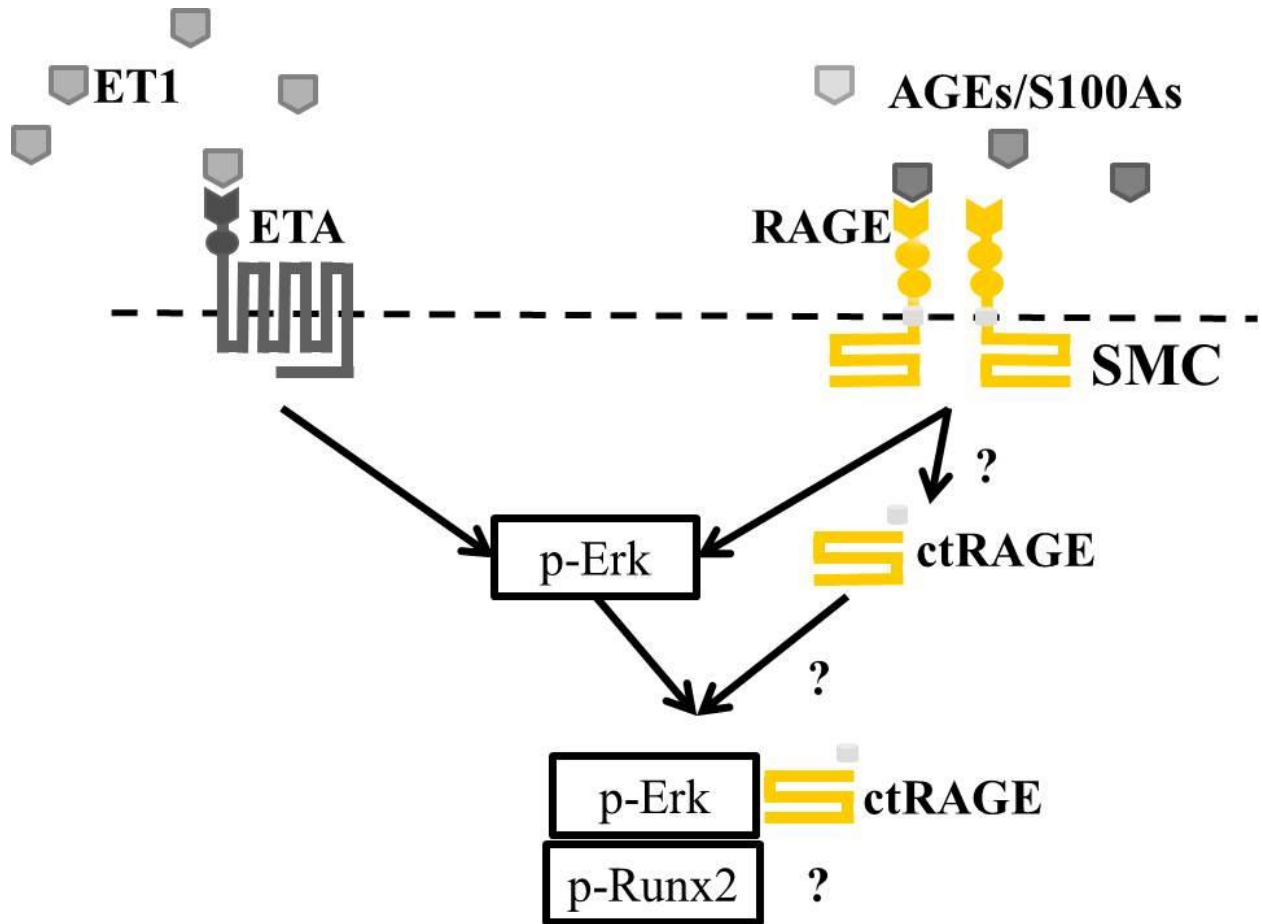
ETA antagonism substantially reduced RAGE expression in procalcific SMC cultures under high glucose conditions. NF $\kappa$ B, increased by ET1 overexpression,<sup>62,70</sup> has been shown to upregulate RAGE<sup>46,90</sup> It is possible that ET1-ETA signaling activates NF $\kappa$ B, which in turn, upregulates RAGE expression in procalcific high glucose conditions.<sup>40,46</sup>

### 3.2.6. Conclusions

In this aim, we investigated the potential interplay between RAGE and ET1-ETA signaling pathways. Our results suggest that RAGE and ET1-ETA are both required for matrix calcification

of SMCs enhanced by high glucose, and ET1-associated calcification under high glucose conditions was shown to be RAGE-dependent. Additionally, we evaluated the effects of RAGE signaling on ET1-ETA expression, and vice-versa. ETA antagonism reduced RAGE expression, and ET1 expression was not affected by RAGE signaling. However, we have not detected the effects these pathways have on each other downstream of the membrane-bound receptors. Regardless of the effect of ETA antagonism on RAGE expression, it is possible that the connection between the two pathways in the context of glucose-associated SMC calcification is resultant of a mutually downstream mediator of osteochondrogenic differentiation.

The details of this interaction between ET1-ETA and RAGE remain undefined, but it is likely that SMC calcification is mediated through downstream activation of Runx2. RAGE and ETA mutually activate Erk1/2.<sup>83,90</sup> Additionally, ctRAGE has been shown to bind directly to Erk1/2,<sup>52,91</sup> and p-Erk1/2 is known to activate Runx2.<sup>35</sup> Theoretically, binding multiple components would thermodynamically stabilize the resulting structure, thus enhancing or prolonging Runx2 activity. Thus, given the results of this study, we hypothesize that RAGE works in conjunction with ETA to activate and stabilize Runx2 (**Figure 22**). This model of ETA and RAGE signaling could explain the dependence upon high glucose and RAGE for ET1-ETA mediated effects of SMC matrix calcification.



**Figure 22:** Possible pathway downstream of ETA and RAGE signaling, including an active Runx2 complex formed with ctRAGE and p-Erk1/2.

## **Chapter 4: Future directions**

### **4.1. Investigation of kinase pathways downstream of ET1-ETA and RAGE**

We determined that both RAGE and ET1-ETA signaling have an effect on matrix calcification and osteochondrogenic differentiation of SMCs under procalcific conditions with high glucose, and our results suggest interaction between the ET1-ETA and RAGE pathways. However, their combined downstream effect on Runx2 transcriptional activity is most likely the main cause of the enhanced calcification observed in high glucose conditions. The downstream effects of both of these pathways have yet to be examined in our system, obscuring the link between the membrane-bound receptors and osteochondrogenic gene regulation. Various MAPKs have been shown to lead to greater Runx2 expression and transcriptional activity, especially p-Erk1/2.<sup>35</sup> We will investigate Erk along with other MAPK pathways, such as p38 and Akt, which have been shown to be downstream of both RAGE and ET1-ETA in various cell types.<sup>51,83,90-93</sup> Using specific kinase

inhibitors, and atrasentan to block ETA signaling or our shRAGE SMCs to inhibit RAGE signaling, we will show what combination of activated pathways leads to Runx2 expression and/or transcriptional activity. Runx2 expression will be determined through western blot, and Runx2 transcriptional activity will be quantified with an Osteoblast-Specific Element 2 (OSE2) luciferase reporter assay. Cells will be transiently transfected with a p6OSE2-luc construct along with a transfection control via electroporation (Amaxa Basic Nucleofector Kit for Primary Mammalian SMCs) or lipofectamine, and Runx2 activity will be induced with 3.0 mM inorganic phosphate for 2 to 3 days. A Promega dual reporter luciferase kit will be used to measure both firefly luciferase and a renilla luciferase control of the cell lysates. We expect that Erk1/2 signaling will be most important for Runx2 activation, but these experiments will clarify the signaling downstream of membrane-bound ETA and RAGE and improve our understanding of how signaling through these pathways intensifies osteochondrogenic differentiation of SMCs in high glucose conditions.

#### **4.2. Investigation of a possible Erk-Runx2-ctRAGE complex**

We have observed prolonged and higher expression of Runx2 in procalcific conditions with high glucose concentrations. It is uncertain whether Runx2 activity is further stimulated and/or stabilized in this condition. Additionally, ETA antagonism blocked the prolonged expression. We will investigate whether signaling through RAGE works in conjunction with ETA to activate and stabilize Runx2. RAGE and ETA mutually activate Erk1/2,<sup>83,90</sup> and ctRAGE has been shown to bind directly to Erk1/2.<sup>52,91</sup> Additionally, p-Erk1/2 is known to activate Runx2.<sup>35</sup> Theoretically, binding multiple components would thermodynamically stabilize the resulting structure, resulting in prolonged transcriptional activity of Runx2. We will use a co-immunoprecipitation technique to detect complex formation between these components (Erk-Runx2-ctRAGE). Magnetic beads coated with agarose resin coupled with Protein G (Invitrogen Dynabeads Protein G for

Immunoprecipitation) will be used with a p-Erk1/2 antibody to isolate all complexes formed with p-Erk1/2. All proteins associated with p-Erk1/2 will be separated via electrophoresis, and RAGE and Runx2 protein will be measured through Western blotting. To further investigate the Erk-Runx2-ctRAGE complex formation in calcifying blood vessels, we will utilize a proximity ligation assay. Two primary antibodies from different species will be used to recognize p-Erk1/2 and RAGE, and Runx2 and RAGE. Species-specific secondary antibodies conjugated with a short DNA strand will bind to the primary antibodies. When the PLA probes are in close proximity, the DNA strands can interact. After joining of the oligonucleotides, they will be amplified with a polymerase and quantified. Pending the outcome of the experiments investigating the role of other MAPKs in ETA and RAGE signaling, complexes formed with other MAPKs may be investigated.

#### **4.3. Does ETA antagonism inhibit T2DM-related vascular calcification *in vivo*?**

We determined that atrasentan is an effective inhibitor of osteochondrogenic differentiation and matrix calcification of SMCs in a high glucose setting *in vitro*. Using LDLr<sup>-/-</sup> mice fed with a diabetogenic, procalcific diet (HFCD), we will investigate whether ETA antagonism can inhibit T2DM-associated calcification. After the mice on HFCD diet become obese and develop hyperinsulinemia and hyperglycemia, we will deliver atrasentan to half of them via their drinking water. After the untreated mice begin to develop vascular calcification, we will begin to quantify the effects of atrasentan on vascular calcification. We will compare aortic calcium content between atrasentan-treated and untreated mice. Additionally, serum insulin, glucose, and ET1 levels will be measured. Immunohistochemistry for SMC markers, osteochondrogenic markers, ET1, ETA, RAGE, and p-Erk1/2 will be completed for aortic arch sections from atrasentan-treated and untreated groups. Mice fed with NC and HFD will serve as controls. If we observe that atrasentan

is an effective inhibitor of T2DM-related vascular calcification *in vivo*, ETA antagonism may have a clinical application in prevention of vascular calcification in patients with T2DM.

Overall, these studies will elucidate a mechanism by which T2DM enhances vascular calcification and identify a potential target for pharmaceutical intervention for the prevention of vascular calcification in patients with T2DM.



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