INVITED REVIEW

The pigeon (*Columba livia*) model of spontaneous atherosclerosis

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ABSTRACT Multiple animal models have been employed to study human atherosclerosis, the principal cause of mortality in the United States. Each model has individual advantages related to specific pathologies. Initiation, the earliest disease phase, is best modeled by the White Carneau (WC-As) pigeon. Atherosclerosis develops spontaneously in the WC-As without either external manipulation or known risk factors. Furthermore, susceptibility is caused by a single gene defect inherited in an autosomal recessive manner. The Show Racer (SR-Ar) pigeon is resistant to atherosclerosis. Breed differences in the biochemistry and metabolism of celiac foci cells have been described. For example, WC-As have lower oxidative metabolism but higher amounts of chondroitin-6-sulfate and nonesterified fatty acids compared with SR-Ar. Gene expression in aortic smooth muscle cells was compared between breeds using representational difference analysis and microarray analysis. Energy metabolism and cellular phenotype were the chief gene expression differences. Glycolysis and synthetic cell types were related to the WC-As but oxidative metabolism and contractile cell types were related to the SR-Ar. Rosiglitazone, a PPARγ agonist, blocked RNA binding motif (*RBMS1*) expression in WC-As cells. The drug may act through the c-*myc* oncogene as RBMS1 is a c-*myc* target. Proteomic tests of aortic smooth muscle cells supported greater glycosylation in the WC-As and a transforming growth factor β effect in SR-Ar. Unoxidized fatty acids build up in WC-As cells because of their metabolic deficiency, ultimately preventing the contractile phenotype in these cells. The single gene responsible for the disease is likely regulatory in nature.

Key words: genetics, oxidative metabolism, glycolysis, contractile phenotype, synthetic phenotype

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INTRODUCTION

Atherosclerosis is the leading cause of death in the United States, contributing to both heart disease and stroke. The disease typically develops at arterial bifurcations, such as those in the carotid and coronary arteries and at the celiac focus of the aorta (Kjaernes et al., 1981; Bassiouny et al., 1994). Atherosclerotic plaques begin early in life as fatty streaks composed of lipid-filled foam cells originating from smooth muscle (Stary, 1989; Napoli et al., 2002). These initial lesions progress slowly to mature plaques which contain many types of lipid-filled cells, extracellular matrix (**ECM**) components (proteoglycans, collagen, elastin), calcium, cholesterol esters, and cellular debris.

Several hypotheses attempt to explain these phenomena in humans including response to injury, response to retention, phenotypic reversion, and, to a lesser extent, mitochondrial dysfunction. These hypotheses are reviewed elsewhere (Anderson et al., 2012a). Once formed, atherosclerotic plaques decrease the size of the arterial lumen; raise blood pressure; and rupture, initiate clots which may block the entire passage, or both, thus affecting blood flow to downstream tissues and organs.

The major focus of atherosclerosis research has been on delaying plaque progression rather than preventing plaque initiation because symptoms of the disease manifest slowly over a person's lifetime (Munro and Cotran, 1988; Stary, 1989). One clue in early detection could be the role of inheritance; many genes associated with plaque progression and potential rupture have been identified. Despite such progress, genes that directly affect susceptibility and foam cell initiation remain unknown.

Animal models are useful for experimentation, and usually fall into 1 of 4 categories: induced models, spontaneous or natural models, negative or nonreactive models, and orphan models without a human disease counterpart (Davidson et al., 1987). In addition to categorizing the model, several criteria should be met when choosing an animal model of human disease. These range from practical concerns such as cost, avail-

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Table 1. Comparison of human and pigeon atherosclerosis

Item	Human	Pigeon
Primary cell type	SMC	SMC
Location of primary lesions/foam cells	Coronary, celiac branch	Coronary, celiac branch
Characteristics		
Spontaneous	Yes	Yes
Diet-induced	Yes	Yes
Thrombosis	Yes	Yes
Myocardial infarction	Yes	Yes
Lipoprotein profiles		
Predominant form	LDL	HDL
ApoE	Yes	No
ApoB100	Yes	Yes
ApoB-48	Yes	No
CETP	Yes	Yes
LDLR	Yes	No
Genetic susceptibility	Multifactorial	Single gene, autosomal recessive
Genome size (Gbp)	3.40	1.47

ability, and ease of manipulation to anatomical and physiological similarities such as pathological location, progression, and genetic profile (Davidson et al., 1987).

Mice, especially transgenic mice, and rabbits are the most-widely employed models of human atherosclerosis today, but rats, dogs, miniature swine, primates, and pigeons have also have also been used to elucidate the role of multiple proteins and genes involved in disease pathology. No single model is suitable for all phases of the human atherosclerosis. Some of these animals are ideal for investigating plaque progression and disease end points, but several authors (Cornhill and Nerem, 1980; St. Clair, 1998; Moghadasian et al., 2001) propose the White Carneau (**WC-As**) pigeon model as most appropriate for the investigation of the earliest events in human atherogenesis, the initiation stage. Table 1 compares human atherosclerosis to the disease in pigeons.

Pigeons

The pigeon (*Columba livia*) model of atherogenesis is valuable for several reasons. Atherosclerosis occurs spontaneously in susceptible pigeons in the absence of any known risk factors, such as elevated blood pressure and serum cholesterol (Wagner, 1978; Wagner et al., 1979). In the early stages, the disease is remarkably similar to the human condition in anatomical location, morphology, ultrastructure, and biochemistry. Susceptibility to pigeon atherosclerosis resides at the level of the arterial wall (Wagner et al., 1973, 1979; Fronek and Alexander, 1981; St. Clair et al., 1986) and is inherited in a manner consistent with an autosomal recessive single gene trait (Smith et al., 2001). Without experimental manipulation, the WC-As develops fatty streaks in the coronary arteries and celiac bifurcation of the aorta (Clarkson et al., 1959; Prichard et al., 1964; Cornhill et al., 1980; Cornhill and Nerem, 1980; Hadjiisky et al., 1991). Early lesions at the aorta's celiac bifurcation progress in a predictable sequence becoming visually unmistakable by 3 yr of age (Nicolosi et al., 1972; Santerre et al., 1972).

While consuming a common diet with housing under similar conditions, the Show Racer pigeon (**SR-Ar**) does not develop the disease, serving as a negative (nonreactive) control for the naturally susceptible WC-As. Pigeon atherosclerosis is independent of plasma cholesterol levels (Wagner, 1978). In humans, high cholesterol levels, especially the low-density lipoprotein (**LDL**) fraction, are associated with increased occurrence of atherosclerosis. Although both pigeon breeds are hypercholesterolemic compared with humans, composite data from several hundred birds aged 6 mo to 3 yr have shown that total cholesterol levels average 220 mg/dL. These values neither differ significantly between the breeds nor change during disease progression in the susceptible breed (Anderson et al., 2012a). Although pigeons circulate most cholesterol in the high-density lipoprotein (**HDL**) form, the LDL/HDL fractions are also similar between breeds and do not contribute to foam cell development. This is important because it indicates a pathogenic mechanism other than elevated cholesterol in the WC-As circulation, a risk factor that has been well studied in humans and other animal models of atherosclerosis. However, more than half of heart attacks occur in people with normal cholesterol levels and no other risk factors (Gurr, 1992; Ridker, 2000), so the pigeon may be useful in elucidating these understudied pathological pathways.

The celiac bifurcation of the pigeon aorta is the most commonly investigated site of spontaneous atherosclerotic plaques. Cushions are smooth muscle cell (**SMC**) aggregations surrounding the arterial openings at branch points. These structures are a remnant of embryological development of the circulatory system (Santerre et al., 1972). At 1 d of age, cushions are evident at the celiac bifurcation in both susceptible and resistant pigeon aortas, but in susceptible pigeons the SMC remain in a synthetic phenotype, proliferate, and do not mature to the contractile phenotype seen in re-

sistant aortas. By 6 wk of age, synthetic SMC begin to accumulate lipid vacuoles to form foam cells and produce excessive amounts of ECM proteins: proteoglycans, collagen, and elastin. The cellular pathology is quite apparent under microscopic examination by 6 mo of age (Nicolosi et al., 1972; Santerre et al., 1972).

The anatomic location of plaque development in the spontaneous WC-As model contrasts with that in the diet-induced model in which lesions appear at multiple and variable sites along the aorta (Wagner, 1978; Gosselin, 1979; Jerome and Lewis, 1985) and demonstrate different pathology than the spontaneous lesion. Smooth muscle cells are the primary cell type in spontaneous foam cells (Cooke and Smith, 1968; St. Clair, 1983), whereas diet-induced foam cells are macrophage-derived (Gosselin, 1979; St. Clair, 1983; Jerome and Lewis, 1984; Denholm and Lewis, 1987). In addition, although plaques induced by a cholesterol-rich diet develop faster in the pigeon (Jerome and Lewis, 1984; Xu, 2004), foam cell initiation occurs by a different mechanism (Santerre et al., 1972; St. Clair, 1983). Therefore, this review is confined to spontaneous atherosclerosis in the WC-As so as not to confound the interpretation of atherogenic events with an artificial diet.

Primary aortic SMC cultures, prepared from 18-d-old embryos (just before hatching) or 1-d-old squabs, exhibit an identical sequence of events but at a greatly accelerated rate (7 to 10 d) (Smith et al., 1965; Cooke and Smith, 1968; Wight et al., 1977; Wight, 1980). The subculturing process can change the cellular phenotype (Worth et al., 2001), but this has not been observed in either breed. Smooth muscle cells from WC-As grown in vitro show degeneration similar to those cells observed in the celiac focus in vivo (Cooke and Smith, 1968; Wight et al., 1977; Anderson et al., 2012a).

There are significantly more SMC lipid vacuoles in the WC-As than the SR-Ar in vitro (Cooke and Smith, 1968). When grown in coculture or on spent SR-Ar medium (Table 2), the amount of WC-As cells occupied by lipid did not change. However, SR-Ar cells in coculture showed significant lipid increase not only over the controls, but also above the level of the WC-As control cells. In addition, Table 2 shows that SR-Ar cells grown on spent WC-As medium showed increased lipid over SR-Ar controls, bringing their vacuole grade closer to that of the susceptible WC-As control cells. Taken together, these results suggest that the WC-As cells produce or secrete a factor that overrides the SR-Ar cells' natural resistance to lipid accretion. The reciprocal effect was not observed.

Biochemical Differences

Many biochemical and metabolic differences have been identified between the celiac foci of susceptible and resistant pigeons before WC-As foam cell development. The most prominent differences identified include increased levels of nonesterified fatty acids (**NEFA**) and chondroitin-6-sulfate (**C_6S**) and decreased oxidative metabolism in the WC-As.

Increased NEFA levels in the celiac focus of WC-As squabs (Hajjar et al., 1980) have been measured as early as 1 d of age (0.21 μg of NEFA per μg of DNA versus 0.05 in the SR-Ar). Levels of NEFA increase with age at this site in both breeds, but the WC-As amount is consistently higher. By 6 mo of age, WC-As contain an average of 6.01 μg of NEFA per μg of DNA, whereas the SR-Ar is approximately half that value, at 3.13 (Table 3). By this age, cholesterol esters (**CE**) are predominant in WC-As cells, and linoleic acid (18:2) is the primary fatty acid in these CE. When added to primary cell cultures at the physiological level in pigeon serum (100 μ*M*, 300 μ*M*), the lipid vacuole grades from both pigeon breeds increased in a dose-dependent response.

Furthermore, rosiglitazone blocked the increased vacuole grades in WC-As and SR-Ar (Anderson et al., 2014). The WC-As lipid classes that were elevated in the presence of 100 μ*M* 18:2 include NEFA, as would be expected, as well as CE. Triacylglycerol (**TAG**) content actually decreased with 18:2 treatment in WC-As. This apparent dichotomy suggests that NEFA is being used for increased synthesis of CE rather than TAG.

By 6 wk, WC-As are synthesizing more C_6S than the SR-Ar at the celiac cushion in vivo (Curwen and Smith, 1977). Increased C_6S is also detectable in vitro (Wight, 1980), where levels are 3 to 4 times higher in WC-As aortic SMC and appear to correlate with lipid vacuole scores. The WC-As SMC score a 2.19 whereas SR-Ar cells score a 1.73 (Anderson et al., 2014).

Human and pigeon SMC synthesize C_6S for the ECM (Wight, 1985; Edwards et al., 1995). This proteoglycan

Table 2. Comparison of vacuole grades[1] from atherosclerosis-susceptible White Carneau (WC-As) and atherosclerosis-resistant Show Racer (SR-Ar) pigeon vascular smooth muscle cells (SMC) grown in coculture[2] and on exchanged spent media

Item	WC-As SMC[3]	SR-Ar SMC[3]
Control	2.19 ± 0.04 (12)^{B,a}	1.75 ± 0.03 (19)^{C,b}
Coculture	2.12 ± 0.07 (4)^{B}	2.43 ± 0.33 (3)^{A}
Spent medium exchange[4]		
WC-As Medium	nd	2.13 ± 0.03 (14)^{a}
SR-Ar Medium	2.24 ± 0.04 (10)^{a}	nd

$^{A–C}$Control and coculture means having no common uppercase letter differ significantly ($P < 0.001$).

^{a,b}Means within a breed cell type having no common lowercase letter differ significantly ($P < 0.001$).

[1]Vacuole grades: A vacuole grade of 1 indicates a lack of ORO stained droplets. A vacuole grade of 2, 3 and 4 indicates that 1/3 and 2/3 and the entire cytoplasm, respectively are occupied by ORO positive droplets (Smith et al., 2001).

[2]Coculture: WC-As and SR-Ar explants grown on different areas of the same coverslip, receiving the same fresh media.

[3]Number in parentheses= # flasks; nd = not done.

[4]Spent medium exchange: WC-As and SR-Ar explants grown on separate coverslips, fed with a solution of 50% spent media from the opposite breed, and 50% fresh media.

Table 3. Lipid content and classes of atherosclerosis-susceptible White Carneau (WC-As) and atherosclerosis-resistant Show Racer (SR-Ar) pigeons in vivo and in vitro (Nicolosi et al., 1972; Hajjar et al., 1980)

	WC-As		SR-Ar	
Item	(μg of lipid/μg of DNA)	(%)	(μg of lipid/μg of DNA)	(%)
Celiac foci in vivo (6 mo)				
Cholesterol esters	6.91	16.2	1.44	9.0
Triacylglycerol	7.82	18.3	2.03	12.8
Nonesterified fatty acids	6.01	14.1	3.13	19.7
Cholesterol	7.49	17.5	1.57	9.9
Phospholipids	14.5	33.9	7.75	48.7
Total	42.73		15.92	
Aortic smooth muscle cells in vitro				
Cholesterol esters	12.17	33.8	6.99	28.3
Triacylglycerol	11.30	31.4	7.31	29.6
Nonesterified fatty acids	2.81	7.8	2.10	8.5
Cholesterol	3.89	10.8	3.31	13.4
Phospholipids	5.80	16.1	4.99	20.2
Total	36.0		24.7	

binds to circulating LDL (Wight, 1980; Tovar et al., 1998; Nakashima et al., 2007; Wagner et al., 2007), an association that led to the response to retention hypothesis of atherogenesis (Williams and Tabas, 1995). According to this hypothesis, ECM proteoglycan content and composition influences lipid influx into cells.

Although the role of C_6S on lipid accumulation is not clear in the pigeon, the effect of its addition to cultured SMC is interesting. When added to WC-As, C_6S (10 μg/μL) has no effect on the vacuole grade (2.17). However, C_6S added to SR-Ar cells increased the vacuole score to 2.68 (S. C. Smith, unpublished data), showing that the SR-Ar are accumulating lipid, a property not usually associated with mature, contractile SMC, but rather a synthetic phenotype indicative of the WC-As. The SR-Ar lipid classes contributing to this increase include NEFA, CE, and other sterols. These increased lipid classes brought about by C_6S in the SR-Ar are the same classes altered by 18:2 in the WC-As. Phospholipid and TAG amounts did not change with C_6S treatment.

Energy metabolism is also different between breeds before foam cell formation. At d 1, there is an increase in F_1 ATPase activity (Hajjar, 1978) in the WC-As, and by wk 6, there is a lack of NADH transhydrogenation control in the WC-As (Hajjar and Smith, 1980). Zemplenyi and Rosenstein (1975) showed decreased TCA activity and increased glycolysis in the WC-As by this age. This could account for decreased oxidative phosphorylation and increased NEFA levels, but studies have not shown which event occurs first in pigeon atherogenesis.

By 6 mo of age, the WC-As SMC are hypoxic compared with the SR-Ar (Hajjar et al., 1988), and demonstrate decreased F_1 ATPase activity (Hajjar and Smith, 1980). In addition, lipid vacuoles have clearly formed at the celiac foci by this age (Cooke and Smith, 1968). There is more total lipid (values given in micrograms of lipid per microgram of DNA) in the susceptible WC-As celiac foci at 6 mo (42.73) than in the SR-Ar (15.92), which is consistent with higher amounts of all lipid classes analyzed in the WC-As compared with the SR-Ar in vivo (Table 3). Although circulating cholesterol is not different between breeds, there is more intracellular cholesterol, especially esterified cholesterol in the WC-As (Nicolosi et al., 1972). This fact is important because cholesterol esters are a significant component of foam cells and subsequent plaques in both pigeon and human atherosclerosis. There is more total lipid in WC-As cultured aortic smooth muscle cells compared with SR-Ar (36.0 vs. 24.7). Cholesterol esters were elevated in the WC-As in vitro compared with the SR-Ar, although cholesterol itself was not different between breeds (Table 3).

To further investigate CE composition differences, hydrolase activity was measured and compared between celiac tissue homogenates from both breeds. Both acid cholesterol ester hydrolase (**ACEH**) and neutral cholesterol ester hydrolase activity were higher in the SR-Ar than the WC-As (Fastnacht, 1993). No change in ACEH activity was observed in either breed with the addition of C_6S, although NCEH activity was significantly reduced ($P < 0.01$) in the SR-Ar cells when exposed to this proteoglycan. These combined results suggest that CE accumulation in pigeon SMC is more influenced by hydrolase activity than by influx, and this enzyme's activity appears to be inhibited by C_6S.

Delta-9-tetrahydrocannabinol (**THC**), the psychoactive component of marijuana, has been shown to inhibit cholesterol ester hydrolase (Burstein et al., 1978) by competitive binding (Shoupe et al., 1980). To determine if this compound had an atherogenic effect in pigeons, Verdon (1989) injected THC into 6-mo-old WC-As and SR-Ar twice weekly for 3 mo. Blood was drawn to evaluate postinjection effects, and at the end of the study aortic lesions were graded for degree of occlusion. There was no difference in aortic occlusion in either breed or treatment. However, compared with the controls, THC

Table 4. Differentially expressed genes in atherosclerosis-susceptible White Carneau (WC-As) pigeons identified in 4 experiments

Experiment	Transcripts upregulated in WC-As	Transcripts downregulated in WC-As
In Vivo_1 d_RDA (Anderson et al., 2013)	Lumican (*LUM*)	NADH dehydrogenase subunit 1 (*ND1*)
	Beta actin (*ACTB*)	CCR4-NOT transcription complex (*CNOT2*)
	Caveolin (*CAV1*)	Proteasome maturation factor (*POMP*)
In Vivo_1 d_Microarray (Anderson et al., 2013)	Hemoglobin binding protein (*HBB*)	Heat shock protein 90 (*HSP90*)
	p53 binding protein (*MDM1*)	Acyl CoA dehydrogenase (*ACAD8*)
	Serine protease inhibitor Clade F (*SERPINF2*)	SMAD family member 2 (*SMAD2*)
In Vivo_6 mo_RDA (our unpublished data)	Caveolin (*CAV1*)	Spermine N1-acetyl transferase (*SAT1*)
	Cytochrome oxidase II (*COII*)	Gelsolin (*GSN*)
	NADH dehydrogenase subunit 1 (*ND1*)	Lumican (*LUM*)
In Vitro_RDA (Anderson et al., 2012b)	Caveolin (*CAV1*)	Lumican (*LUM*)
	Enolase (*ENO1*)	Cytochrome *b* (*CYTB*)
	Retinol binding protein 7 (*RBP7*)	Fibulin (*FBLN5*)

administration significantly decreased plasma cholesterol concentration in the WC-As over the course of the study ($P < 0.005$). This decrease did not occur in the SR-Ar. The effect of THC on plasma cholesterol in WC-As but not SR-Ar is another example of an effect that was seen in the susceptible breed but not the resistant, supporting the view that there are significant biochemical differences between the 2 breeds.

Attempts to elucidate a chronological sequence of biochemical events showed reduced utilization of yolk lipids by susceptible embryos with an accumulation of NEFA in the celiac cushion by hatching (Cramer and Smith, 1976; Hajjar et al., 1980). This accumulation became significantly greater by 6 wk of age, at which time there was a marked increase in the production of C_6S proteoglycans. Also at this age, significant reduced mitochondrial oxidative function was seen. Correspondence with literature from other species as well as studies in pigeon aortic cell culture indicate that the increased NEFA stimulated proteoglycan expression, which in turn disrupted cellular maturation and metabolism, maintaining the susceptible SMC in a synthetic state.

This apparent sequence of events is supported by in vitro experimental results in which 18:2 added to aortic SMC cultures from both WC-As and SR-Ar increased the cellular lipid content. The PPARγ antagonist rosiglitazone blocked this effect and reduced the lipid content of WC-As control cells. However, C_6S added to WC-As and SR-Ar cultures increased lipid only in SR-Ar cells and caused these cells to revert to a synthetic phenotype.

Genetic Differences

Many genes contribute to atherosclerosis in humans and, combined with the influences of diet and lifestyle, the disease appears multifactorial in most people. In pigeons, atherosclerosis was once thought to be a polygenic disorder (Goodman and Herndon, 1963) until Smith et al. (2001) showed that spontaneous atherosclerosis is inherited as a single gene disorder, with resistance being the dominant trait (OMIA #8932). Characterization of the gene responsible for susceptibility, and an understanding how this gene influences disease pathology could reveal a mechanism that remains unidentified in more confounded atherogenic models.

Susceptibility and initiation may be a consequence of the same gene or the 2 may be sequential. These alternatives cannot be distinguished at present. Toward that end, many thorough experiments have attempted to identify candidate genes contributing to the atherosclerotic susceptible/resistant phenotype in the pigeon. The top 3 differentially expressed transcripts resulting from each experiment are presented in Table 4.

Gene expression at the celiac foci at 1 d of age in vivo was compared using 2 methods (Anderson et al., 2013). The first, representational difference analysis (**RDA**), is an open system that allows for gene discovery, and is dependent on the presence of internal restriction sites (Hubank and Schatz, 1994). Microarray analysis, a closed system, is dependent on pigeon/chicken homology as well as the presence of an expressed transcript tag (**EST**) and its corresponding transcript.

In the RDA experiment, 25 genes were expressed exclusively in the WC-As, including β-actin (***ACTB***), and caveolin (***CAV1***). Fifteen genes were exclusive to the SR-Ar, including CCR4-NOT transcription complex (***CNOT2***) and proteasome maturation protein (***POMP***). Some genes were expressed in both breeds, but NADH dehydrogenase subunit 1 (***ND1***) and lumican (***LUM***), a dermatan sulfate proteoglycan, were significantly different between the SR-Ar and WC-As, respectively. Putative pathways inferred through bioinformatic analysis that were predominating in the WC-As included transforming growth factor-β (**TGFB**)-dependent cytoskeletal remodeling and cell adhesion. In the SR-Ar, oxidative phosphorylation and cytoskeletal remodeling were indicated by the data set, meaning that the WC-As downregulated these pathways.

Microarray analysis revealed 48 differentially expressed genes. Seventeen of these genes demonstrated significant fold increases in the WC-As and included hemoglobin β chain (***HBB***), p53 binding protein (***MDM1***), and serine proteinase inhibitor, clade F (***SERPINF2***). Thirty one genes were downregulated in the WC-As and included heat shock protein 90 (***HSP90***), acyl CoA dehydrogenase (***ACAD8***), and SMAD Family Member 2 (***SMAD2***), a TGFB transcription factor. Putative pathways for cytoskeletal re-

modeling, lipid metabolism, and the immune response were downregulated in the susceptible WC-As.

Further reciprocal RDA experiments were conducted at 6 mo of age to examine the gene expression profile at the celiac focus in both breeds once foam cells have formed in the WC-As (J. L. Anderson, unpublished data). In these experiments, *CAV1* was exclusively expressed by the WC-As and comprised 34% of the library (37 out of 109 EST). Cytochrome oxidase II (***COII***) was also upregulated in the WC-As library at this age, as was NADH subunit 4 (***ND4***). Spermine N1-acetyl transferase (***SAT1***) was significantly downregulated in the WC-As library, along with gelsolin (***GSN***), prohibitin 2 (***PHB2***), ubiquinone (***NDUFA10***), and *LUM*.

Four replicate RDA experiments were conducted on primary cultured SMC harvested before foam cell formation in the WC-As (Anderson et al., 2012b). Multiple transcripts were significantly different between breeds. In the WC-As, *CAV1*, enolase (***ENO1***), and retinol binding protein 7 (***RBP7***) were present in the highest copy number, but chemokine ligand 12 (***CXCL12***), ribophorin (***RPN1***), and *ACTB* were also significantly different. Cytochrome *b* (***CYTB***) and *LUM* were absent in the WC-As. Other significant transcripts downregulated in the WC-As included fibulin (***FBLN5***), α actin (***ACTA2***), the TGFB receptor activin (***ACVR1***), myosin light chain kinase (***MYLK***) and *POMP*. Predominant pathway differences between breeds were those involved in contractility and energy metabolism. Genes associated with a synthetic phenotype and glycolysis were expressed in the WC-As SMC whereas SR-Ar SMC expressed genes indicative of a contractile phenotype and mitochondrial oxidation.

Finally, RDA was used to compare gene expression of rosiglitazone-treated WC-As SMC in vitro (Anderson et al., 2014). Rosiglitazone was selected as a PPARγ agonist, but that compound appeared to exert some of its effects through c-*myc*, an oncogene that contributes to SMC proliferation. The most significant transcript expressed in WC-As control cells, RNA binding motif (***RBMS1***) is a downstream target of c-*myc*, and its expression was blocked by rosiglitazone treatment. Activin receptor 1 (*ACVR1*), *LUM* and *CAV1* were also blocked by rosiglitazone. The primary transcripts expressed in rosiglitazone-treated WC-As were cytochrome P450 α hydroxylase (***CYP17A1***) and *ENO1*.

The most persistent differentially expressed gene, *CAV1*, was identified across the in vivo and in vitro experiments. The gene was exclusively expressed in the WC-As in vivo at both 1 d and 6 mo and, in vitro, WC-As transcripts vastly outnumbered SR-Ar transcripts ($P < 0.0001$). Also in vitro, rosiglitazone blocked *CAV1* expression in WC-As. Although it is unclear whether in response to PPARγ or c-*myc*, *CAV1* downregulation was concomitant with a decreased lipid vacuole score (Anderson et al., 2014).

Lipid classes were not analyzed in this experiment, but it is known that *CAV1* participates in cellular lipid metabolism especially that of cholesterol (Cohen et al., 2004). The *CAV1* gene is probably not participating in LDL influx, as suggested by the response to retention hypothesis because circulating cholesterol is not different between breeds. However, it could be preventing CE hydrolysis and cholesterol efflux because intracellular lipid amounts, particularly CE, are different between breeds (Table 3).

The role of *CAV1* in atherosclerosis has been investigated in mice (Frank et al., 2004), and rabbits (Lin et al., 2004). Its role in transmembrane signaling has been implicated in multiple human diseases, including type II diabetes (Cohen et al., 2004) and atherosclerosis (Schwencke et al., 2006). Further investigation of the structure, regulation, and function of *CAV1* in pigeons is warranted.

Pigeon expression patterns for *LUM* are quite interesting. At 1 d in vivo, *LUM* is slightly upregulated in the WC-As, but at 6 mo and in vitro, it was significantly downregulated. Expression of *LUM* in WC-As in vitro, although minimal compared with SR-Ar, was significantly reduced by rosiglitazone. One explanation for the seemingly contradictory *LUM* expression profile is the phenotypic reversion hypothesis (Schwartz et al., 1986; Thyberg et al., 1990; Owens et al., 2004). This hypothesis states that atherogenic human SMC do not differentiate from the lipid-accumulating synthetic phenotype to the more quiescent contractile phenotype of a mature SMC. Instead, they maintain the synthetic state, continue to accrue lipid, primarily cholesterol esters, and proliferate. During this transition, the proteoglycan profile shifts. In the pigeon, both WC-As and Sr-Ar SMC make *LUM*, a protective ECM proteoglycan (Troup et al., 2003; Shao et al., 2012) initially, but by 6 mo, *LUM* production has ceased in the WC-As. The reason for this downregulation is unknown, but it does not occur in the SR-Ar, where *LUM* production continues unhindered. Future in vivo investigation could focus on tracking *LUM* expression in the WC-As.

It is interesting that a versican transcript, the prominent ECM C_6S proteoglycan (Lundstam et al., 1999), was not identified in any of the analyses, because C_6S itself is one of the first biochemical differences between breeds. Core protein production may not differ between breeds, but the glycosylation status, and subsequent cellular effects, are different. Ribophorin is responsible for the glycosylation of nascent proteoglycans (Wilson and High, 2007), and its expression was detected in the WC-As in vitro. Increased glycosylation has been associated with the response to retention hypothesis, as it increases the binding affinity for LDL.

Genes related to energy metabolism in vivo also exhibit seemingly disparate expression patterns. The NADH1 (*ND1*) transcript was downregulated at one day in the WC-As (Anderson et al., 2013), whereas *ND4* and cytochrome oxidase II (*COII*) were significantly upregulated at 6 mo (J. L. Anderson, unpublished data). However, ubiquinone (*NDUFA10*) was downregulated in the WC-As. It could be that the lack of ubiquinone in the WC-As is causing a compensatory

increase in *COII*, but this is unclear. The increase in *ND4* production could also be compensatory because of the lack of NADH transhydrogenation that occurs by 6 wk of age.

Differences in energy production were much clearer in vitro, where the analysis is limited those genes expressed by vascular smooth muscle cells. Glycolytic enzymes including *ENO1* were exclusively expressed in WC-As compared with SR-Ar (Anderson et al., 2012b), and the addition of rosiglitazone increased *ENO1* expression (Anderson et al., 2014). This result has special interest because in addition to glycolysis, *ENO1* has a dual role as a transcription factor (Nikitin et al., 2003; Kim and Dang, 2005). In the SR-Ar, *CYTB* was uniquely expressed and other genes associated with oxidative phosphorylation had higher copy numbers in the SR-Ar (Anderson et al., 2012b). These findings support the energy differences previously reported by Zemplenyi and Rosenstein (1975) and, along with the biochemical and morphological data, support the atherogenic hypothesis of mitochondrial dysfunction (Scheckhuber et al., 2005; Yu et al., 2012).

The lack of efficient oxidation and downregulation of *ACAD8* in the WC-As could cause the measured increase in NEFA, but also has implications in the SMC phenotype. Weiss et al. (2006) found that ATP produced in the mitochondria drives the contractile machinery, whereas ATP produced in the cytoplasm is more suited for membrane pumps. Phenotypic stages can be distinguished by the type of actin produced by the SMC (Gabbiani et al., 1984). β-Actin (ACTB) is associated with a synthetic phenotype and was significantly different between breeds in vivo at one day (Anderson et al., 2013) and in vitro. α-Actin is associated with a contractile phenotype and was exclusively expressed in SR-Ar in vitro. In addition, gelsolin (GSN) is a major regulator of actin assembly (Wang et al., 2009), and was exclusively expressed by the SR-Ar at 6 mo of age (J. L. Anderson, unpublished data). Differential expression of genes is consistent with the biochemical phenomena observed. The WC-As expressed genes associated with a synthetic phenotype and glycolytic energy production, whereas SR-Ar expressed genes associated with a contractile phenotype and oxidative metabolism.

Protein Differences

Soluble proteins were extracted from primary WC-As and SR-Ar SMC in vitro, separated on 2-dimensional electrophoretic gels, and arrayed on a map (Smith et al., 2008). Many proteins were differentially expressed, but only 8 were successfully annotated from each breed.

In the WC-As, these proteins included heat shock protein (**HSP70**), cyclin (**CCND2**), LUM, and RPN1. Ribophorin was also found in the RDA in vitro experiment, and further suggests increased glycosylation in the WC-As. It is interesting that LUM itself was greater in the WC-As proteomic analysis, whereas *LUM* transcription was elevated in the SR-Ar genetic analysis. There is no direct correlation between mRNA synthesis and protein abundance (Gygi et al., 1999), but this contradictory finding further complicates the role of *LUM* in pigeon atherogenesis.

Serine threonine kinase (**STK**), myosin phosphatase (**MYPT1**), activin binding protein (**FST**) and fatty acid binding protein (**FABP**) were identified in the SR-Ar. Activin binding protein is of interest because the activin receptor transcript was downregulated in the WC-As in vitro, and strongly suggests the influence of TGFβ, as did the expression of *SMAD2* in vivo at one day of age. Wagner et al. (2007) found that WC-As and SR-Ar SMC respond differently to TGFβ, and the binding proteins, receptors, and transcription factors identified in the reviewed experiments could explain the differences in cellular proliferation. Khanna (2004) showed that c-*myc* expression increases when TGFβ is inhibited, and Igarashi et al. (2009) demonstrated that TGFB downregulates *CAV1*.

Concluding Remarks

Susceptible WC-As aortic SMC lack the metabolic pathways to oxidize fatty acids that accumulate and appear to alter the expression of proteoglycan genes. This lack of mitochondrial oxidation also does not produce enough ATP for SMC to develop and maintain the contractile phenotype. Increased abnormal proteoglycan production with an excess of chondroitin sulfate by these cells in the synthetic state lead to 2 subsequent aspects of lesion development: 1) decreased intracellular hydrolysis of cholesterol esters preventing their efflux from the cell; and 2) accumulation of large amounts of proteoglycans in the ECM. Both of these features are characteristic of the early stages of atherosclerotic lesion development.

Attempts to identity a single gene responsible for initiation of the WC-As pathological sequence have produced inconclusive results. Numerous genes are differentially expressed in celiac cushions as well as aortic SMC from WC-As and SR-Ar pigeons. Therefore, at the present state of our knowledge, the single gene autosomal recessive inheritance pattern for atherosclerotic susceptibility might be postulated to be due to a regulatory gene. Current candidates include *PPARγ*, c-*myc*, and *TGFβ*. Rosiglitazone, a *PPARγ* antagonist, altered c-*myc* gene expression in WC-As and blocked expression of genes responsible for abnormal metabolism and increased expression of genes similar to SR-Ar.

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