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TRANSCRIPTIONAL PROFILING OF EARLY ONSET DIET-INDUCED ATHEROSCLEROSIS IN APOLIPOPROTEIN E-DEFICIENT MICE

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1. ABSTRACT

Excessive dietary fat and cholesterol exacerbate atherosclerosis. To obtain unbiased insight into the early pathological changes induced by fat feeding in the artery wall, we used high-density microarrays to generate transcriptional profiles of aortic tissue from two groups of atherosclerosis-prone apolipoprotein E-null mice: controls maintained on standard chow and experimental animals exposed short-term to a Western-type diet, a regimen which produced severe hypercholesterolemia without significant development of atheromas. By applying rigorous selection criteria, we identified 311 genes differentially regulated by these dietary conditions. The set of diet-regulated genes exhibited striking functional relationships and represented both novel and known regulatory networks implicated in injury of the artery wall, including cell adhesion genes, histocompatibility antigen and major histocompatibility complex genes, flavin-containing monooxygenases, interferon-regulated genes, small inducible cytokines, collagen and procollagen genes, and complement system components. Further examination of genes identified by this study will provide insights into the molecular mechanisms by which high-fat cholesterol-rich dietary regime initiates pathological alterations in healthy arteries.

2. INTRODUCTION

Atherosclerosis is a chronic inflammatory disease of middle-sized and large-caliber arteries that normally progresses over several decades and may remain silent until fatal manifestations occur at advanced disease stages. It is widely accepted that several pathological stimuli (hyperlipemia, hypertension, diabetes, smoking, etc) initiate and sustain atherogenesis by causing endothelial damage (1,2). Circulating leukocytes adhere to the injured endothelium and migrate towards the subendothelial space, where resident monocytes differentiate into macrophages that avidly absorb modified low-density lipoproteins (LDLs) to form the lipid-laden foam cells characteristic of early fatty streaks. Activated neointimal macrophages and lymphocytes produce inflammatory mediators that induce the proliferation of vascular smooth muscle cells (VSMCs) and their migration towards the growing atherosclerotic lesion (1-3). Rupture or erosion of advanced atheromatous plaques can lead to thrombus formation and acute ischemic events e.g., myocardial infarction and stroke.

Atherothrombosis and associated cardiovascular disease (CVD) constitute the major cause of mortality in industrialized nations, and their incidence in developing countries is increasing at an alarming rate. Thus, it is of utmost importance to develop novel preventive and therapeutic strategies to reduce the social and health-care burden of CVD. Both genetic factors and excessive dietary intake of saturated fat and cholesterol can provoke hypercholesterolemia, a major risk factor for the development of atherosclerosis (1,2). Although recent decades of research have yielded progress towards an understanding of the molecular basis of

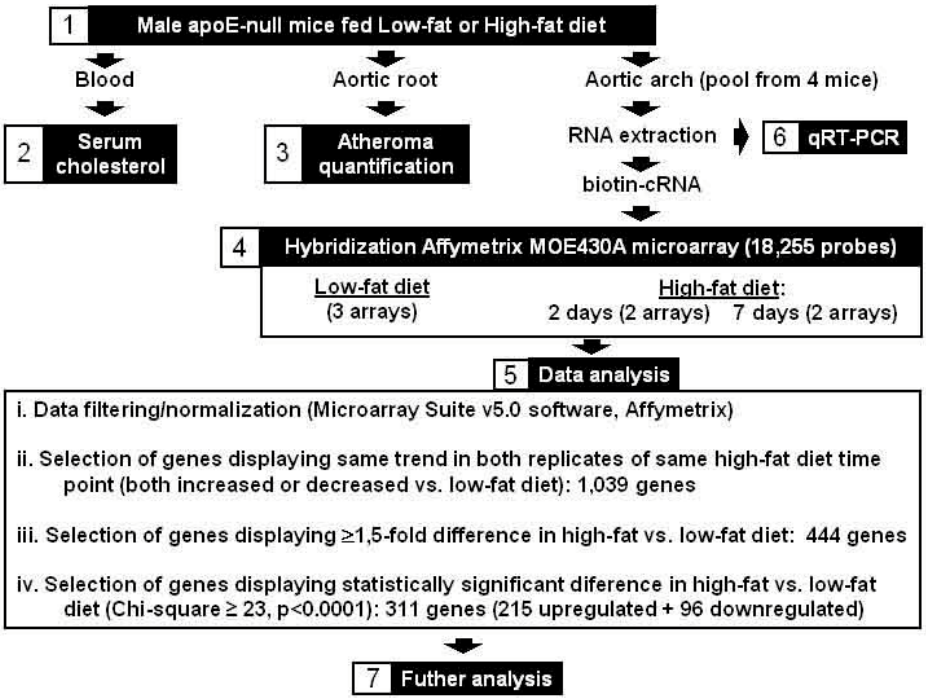

Figure 1. Study design. Two-month-old male apoE-deficient mice were fed standard LF-diet or HFC-diet for 2, 7 and 30 days (HFC-2d, HFC-7d, HFC-30d, respectively). Blood was collected to measure serum cholesterol and the heart and aortic arch was harvested for atheroma quantification in cross-sections of the aortic root. For microarray analysis, the aortic arch from four mice of each LF-diet, HFC-2d and HFC-7d groups was pooled and total RNA was obtained. RNA was reverse transcribed, and biotinylated cRNA was prepared for large-scale transcriptional profiling using the Affymetrix MOE430A oligonucleotide microarray. The procedure for data analysis is schematized (see text for details).

hypercholesterolemia-induced vessel damage, identification of the earliest gene expression changes induced by this atherogenic stimulus on the 'healthy' artery wall remains an important objective. The use of high-density microarray technology is emerging as a powerful tool to identify new genes and signaling pathways that are central to human disorders, including CVD (4-7).

Here we utilized high-density cDNA microarrays representing over 18,000 murine genes and ESTs to analyze gene expression changes in the aortic arch of fat-fed apolipoprotein E (apoE)-deficient mice, a widely used model that has permitted major advances in understanding how hypercholesterolemia promotes atheroma formation (8-10). apoE-null mice spontaneously develop elevated plasma cholesterol and complex atherosclerotic lesions resembling those observed in humans, a process that can be accelerated upon exposure to a high-fat cholesterol-rich diet. In our study, vessels were harvested after a very brief exposure (2 and 7 days) to an atherogenic diet, when severe hypercholesterolemia was manifest but atheromatous lesions were still largely absent. By applying rigorous statistical analysis to resulting data sets, we identified 311 genes whose expression in the aortic arch was significantly altered at this early disease stage (215 upregulated, 96 downregulated, $p<0.0001$). We have examined the functional relationships amongst differentially-regulated genes. Collectively, our observations identify novel genes potentially involved in the earliest phases of vessel injury induced by dietary fat, thus establishing a strong foundation for further expression and functional studies to assess novel hypothesis on the initiation of vessel damage induced by hypercholesterolemia.

3. MATERIALS AND METHODS

3.1. Animals, diet and study design

An overview of the experimental design is provided in figure 1. Male apoE-null mice (C57BL/6J, Charles River) were maintained on a low-fat standard diet after weaning (LF-diet, 2.8% fat, Panlab, Barcelona, Spain). At 2 months of age, mice received a high-fat cholesterol-rich diet (HFC-diet) containing 12% fat, 1.25% cholesterol and 0.5% sodium cholate (S8492-S010, Ssniff, Germany) for varying periods of time. Controls were maintained in LF-diet. Blood was collected from the retroorbital plexus under anesthesia to measure serum cholesterol using an autoanalyzer Cobas Mira (Roche).

3.2.Atheroma quantification and immunohistochemistry

At the moment of sacrifice, the heart and proximal aorta were dissected from mice, perfusion-fixed in situ with 4% paraformaldehyde, paraffin-embedded, and mounted in a Micron microtome. Atherosclerotic lesion size in cross-sections of the aortic root was quantified as the area occupied by Mac-3-immunoreactive cells (anti-Mac-3 antibodies, Santa Cruz Biotechnology, sc-19991, 1/100) using computer-assisted planimetry essentially as previously described (11).

3.3. RNA preparation and microarray analysis

Progenika (Spain) carried out RNA extraction and microarray analysis following the protocol suggested in the Expression Analysis Technical Manual (Affymetrix). Total RNA from the pooled aortic arch from 4 mice was extracted with TRIzol reagent (Invitrogene) followed by purification using the RNeasy kit (Qiagen). First-strand cDNA synthesis was performed using 4-6 microgram of RNA template and the SuperScript Choice System kit (Life Technologies). Array High Yield RNA transcript labeling (T7) (Enzo) was used for synthesis of biotinylated cRNA, which was hybridized to a mouse gene expression array containing representation of more than 18,000 probe sets of mouse cDNAs and ESTs (MOE430A, Affymetrix) (3 arrays for LF-diet and 2 arrays for each HFC-diet group). The P call% of the chips analyzed ranged from 44.0 to 56.9. All genes on the DNA chips were visually inspected for data quality, examination of graphical and numerical summaries of expression and outlier assessment according to Affymetrix quality control standards. Scaling/normalization was done with the "Selected Probe Sets" method using the Microarray Suite 5 (MAS 5) software (Affymetrix). Signal Log ratio was calculated by One-Step Tukey´s algorithm. Features with reference values of <3 SDs above average background were eliminated. For significance analysis p-values were computed for each probe set using a Wilcoxon´s Signed Rank test (GeneChip Expression analysis, Affymetrix)

3.4. Statistical analysis and selection of differentially regulated transcripts

We selected genes displaying the same trend in both replicates of each HFC-diet time point (both increased or decreased versus LF-diet) and whose expression differed ≥1.5-fold versus LF-diet. Expression of genes fulfilling these criteria was compared in LF-diet and HFC-diet using the Chi-square test, which combines probabilities from independent tests of significance. Differentially-expressed genes displaying Chi-square≥23.5 (2k degree of freedom) and $p<0.0001$ were analyzed with J-Express (www.ii.uib.no/~bjarted/jexpress), a java application for the analysis of gene expression and K-means clusters.

3.5. Quantitative real-time RT-PCR (qRT-PCR).

DNase-treated total RNA (2 microgram) was reverse transcribed with RNAse H Minus (Promega) and dT_{12} as primer. Assays-on-Demand kits containing primers and TaqMan probes for C3, CCL6, MEF2C and TGF beta 3 and the TaqMan universal PCR mix were purchased from Applied Biosystems to perform two independent experiments in duplicate according to the manufacturer's recommendations. Negative controls consisted of reactions without template. In order to normalize for template input, an internal control consisting of GAPDH transcript level was measured for each sample and utilized to calculate the threshold cycle number (Ct). Fold change values were calculated as the ratio of the Δ^{Ct} sample averages. The results represent the mean ± SEM.

4. RESULTS

4.1. Characterization of the experimental model

Because we sought to investigate changes in the transcriptional profile at initial stages of hypercholesterolemia-induced vessel injury, we first performed pilot studies in apoE-null mice receiving either LF-diet or HFC-diet for 2, 7 and 30 days (HFC-2d, HFC-7d and HFC-30d, respectively). Serum cholesterol was significantly elevated from 372±34mg/dL in LF-diet to 1346±49 mg/dL in HFC-2d ($p<0.0001$), and further increased to 3,002±111 mg/dL in HFC-7d and 2,749±100 mg/dL in HFC-30d ($p<0.0001$ versus both LF-diet and HFC-2d) (figure 2A). We next quantified atheroma size in cross-sections of the aortic root (figure 2B,C), a highly atherogenic region in this animal model. As revealed by immunohistochemistry for macrophage-specific Mac-3 (12), atheromatous lesions were not observed in LF-diet mice (n=6). Likewise, atheromas were largely absent in three HFC-2d and three HFC-7d mice, and three mice in each of these groups presented incipient lesions overtly smaller than those detected in HFC-30d. Thus, 2 and 7 days of fat feeding appeared appropriate to identify changes in gene expression induced by fat-feeding prior to significant macrophage recruitment within the artery wall.

4.2. Large-scale microarray study of aortic tissue from apoE-null mice exposed short-term to HFC-diet

We next performed large-scale microarray analysis using RNA isolated from the aortic arch of LF-diet, HFC-2d and HFC-7d of apoE-null mice (see experimental design in figure 1). The oligonucleotide chips used in these studies contain probe sets for 18,255 murine cDNAs and ESTs. We examined three microarrays for LF-diet and two microarrays for each HFC-2d and HFC-7d. Each array was hybridized with RNA pooled from four mice. Transcript levels were estimated using the Microarray Suite v5.0 software (MAS 5.0, Affymetrix). Absolute call (present, marginal, or absent) and average difference (increased and decreased in HFC-diet versus LF-diet) were used as gene expression measures. We first selected 1,039 transcripts displaying the same tendency in both replicates of each HFC-diet time point (increased or decreased in both duplicates of HFC-diet versus LF-diet). Among these 1,039 transcripts, we selected 444 genes displaying ≥1.5-fold difference in HFC-diet versus LF-diet and compared their expression in LF-diet versus HFC-diet using the Chi-square test. By applying all these criteria, we identified 311 differentially-regulated genes (≥1.5-fold difference) with Chi-square≥23.5 and $p<0.0001$ (Table 1 and Table 2).

To generally validate the results of microarray analysis, we examined the expression of C3, CCL6, MEF2C and TGFbeta 3 genes by qRT-PCR using tissue obtained from a new set of mice. For each gene, two independent RT-PCR assays were performed with total RNA purified from aortic arch pooled from 3-4 mice.

Overall, the four genes examined disclosed a good correlation between the two methods, with the qRT-PCR data showing slightly greater fold-changes in some instances (figure 3).

4.3. Temporal profile and functional clustering of genes regulated in the aortic arch of apoE-null mice by short-term HFC-diet

The 311 genes regulated by HFC-diet were classified in six clusters based on their temporal expression pattern (Up: up-regulated, Down: down-regulated, NC: no change) (figure 4A). Using Affymetrix (www.affymetrix.com/analysis)

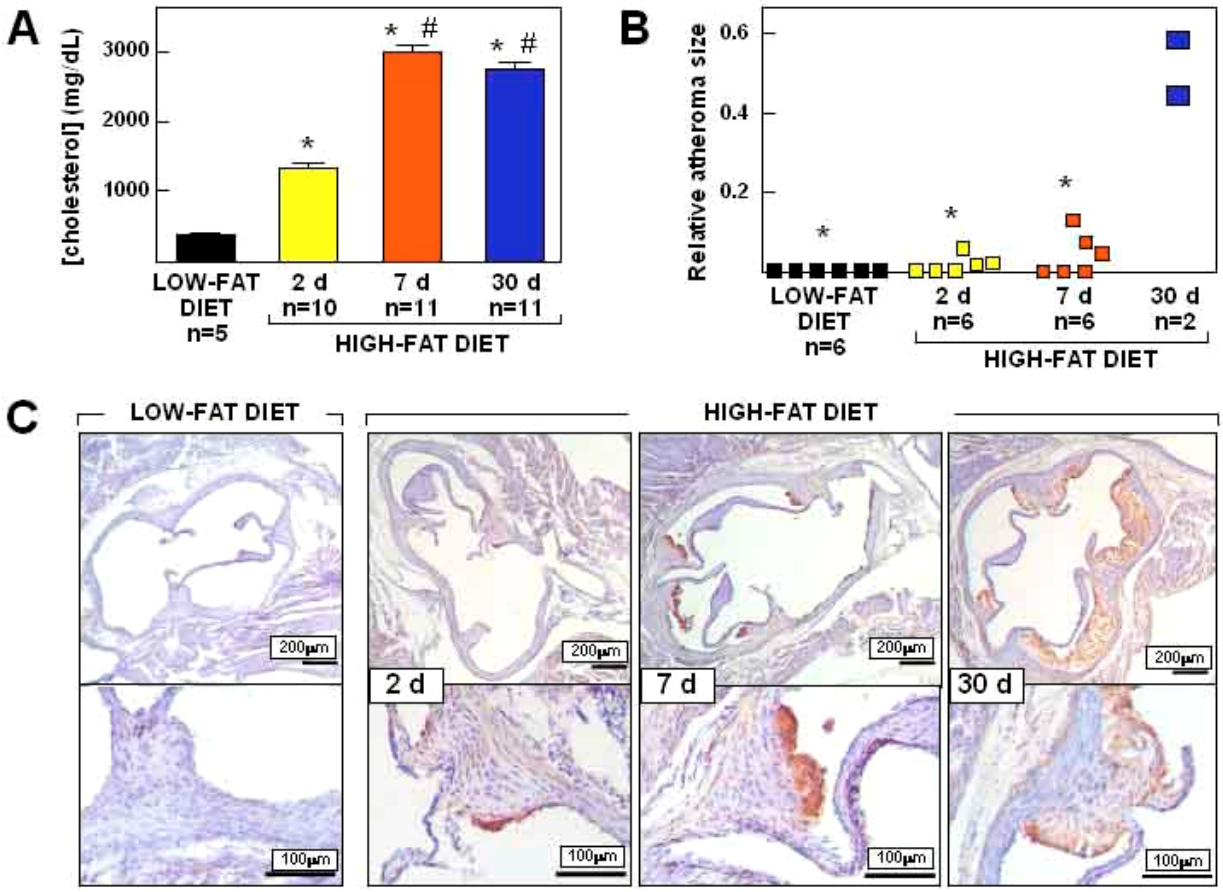

Figure 2. Characterization of the experimental model. A. Effect of atherogenic diet on serum cholesterol level. *, p<0.0001 versus LF-diet; #, p<0.0001 versus HFC-2d. B. Atheroma development in the aortic root region quantified by computer-assisted planimetry as the area occupied by Mac-3-expressing macrophages. Lesions at 2 and 7 days of HFC-diet were absent in half of the mice or just incipient in the other half (confer prominent lesions after 30 days of HFC-diet). *, p<0.0001 versus HFC-30d. C. Representative photomicrographs showing Mac-3 immunoreactivity (red staining) and haematoxylin counterstaining. Differences among groups were analyzed by ANOVA and Fisher's post-hoc test.

Table 1. Genes up-regulated by high-fat versus low-fat diet in aortic arch of apoE-null mice (215 genes): Clusters I+II+III [1]

Function [2]	GeneBank	Description	Cluster	2 days	7 days
				Fold change (High-fat versus Low-fat) [3]	
Metabolism	NM_007876	dipeptidase 1 (renal)	I	1.41[5]	1.68[4]
	NM_017370	Haptoglobin	I	1.74[6]	3.14[6]
	AW208566	lysozyme	I	1.37[5]	2.64[5]
	AK002700	sulfotransferase family 1A	I	2.14[6]	1.8[6]
	NM_009252	SerpinA3 [8]	I	4[6]	6.73[5]
	BC013477	alcohol dehydrogenase 1	II		2.64[4]
	AK003671	carbonic anhydrase 3	II		1.62[4]
	X61397.1	carbonic anhydrase-related polypeptide	II		2.46[4]
	AI315015	carboxylesterase 3	II		1.8[5]
	NM_007940	epoxide hydrolase 2, cytoplasmic	II		1.93[5]
	NM_133806	expressed sequence AA420407	II		1.57[4]
	U09114	glutamate-ammonia ligase	II		2.93[4]
	AI391218	glutamine synthetase	II		2.22[5]
	NM_010362	glutathione S-transferase omega 1	II		1.62[4]
	NM_010809	matrix metalloproteinase 3	II		2.14[5]
	AB022340	SA rat hypertension-associated homolog	II		1.87[4]

	BC02593	Similar to 10-formyltetrahydrofolatedehydrogenase	II		2.14[4]
	NM_009121	spermidinespermine N1-acetyl transferase	II		2[4]
	BC026584	Unknown (protein for MGC:37234)	II		2.54[4]
	AF031467	branched-chain amino acid aminotransferase	III	1.8[5]	
	BC003264	pyrophosphatasephosphodiesterase2	III	1.62[6]	
	BG076333	MTHFdehydrogenase (NAD+ dependent)	III	1.52[5]	
	NM_008713	nitric oxide synthase 3, endothelial cell [8]	III	1.54[5]	
	BM207712	phosphoribosylaminoimidazole carboxylase	III	1.52[4]	
	D87867	UDP-glucuronosyltransferase	III	2.64[6]	
Lipid metabolism and transport	NM_009605	adipocyte complement related protein	I	2.3[5]	2.55[6]
	NM_080575	acetyl-Coenzyme A synthetase 2	II		1.93[4]
	NM_026384	diacylglycerol O-acyltransferase 2	II		1.93[5]
	AK017272	lipoprotein lipase [7 8]	II		3.03[5]
	BB305534	ATP-binding cassette, sub-family A [8]	II		2.14[5]
	AW413978	ATP-binding cassette, sub-family G1	II		2.55[5]
	NM_010174	fatty acid binding protein 3, muscle and heart	II		1.74[4]
	BC013442	solute carrier family 27 (fatty acid transporter)	II		4.76[5]
	NM_015729	acyl-Coenzyme A oxidase 1, palmitoyl	III	1.74[5]	
	BC026209	arachidonate 5-lipoxygenase activating protein	III	2[6]	
	NM_010191	farnesyl diphosphate farnesyl transferase 1	III	2.3[5]	
Transport	NM_009994	cytochrome P450, 1b1, benz(a)anthracene	I	1.93[6]	1.52[5]
	NM_021282	cytochrome P450, 2e1, ethanol inducible	I	3.25[6]	2.93[6]
	AK004616	solute carrier family 21	I	1.62[5]	1.87[5]
	AV286265	xanthine dehydrogenase	I	2.46[5]	2.3[5]
	BC015260	FK506 binding protein 5 (51 kDa)	I	2.73[6]	2.93[6]
	NM_008030	flavin containing monooxygenase 3	I	1.74[6]	2.22[6]
	BC011229	favin containing monooxygenase 1	II		1.87[5]
	NM_01888	flavin containing monooxygenase 2	II		1.8[5]
	NM_008898	P450 (cytochrome) oxidoreductase	II		1.93[5]
	BE648080	potassium channel, subfamily K, member 3 [8]	II		2.14[4]
	BC011293	Similar to RIKEN cDNA 5730438N18 gene	II		2.14[4]
	NM_054098	Tnfa-induced adipose-related protein (Tiarp-pending)	II		2.07[4]
	BC01270	Ucp1	II		1.62[4]
	NM_008049	ferritin light chain 2	III	1.52[5]	
	NM_008218	hemoglobin alpha, adult chain 1	III	1.68[4]	
	AK011116	hemoglobin, beta adult major chain	III	1.57[5]	
	BC027434	hemoglobin, beta adult minor chain	III	1.52[5]	
	BC028831	Similar to dihydropyrimidine dehydrogenase	III	2.46[4]	
	AF440692	transferrin	III	1.93[4]	
	NM_031188	major urinary protein 1	III	3.25[4]	
Immune response	BC002073.	chemokine c-c ligand 6	I	3.14[4]	5.28[6]
	NM_007651	CD53 antigen	I	1.8[5]	3.25[6]
	NM_021443	small inducible cytokine A8	I	2.14[5]	3.86[6]
	NM_011338	small inducible cytokine A9	I	1.93[5]	2.93[6]
	NM_011888	small inducible cytokine A19	II		1.93[4]
	NM_019932	platelet factor 4	II		1.74[4]
	NM_053094	CD163 antigen	II		1.93[4]
	BF123440	CD24a antigen	III	2.38[5]	
	BC021637	CD68 antigen	III	1.87[5]	
	BE197524	guanylate nucleotide binding protein 2	III	2.38[5]	
	NM_008328	interferon activated gene 203 (Ifi203)	III	1.68[6]	
	NM_008332	interferon-induced protein (Ifit2)	III	1.68[6]	
	M74124	interferon activated gene 205 (Ifi205)	III	4.29[5]	
	NM_008331	interferon-induced protein 1 (Ifit1)	III	3.86[6]	
	NM_010501	interferon-induced protein 3 (Ifit3)	III	2.93[6]	
	NM_013585	proteosome subunit, beta type 9	III	2.07[4]	
	NM_010724	proteosome subunit, beta type 8	III	2.14[6]	

	BC010291	RIKEN cDNA 1110004C05 gene	III	1.8[5]	
	NM_018851	SAM domain and HD domain1	III	1.68[5]	
	BQ033138	2-5 oligoadenylate synthetase-like 2	III	5.1[5]	
Defense	BM224327	Fc receptor, IgG, low affinity Iib	I	1.74[4]	2.46[6]
	BC002070	lymphocyte antigen 6 complex	I	2.22[6]	1.74[5]
	NM_010741	lymphocyte antigen 6 complex, locus C	I	2.55[6]	2.2[5]
	M34962.1	histocompatibility 2, L region	I	1.8[5]	1.52[5]
	L36068.1	MHC class I H2G7 D	I	1.8[4]	1.41[4]
	BC003476	Unknown (protein for MGC:6517)	II		1.57[5]
	NM_008161	glutathione peroxidase 3	II		2.83[5]
	BE688749	histocompatibility 2, class II antigen A alpha	II		1.57[4]
	NM_010382	histocompatibility 2, class II antigen E beta	II		1.68[4]
	S70184	histocompatibility class I antigen H-2Kd	III	1.57[4]	
	NM_010395	histocompatibility 2, T region locus 10	III	1.62[4]	
	NM_010398	histocompatibility 2, T region locus 23	III	2.07[4]	
	M33151	MHC class I H2-L-d glycoprotein	III	1.8[4]	
	M86502	MHC class I protein (H-2Df) mRNA	III	1.74[5]	
	M29881	MHC class I Q89d cell surface antigen	III	2.3[5]	
Cell adhesion	NM_008816	platelet endothelial cell adhesion molecule (Pecam)	I	2.14[6]	1.8[5]
	BE307351	CD36 antigen [8]	II		2[5]
	NM_008404	integrin beta 2 [8]	II		2.22[4]
	X16834	Mac-2 antigen.	II		2.83[5]
	NM_009263	secreted phosphoprotein 1 [8]	II		13.45[5]
	AF361882	endothelial cell-selective adhesion molecule (Ecam)	III	1.57[4]	
	NM_010494	intercellular adhesion molecule 2 (Icam2)	III	1.68[4]	
	NM_011693	vascular cell adhesion molecule 1 (Vcam1) [8]	III	1.68[4]	
	BB493533	integrin alpha 5 (fibronectin receptor alpha)	III	1.6[4]	
	NM_010708.	lectin galactose binding soluble 9	III	2.55[5]	
	NM_010740	lymphocyte antigen 68	III	1.93[4]	
	NM_012050	osteomodulin	III	1.57[4]	
Signaling	NM_007556	bone morphogenetic protein 6	I	2.46[5]	1.93[5]
	NM_013602	metallothionein 1	I	2.46[6]	3.03[6]
	AA796766	metallothionein 2	I	2.38[5]	3.48[5]
	AF199010	PALS2-beta splice variant	I	2.07[4]	2.22[5]
	NM_021400	proteoglycan 4 (megakaryocyte stimulating)	I	2.55[5]	5.46[5]
	AF146523	receptor activity modifying protein 2	I	1.68[5]	2.07[6]
	AF157628	receptor-type protein tyrosine phosphatase	I	1.87[6]	1.52[6]
	BB241535	cytokine inducible SH2-containing protein 3	II		2.55[4]
	AI323359	colony stimulating factor receptor 1	II		1.57[5]
	BM239828	interferon-inducible GTPase	III	2.46[5]	
	NM_008856	protein kinase C eta	III	1.62[4]	
	AF378088	Wrch-1	III	2[4]	
Transcription	NM_010286	glucocorticoid-induced leucine zipper	I	2.07[5]	2.0[4]
	AF201289	TSC22-related inducible leucine zipper 3c	I	2.3[5]	2.38[5]
	BB831146	CCAAT-enhancer binding protein delta [8]	II		1.93[5]
	BC018323	D site albumin promoter binding protein	II		2.07[5]
	NM_011066	homolog 2 (Drosophila) (Per2)	II		3.48[4]
	BB744589	pantophysin	II		2[4]
	NM_011355	SFFV proviral integration 1	II		2.3[5]
	BC017689	Similar to thyrotroph embryonic factor	II		2.07[5]
	U20344	gut-enriched Kruppel-like factor	III	1.8[4]	
	BG069413	Kruppel-like factor 4 (gut)	III	1.52[4]	
	NM_011441	SRY-box containing gene 17	III	2.07[4]	
	NM_009236	SRY-box containing gene 18	III	2[5]	
	BM240719	tripartite motif protein 30	III	1.93[4]	
	AF220015	tripartite motif protein (Trim30Rpt1)	III	2.38[4]	
Complement cascade	NM_007572	complement component 1q	I	1.62[5]	2.73[5]
	NM_009777	complement component 1qb	I	1.8[5]	2.73[5]

	NM_007574	complement component 1qc	I	1.74[6]	2.22[5]
	K02782.1	complement component 3 [7]	I	2.55[6]	2.38[5]
	NM_009780	complement component 4 (within H-2S)	I	2.07[6]	1.93[6]
	NM_009779	complement component 3a receptor 1	II		2.2[4]
	NM_013459	adipsin (Factor D)	II		1.58[5]
Coagulation	NM_028784	factor XIII alpha [8]	I	1.32[5]	1.8[5]
	NM_013473	annexin A8	III	1.8[4]	
	NM_011171	protein C receptor, endothelial [8]	III	1.62[4]	
	AV026492	thrombospondin 1	III	2.93[4]	
Cell cycle	NM_008059	G0/G1 switch gene 2	II		1.87[5]
	NM_008198	histocompatibility 2, cc factor B (H2-Bf)	II		2.83[4]
	NM_016693	mitogen-activated protein kinase kinase kinase 6	II		2[4]
	NM_025427	RIKEN cDNA 1190002H23 gene	II		2.14[4]
	BC011306	clone MGC:7052 IMAGE:3156482	II		1.57[5]
	BC006852	Unknown (protein for MGC:11504)	II		2.07[4]
	AK007630.	cyclin-dependent kinase inhibitor p21 [8]	III	1.68[5]	
Cytoskeleton / development	M76601.	alpha cardiac myosin heavy chain	II		11.71[5]
	L47552DB	cardiac troponin T isoform A2b	II		9.51[5]
	NM_022879	myosin light chain, regulatory A (Mylc2a)	II		7.46[5]
	NM_011619	troponin T2, cardiac (Tnnt2) [8]	II		3.61[5]
	NM_010858	myosin light chain, alkali, embrionic (Myla)	II		2.22[4]
	AF321853	ventroptin-alpha	III	4.59[4]	
Apoptosis	NM_009760	BCL2adenovirus E1B 19 kDa-interacting	I	1.8[5]	1.62[5]
	NM_010907	alpha (Nfkbia)	I	1.32[5]	1.57[5]
	BB221402	fat-specific gene 27	III	3.48[5]	
Not annotated	NM_007796	cytotoxic T lymphocyte-associated protein 2	I	3.14[5]	2.38[5]
	NM_021398	embryonic epithelial gene 1	I	1.46[5]	2.07[5]
	AI467657	expressed sequence AI467657	I	2.14[5]	1.62[5]
	BB667216	expressed sequence AI551257	I	1.68[5]	1.68[5]
	AI551117	expressed sequence AW322500	I	1.68[5]	1.74[5]
	NM_029796	leucine-rich alpha-2-glycoprotein	I	2.46[5]	3.14[6]
	NM_054102	Nd1 (Nd1-pending)	I	3.14[6]	2.14[6]
	NM_011157	proteoglycan, secretory granule	I	1.74[6]	2.0[5]
	BG916808	RIKEN cDNA 0610037M15 gene	I	2.64[5]	1.8[5]
	BC028444	Similar to RIKEN cDNA 2610025P08 gene	I	2.38[5]	1.68[5]
	BC011193	Unknown (protein for MGC:18490)	I	1.52[5]	1.52[5]
	AU067669	ESTs, FEZ1_RAT FASCICULATION	II		1.74[4]
	NM_008016	fibroblast growth factor inducible 15	II		2[4]
	AW212577	fibrosin	II		1.57[4]
	NM_010268	ganglioside-induced differentiation	II		1.8[5]
	NM_008625	mannose receptor, C type 1	II		2.46[5]
	BB409331	Mus musculus, clone IMAGE:4920406	II		1.52[4]
	AI256077	Mus musculus, clone MGC:29256 I	II		3.36[4]
	BB757992	period homolog 3 (Drosophila)	II		3.25[5]
	BC011203	RIKEN cDNA 0610039N19	II		1.8[5]
	NM_134042	RIKEN cDNA 1110038I05 gene	II		1.52[4]
	NM_026835	RIKEN cDNA 1110058E16 gene	II		5.1[4]
	AK007421	RIKEN cDNA 1300003D03	II		1.52[4]
	NM_027209	RIKEN cDNA 1810027D10 gene	II		2.93[4]
	AK015888	RIKEN cDNA 2610318G18 gene	II		1.62[4]
	AK013740	RIKEN cDNA 2900062L11	II		2.07[4]
	BC024581	Similar to HRAS-like suppressor 3	II		1.87[4]
	BC010206	Similar to myosin regulatory light chain	II		1.62[4]
	BC004092	Similar to NS1-binding protein	II		2.38[4]
	BC022943	Similar to plastin 2, L	II		3.14[4]
	BC008107	Similar to tissue inhibitor of metalloproteinase	II		3.14[4]
	NM_009349	thioether S-methyltransferase	II		2.22[5]
	NM_053082	transmembrane 4 superfamily member 7	II		2[5]

	BC024613	Unknown (protein for MGC:25884)	II		2[4]
	BB009037	ceruloplasmin	III	1.62[5]	
	NM_013805	claudin 5	III	1.68[4]	
	BG064656	cytotoxic T lymphocyte-associated 2 beta	III	1.68[4]	
	D63902.1	estrogen-responsive finger protein	III	2.07[4]	
	BB132493	ESTs	III	3.61[5]	
	BM241271	ESTs, similar to A Chain Acyl Thioesterase 1	III	2[4]	
	BB795072	expressed sequence AA959601	III	1.8[4]	
	AY075132	HELICARD	III	1.62[4]	
	NM_008330	interferon gamma inducible protein, 47 kDa	III	3.25[4]	
	AY090098	interferon stimulated gene 12	III	2.14[4]	
	NM_019440	interferon-g induced GTPase (Gtpi-pending)	III	2.14[4]	
	BC02275	interferon-stimulated protein (20 kDa)	III	1.62[4]	
	AI323506	myelin basic protein	III	2[4]	
	NM_011150	peptidylprolyl isomerase C-associated protein	III	1.77[4]	
	NM_023168.	RIKEN cDNA 1110025J15 gene Lag protein	III	1.8[4]	
	BC019452	RIKEN cDNA 1200003C23	III	1.68[4]	
	AK017926	RIKEN cDNA 5830413E08 gene	III	1.87[4]	
	AV244484	RIKEN cDNA 8430417G17 gene	III	2.46[4]	
	NM_018784	sialyltransferase 10 (alpha-2,3-sialyltransferase VI)	III	1.74[4]	
	BC002136	Similar to coronin, actin binding protein 1A	III	2.73[4]	
	NM_011579	T-cell specific GTPase	III	3.73[5]	
	AF173681	thioredoxin interacting factor	III	1.62[4]	
	BB328405	tissue inhibitor of metalloproteinase 4	III	1.93[5]	
	BC011306	Unknown (protein for MGC: 7052)	III	2[4]	
	BC024610	vascular endothelial zinc finger 1	III	1.8[4]	

[1]Clusters I, II, and III are defined in figure 4A. [2]Functional categories assigned using Affymetrix (http:/www.affymetrix.com/analysis) and GeneCards (http:/www.bioinfo.weizmann.ac.il/cards/index.shtml) databases. [3]The Table includes genes displaying at least 1.5-fold up-regulation within the aortic arch at one of the HFC-diet time points versus LF-diet and an associated $p<0.0001$ as determined by the Chi-square test for independence (Chi-square≥23.5, 2k degree freedom) (See Materials and Methods). [4]$p<0.0001$; [5]$p<0.00001$; [6]$p<0.000001$. [7]Genes included in the list of 'Hyperlipemia' genes shown in 'Cardio' (http://cardio.bjmu.edu.cn) (45). [8]Genes included in the list of 'Arteriosclerotic Heart Disease' genes shown in 'Cardio' (http://cardio.bjmu.edu.cn) (45).

Table 2. Genes down-regulated by high-fat versus low-fat diet in aortic arch of apoE-null mice (96 genes): Clusters IV+V+VI [1]

Function [2]	**GeneBank**	**Description.**	**Cluster**	**2 days**	**7 days**
				Fold change (High-fat versus Low-fat) [3]	
Metabolism	BB251523	pyrroline-5-carboxylate synthetase	IV	-1.8[4]	-1.62[4]
	BB276877	expressed sequence AW538652	V	-2.29[5]	
	BB314208	RIKEN cDNA 0610042A05 gene	V	-1.8[4]	
	AK004087	RIKEN cDNA 1300010O06 gene	VI		-1.8[4]
	BF322712	RIKEN cDNA 2310032J20 gene	VI		-1.87[4]
	BG075800	sialyltransferase 1	VI		-1.68[4]
Lipid metabolism	NM_019811	acetyl-Coenzyme A synthetase 1	IV	-2.14[4]	-2.07[4]
	BI247584	farnesyl diphosphate synthetase	V		-2.22[4]
	AF127033	fatty acid synthase	V		-1.62[4]
	NM_010728	lysyl oxidase	V		-2[5]
	AF332052	ATP citrate lyase	VI	-1.86[4]	
Transport	NM_020010	cytochrome P450, 51	V		-4.14[4]
	AV344473	sortin nexin associated golgi protein 1	V		-1.62[4]
	BC027187	Similar to Per1 interacting protein	V		-1.74[4]
	BC003808	Similar to testin	V		-1.57[4]
	BC013068	Unknown (protein for MGC:18501)	V		-2.55[4]
	AB015790	sortilin-related receptor	VI	-1.5[5]	
	NM_007506	ATP synthase, H+ transporting, F0 (Atp5g1)	VI	-1.74[5]	
	BM229554	expressed sequence C76904	VI	-1.51[4]	
Transcription	AV291165	myocyte enhancer factor 2C	IV	-1.6[4]	-1.8[4]
	AF000998	Clock [7]	V		-1.93[4]

	NM_025613	open reading frame 12	V		-1.62[4]
Cell adhesion and extracellular matrix	BB433705	nephronectin	IV	-1.62[4]	-1.52[5]
	D17546	collagen alpha 1 subunit type XV	IV	-1.5[4]	-1.62[5]
	AF011450	collagen type XV	V		-1.93[4]
	AF064749	collagen alpha 3 subunit type VI	V		-1.52[5]
	BF158638	procollagen, type IV, alpha 1	V		-1.57[5]
	AW744319	procollagen, type V, alpha.1	V		-1.52[4]
	BI455189	procollagen, type VI, alpha 2	V		-1.52[4]
	BM202770	cysteine rich protein 61 [7]	V		-1.68[4]
	NM_133721	integrin alpha 9	V		-2.22[5]
	BC004826	Lutheran blood group (Auberger b antigen)	V		-1.62[4]
	NM_028810	ras homolog gene family, member E	V		-1.74[5]
	NM_016894	receptor (calcitonin) activity modifying protein	V		-1.68[5]
	BC003882	regulator of G-protein signaling 4,	V		-2.14[4]
	NM_011607	tenascin C	V		-1.74[4]
	NM_008398	integrin alpha 7	VI	-1.51[4]	
Signaling	BB794656	chondroitin sulfate proteoglycan 4	IV	-2[5]	-1.93[5]
	BC019711	Unknown (protein for MGC:18825)	IV	-1.86[4]	-2.22[5]
	BB329489	Serpin clade H	IV	-1.5[4]	-1.41[4]
	AW491150	expressed sequence AW491150	V		-1.62[4]
	NM_016719	growth factor receptor bound protein 14	V		-1.68[4]
	NM_008520	transforming growth factor beta binding 3 [7]	V		-1.52[4]
	NM_011160	protein kinase, cGMP-dependent, type I	V		-1.8[4]
	BB703307	Ras association domain family 3 protein	V		-1.8[5]
	BC008101	Similar to hypothetical protein MGC2827	V		-1.74[6]
	BC002298	Unknown (protein for IMAGE:3590815)	V		-1.87[5]
	BB371406	frizzled homolog 2 (Drosophila)	VI	-1.62[5]	
	NM_013569	potassium voltage-gated channel H 2	VI	-2[4]	
	BC021308	Similar to LGN protein	VI	-1.74[4]	
Cell proliferation	BC014690.1	Transforming growth factor beta 3	V		-1.74[5]
	AV310588	cyclin D2	VI	-1.5[4]	
Cytoskeleton	NM_007925	elastin [7]	V		-2.3[5]
	NM_008305	perlecan (heparan sulfate proteoglycan 2) [7]	V		-1.62[4]
	AF237627	smooth muscle leiomodin	V		-1.52[4]
	BF578669	smoothelin	VI	-1.5[4]	
Development	BM121216	actin, alpha-2, smooth muscle, aorta	V		-1.87[5]
	AF348968	polypeptide GalNAc transferase-T2	V		-1.62[4]
	NM_009153	immunoglobulin domain (Ig) (semaphorin) 3B	V		-1.8[4]
	BM232388	tropomyosin -1, alpha	V		-1.74[4]
	AV016515	crystallin, alpha B	VI	-2.1[5]	
Not annotated	BB704811	small EDRK-rich factor 2	IV	-1.5[5]	-1.41[4]
	U63408	MRVI1a protein	IV	-1.6[4]	-1.62[4]
	AV330806	RIKEN cDNA 1200014F01 gene	IV	-1.74[5]	-1.62[4]
	BF168366	RIKEN cDNA 2310067E08	IV	-1.74[5]	-2.22[5]
	BC005446	adenylyl cyclase-associated CAP protein	V		-1.93[5]
	BG965405	B-cell translocation gene -2, anti-proliferative	V		-1.52 [4]
	BG967663	creatine kinase, brain	V		-1.8[4]
	NM_009468	dihydropyrimidinase-like 3	V		-2.07[4]
	BB369191	DNA segment, human D4S114	V		-1.57[4]
	BM118398	ESTs	V		-1.62[4]
	BB323985	expressed sequence AI115348	V		-2.46[5]
	AI849305	Mus musculus, clone IMAGE:3590815	V		-2.22[5]
	BB523906	RIKEN cDNA 2010005I16	V		-1.52[5]
	BB403233	RIKEN cDNA 2010015J01 gene	V		-2[4]
	BB556671	RIKEN cDNA 2610528K11 gene	V		-1.52[4]
	BB429147	RIKEN cDNA 2700063G02 gene	V		-1.68[5]
	BB392869	RIKEN cDNA 3230401N03 gene	V		-1.52[4]
	NM_009255	SerpinE2 [7]	V		-1.52[4]

	BC019124	Similar to LIM and cysteine-rich domains	V		-2[5]
	AF343349	TFII-I repeat domain-containing protein 3	V		-1.62[4]
	AF378762	tumor endothelial marker 8 precursor	V		-1.87[4]
	BC022157	Unknown (protein for IMAGE:5134400)	V		-2.46[5]
	M58566	zinc finger protein 36, C3H type-like 1	V		-1.87[4]
	C77389	DNA segment, Chr 1-1, ERATO Doi 99	VI	-1.6[5]	
	BB145729	expressed sequence AU018702	VI	-1.6[4]	
	BB519728	expressed sequence AW546128	VI	-4[5]	
	NM_011838	Ly6/neurotoxin 1	VI	-1.6[4]	
	BB311034	Mus musculus, clone MGC:36474	VI	-2.46[5]	
	BB811478	nucleoplasmin 3	VI	-1.51[4]	
	NM_025368	RIKEN cDNA 1110007C05	VI	-1.8[4]	
	NM_026063	RIKEN cDNA 2900010M23	VI	-1.5[4]	
	NM_133687	RIKEN cDNA 4930415K17	VI	-1.7[4]	
	BB212560	sclerostin	VI	-1.7[4]	
	BC025602	Similar to hypothetical protein LOC57333	VI	-1.74[4]	
	BB453609	WD repeat domain 6	VI	-1.51[4]	

[1]Clusters IV, V, and VI are defined in figure 4A. [2] Functional categories assigned using Affymetrix (http:/www.affymetrix.com/analysis) and GeneCards (http:/www.bioinfo.weizmann.ac.il/cards/index.shtml) databases. [3] The Table includes genes displaying at least 1.5-fold down-regulation within the aortic arch at one of the HFC-diet time points versus LF-diet and an associated $p<0.0001$ as determined by the Chi-square test for independence (Chi-square≥23.5, 2k degree freedom) (See Materials and Methods). [4] $p<0.0001$; [5] $p<0.00001$; [6] $p<0.000001$. [7]Genes included in the list of 'Arteriosclerotic Heart Disease' genes shown in 'Cardio' (http://cardio.bjmu.edu.cn) (45).

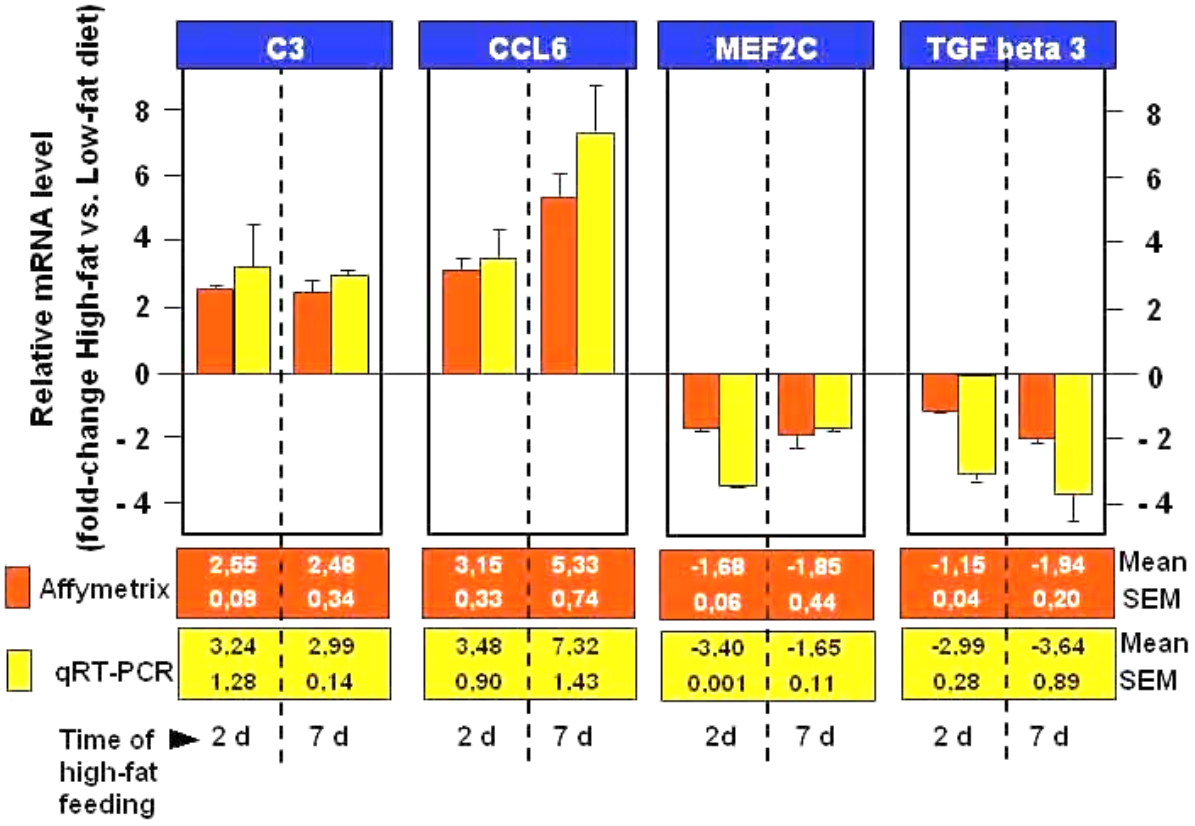

Figure 3. Comparison of qRT-PCR and microarray analysis for selected genes regulated by high-fat diet. qRT-PCR was performed for complement component 3 (C3), chemokine c-c ligand-6 (CCL6), myocyte enhancer factor-2C (MEF2C), and transforming growth factor-beta 3 (TGF beta 3). RNA for qRT-PCR was obtained from the pool of 3-4 aortic arches and results represent the mean ± SEM of 2 independent experiments (See Methods).

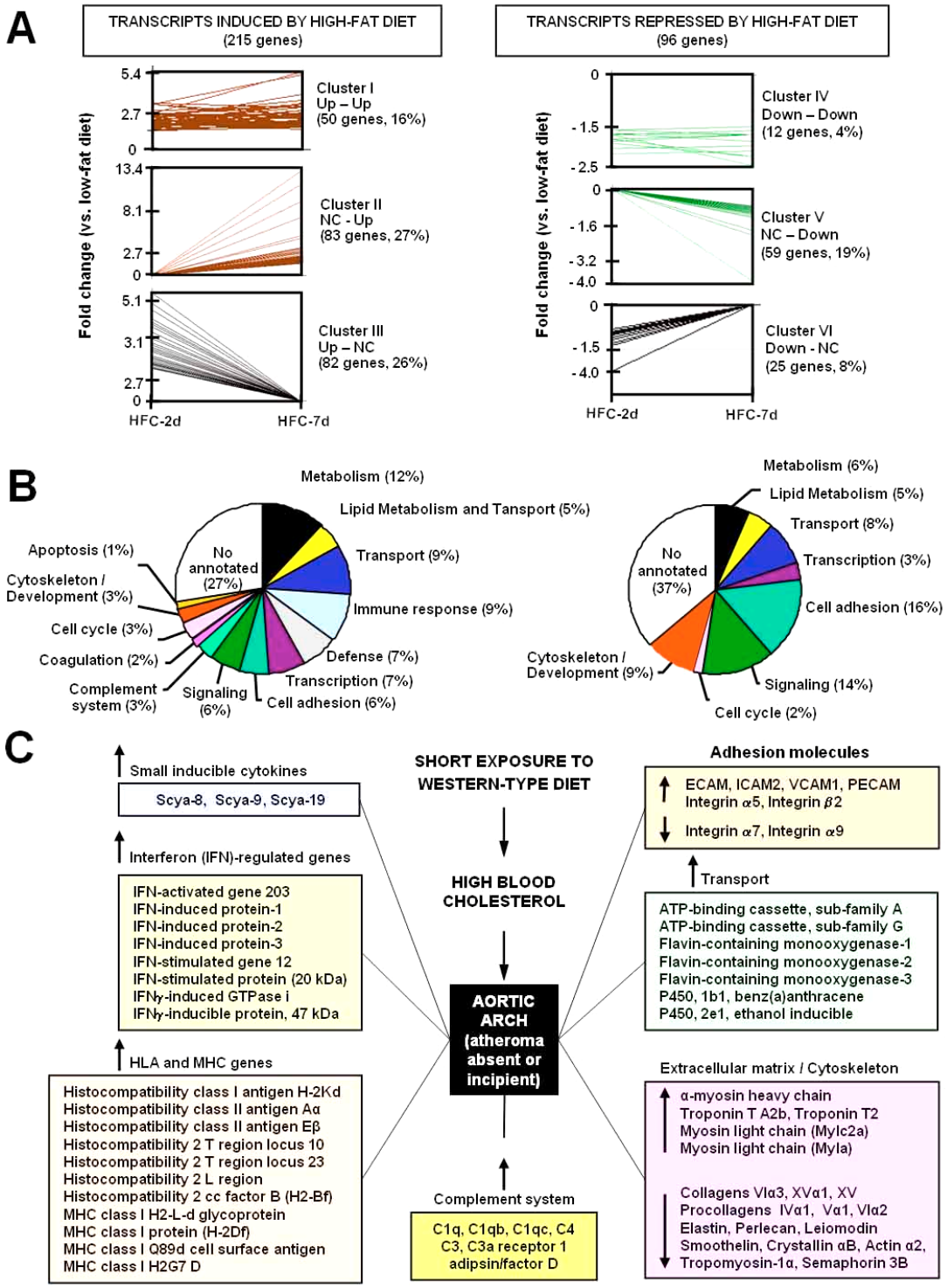

Figure 4. Temporal profile and functional clustering of genes differentially regulated in the aortic arch of fat-fed versus control apoE-null mice. A. K-means clustering of 311 genes displaying diet-induced regulation obtained with the JExpress software using their fold-change in HFC-diet versus LF-diet. Six clusters were generated according to the temporal pattern of gene expression (Up: up-regulated; Down: down-regulated; NC: No Change). Each line represents one gene. B. Classification of the 215 up-regulated (Cluster I+II+III, left) and 96 down-regulated (Cluster IV+V+VI, right) genes by functional category using the Affymetrix and GeneCards databases. Genes for which annotation information was not available are shown as "Not annotated". C. HFC-diet elicits rapid and coordinated changes in the expression of functionally-related murine genes in the aortic arch before significant atheroma formation. Up and down arrows indicate genes displaying diet-dependent up-regulation and down-regulation, respectively.

and GeneCards (www.bioinfo.weizmann.ac.il/cards/index.shtml) databases, we ascribed biological functions to genes displaying up-regulation (Clusters I+II+III; figure 4B, left) and down-regulation (Clusters IV+V+VI; figure 4B, right). Genes significantly changed in both up-regulated and down-regulated clusters are involved in metabolism and transport (26% Up, 19% Down), cell adhesion (6% Up, 16% Down) and signaling (6% Up, 16% Down). Biological processes substantially represented by up-regulated genes included immune response (9%), defense (7%), and transcription (7%), whereas cytoskeletal and developmental genes represented 9% of down-regulated genes. A more precise analysis revealed striking examples of concerted regulation of functionally-related genes (figure 4C), including eight genes involved in cell adhesion (ECAM, ICAM2, VCAM1, PECAM, integrins alpha 5, alpha 7, alpha 9 and beta 2), six interferon-regulated genes, nine histocompatibility genes, two ATP-binding cassette genes (ABCA1 and ABCG1-WHITE), three flavin-containing monooxygenases (Fmo-1, Fmo-2 and Fmo–3), three small inducible cytokines (Scya-8, Scya-9 and Scya-19), six collagen and procollagen genes (collagens VI alpha 3, XV alpha 1, XV; procollagens IV alpha 1, V alpha 1, VI alpha 2), and seven components of the complement system (C1q, C1qb, C1qc, C3, C3ar1, C4 and adipsin/Factor D).

5. DISCUSSION

Previous studies using microarrays have examined gene expression alterations associated with advanced stages of human atherosclerosis by comparing carotid fibrous cap versus adjacent media (13), primary versus recurrent carotid stenotic lesions (14), coronary atheroma from patients with stable versus unstable angina (15), and advanced stable versus ruptured atherosclerotic plaques (16). Transcriptional profiling has also been performed in aorta with established atheromaa versus non-atherosclerotic aorta of apoE-null mice (17,18). These studies have identified novel genes and signaling networks that may play important roles at advanced stages of atherosclerosis when prominent atheromatous plaques are present. In our study, we transcriptionally profiled more than 18,000 murine genes to identify changes in gene expression that may mediate early pathological processes in the aortic arch of apoE-null mice maintained short-term on a Western type atherogenic diet, when severe hypercholesterolemia is manifest but before or at the onset of atheroma formation.

Our results and those from previous transcriptional profiling studies (13-19) are consistent with the notion that early and advanced human and murine atherosclerosis is a complex multifactorial disease involving different cellular processes and regulatory networks (2,3) (figure 4B). Among the genes displaying diet-dependent regulation in our study, we noted striking examples of coordinated regulation of functionally-related genes (figure 4C), such as several histocompatibility (HLA and MHC) genes, interferon-regulated genes, cell adhesion molecules, flavin-containing monooxygenases, small inducible cytokines, extracellular matrix components, cytoskeletal genes and components of the complement system. Differentially-regulated transcripts represented both novel genes potentially involved in the onset of vessel damage, and genes whose role in different aspects of the atherogenic process is well recognized, e.g., ABCA1 and ABCG1 (20-22), adhesion molecules VCAM1, ICAM2, ECAM, PECAM and several integrins (23-27), CD163, CD68 and scavenger receptor CD36 (28-30), and several collagens and procollagens (23,31-34). Our studies revealed a rapid and coordinated up-regulation of several murine complement components (C1q, C1qb, C1qc, C3, C3ar1, C4 and adipsin/Factor D) in the aortic arch of mice shortly exposed to HFC-diet before significant lesion development occurs. Of note in this regard, advanced human and murine atheromas abundantly express early and terminal complement components and complement regulatory proteins (i.e., C3d, C5b-9, C1q, gC1q-R, decay accelerating factor, and factor H), which may be triggered by immunocomplexes, C-reactive protein, oxidized lipoproteins, cholesterol crystals and apoptotic cells (35-44). Thus, complement activation may contribute to both early and advanced stages of atherosclerosis.

Of the 311 genes shown in Table 1 and Table 2, only C3 and lipoprotein lipase are present in the list of 72 'Hyperlipemia' genes shown in 'Cardio', a web-based knowledge resource of genes and proteins related to CVD recently created after a thorough survey of published reports on experimental and human cardiovascular disease models (http://cardio.bjmu.edu.cn) (45). Moreover, only 20 genes in Table 1 and Table 2 are amongst the list of 281 'Arteriosclerotic Heart Disease' genes shown in 'Cardio'. Aortic upregulation of VCAM1, PECAM, glutathione peroxidase 3, interferon-induced protein 1, and gut-enriched Kruppel-like factor was found in apoE-null mice fed HFC-diet either for 2-7 days (this study) or 10-20 weeks (17), suggesting the involvement of these genes in both early and advanced stages of atherosclerosis. Conversely, aortic induction of H2D1, Pdgfc, Cd47, Agpt2, Mglap, Xdh, Th, and Ctsc was observed in apoE-null mice fed HFC-diet for 4-40 weeks (18), but not after short exposure to HFC-diet (this study), suggesting that these genes participate only in advanced phases of atherosclerosis.

Napoli *et al*. (19) demonstrated that maternal hypercholesterolemia in LDL receptor (LDLR)-deficient mice more than doubled atheroma size in the aortic origin of their normocholesterolemic chow-fed offspring at 3 months. The authors subsequently performed microarray analysis of 11,000 murine genes in the nonatherosclerotic descending aorta and identified 139 genes displaying differential expression in normocholesterolemic offspring of hypercholesterolemic LDLR-null mothers. Although important differences in the experimental design exist between our study and that of Napoli et al. (19), we observed comparable profiles for flavin-containing monooxygenase-3 (up-regulation) and integrin alpha-7 (downregulation).

To the best of our knowledge, this is the first large-scale microarray study reporting rapid alterations in gene expression induced in the artery wall by HFC-diet, when

severe hypercholesterolemia has developed but prior to or at the onset of atheromatous lesion formation. We have identified 311 genes that are potentially involved in the initiation of vessel injury induced by dietary fat and cholesterol, most of which have not been previously associated to hyperlipemia or atherogenesis. These candidate genes have been grouped on the basis of their temporal pattern of expression and their assigned biological functions. Our studies establish a strong foundation for further expression and functional studies to assess novel hypothesis on the initiation of vessel damage induced by hypercholesterolemia. Future studies with differentially regulated genes identified by our study will further our basic knowledge of atheroma initiation, thus providing a rational basis for the development of novel diagnostic tools and for implementation of drug-discovery programs.

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Abbreviations: apoE: apoliporotein E; CVD: cardiovascular disease; HFC-diet: high-fat cholesterol-rich diet**;** LDL: low-density lipoprotein; LDLR: LDL receptor; LF-diet: low-fat standard diet; VSMC: vascular smooth muscle cell.

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