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Homocysteine induces inflammatory transcriptional signaling in monocytes

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1. ABSTRACT

Hyperhomocysteinemia (HHcy) is an independent risk factor for cardiovascular disease. Here, we studied transcriptional regulation in homocysteine (Hcy)-induced gene expression in monocytes (MC). We identified 11 Hcy-induced genes, 17 anti-inflammatory cytokine interleukin 10-induced, 8 pro-inflammatory cytokine interferon gamma (IFN gamma)-induced and 8 pro-inflammatory cytokine tumor necrosis factor alpha (TNF alpha)-induced genes through literature search. Binding frequency of 36 transcription factors (TFs) implicated in inflammation and MC differentiation were analyzed within core promoter regions of identified genes, and classified into 3 classes based on the significant binding frequency to the promoter of Hcy-induced genes. Class 1 TFs exert high significant binding frequency in Hcy-induced genes. Class 2 and 3 TFs have low and no significant binding frequency, respectively. Class 1 TF binding occurrence in Hcy-induced genes is similar to that in IFN gamma -induced genes, but not that in TNF alpha -induced. We conclude that Hcy is a pro-inflammatory amino acid and induces inflammatory transcriptional signal pathways mediated by class 1 TF. We term class 1 TF as putative Hcy-responsive TFs.

2. INTRODUCTION

Hyperhomocysteinemia (HHcy) has been established as an independent risk factor for cardiovascular disease (CVD) (1, 2). Meta-analysis shows that an increase of 5 μmol/L in plasma homocysteine (Hcy) levels enhances the risk of CVD by 1.6- to 1.8-fold, which is similar to the risk seen with an increase of 20 mg/dL (0.52 mmol/L) in cholesterol concentration (3).

The causative role of HHcy in human CVD remains controversial. Several secondary prevention trials of Hcy-lowering therapy were reported to have no effect on combined endpoints of cardiovascular events (4). Hcy-lowering is found beneficial to reduce the risk of overall stroke in the HOPE 2 Trial. Indirect evidence for such a benefit was recently obtained a large population-based cohort study, which demonstrated that Hcy-lowering due to folic acid fortification significantly (5, 6).

Reports from our laboratory and others have established that HHcy accelerates vascular inflammation and atherosclerosis in mice (7-9). It has been suggested that HHcy accelerates atherosclerosis via inhibiting endothelial cell growth and impairing post-injury endothelialization (2,

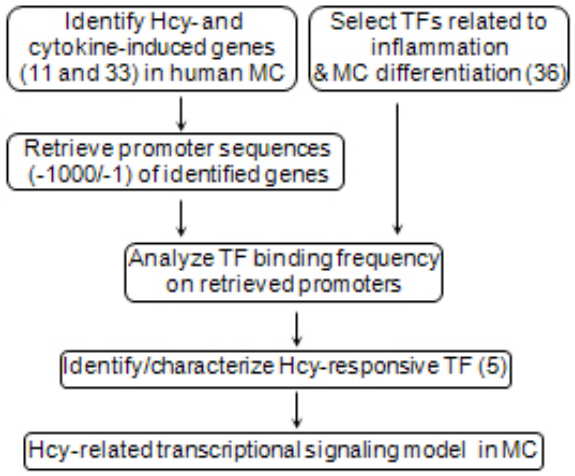

Figure 1. Flow chart of database mining and TF binding pattern analysis in the promoters of Hcy- and cytokines-induced genes. Genes induced by Hcy and 20 cytokines, including pro-inflammatory cytokines TNFα and IFNγ, and anti-inflammatory cytokine IL-10, were identified by literature search. Promoter regions (-1000/-1) of identified genes were retrieved from the NIH/NCBI gene database (http://www.ncbi.nlm.nih.gov/gene/). Binding sites of 36 inflammation & MC differentiation-related TFs were analyzed using TESS (http://www.cbil.upenn.edu/cgi-bin/tess/tess). Putative Hcy-responsive TF were identified and characterized. Hypothetical transcriptional signal pathways of Hcy-induced MC inflammation/differentiation were proposed. MC, monocyte; TF, transcription factor; Hcy, homocysteine; TNFα, tumor necrosis factor α; IFNγ, interferon γ; IL-10, interleukin 10; TESS, transcription element search system.

10, 11), promoting vascular smooth muscle proliferation (12) and inflammatory monocyte (MC) differentiation (13).

MC activation and its recruitment into the artery wall are key cellular events during the development of atherosclerosis. MC can differentiate into macrophages and become lipid-laden foam cells in the arterial walls. Recent notions indicate that MCs can differentiate into inflammatory MC subsets and contribute to vessel wall inflammation (14). We recently reported that HHcy increases the differentiation of the inflammatory $Ly6C^{high/middle}$ MC subsets, promotes the accumulation of inflammatory MCs/macrophages in the vessel wall, and accelerates atherosclerosis in a HHcy-enhanced atherosclerotic mouse model of HHcy and hyperlipidemia (13). However, how HHcy promotes the differentiation of non-inflammatory MCs into inflammatory subsets remains unknown; and transcriptional mechanisms underlying HHcy-induced pro-inflammatory effects and MC differentiation has not been studied.

Several studies on the transcriptome (all the mRNAs being expressed) have showed that Hcy (≥100 μM), induces pro-inflammatory gene expression in endothelial cells (15, 16). The effect of Hcy on inflammatory gene expression has been investigated by several groups (17-21). These individual studies revealed that Hcy induces inflammatory gene expression in MC. However, a summarized analysis of Hcy's effects on inflammatory gene expression in MC is missing.

Transcription factors (TFs) are master genes that regulate gene expression and impact their associated pathways (22). Research to identify TF binding profile has become emerging frontier and fulfills an urgent need for the identification of therapeutic molecular targets. Transcriptional regulation of Hcy-modulated gene expression has not been studied in a systemic manner.

The complete human genome DNA sequence has been deposited in the National Institutes of Health (NIH) database, making the retrieval of experimentally-identified gene promoter sequences possible. In addition, nearly 2,000 TFs and their binding sequences have been experimentally characterized and placed in the searchable web-based transcription element search system (TESS) and other databases (23). These advances make the identification of TF binding patterns in the promoters of genes feasible.

In this study, we summarized Hcy- and cytokine-induced genes in human MC through extensive literature and data base search, analyzed their relevant TF binding profile, identified putative Hcy-responsive TFs and established a hypothetic model of Hcy transcriptional signaling (Figure 1). Our study is the first to use dynamic approaches to systemically summarize gene regulation findings in the literature, combining with database mining and bioinformatics analysis, leading to the identification of TF binding profiling and models of transcriptional signaling.

3. MATERIALS AND METHODS

3.1. Identification of Hcy- and cytokine-induced genes in human MCs

Extensive literature search was performed using the NIH/PubMed database (http://www.ncbi.nlm.nih.gov/pubmed/) to identify Hcy- and cytokines-induced genes. All Genes induced by Hcy, pro-inflammatory cytokine tumor necrosis factor α (TNFα), pro-inflammatory cytokine interferon γ (IFNγ), and anti-inflammatory cytokine interleukin 10 (IL-10) in human MC were identified and listed in Figure 2. All selected genes were validated for their transcriptional regulation in their original publication. The Gene ID numbers of the identified genes were obtained from the NIH/National Center of Biotechnology Information (NCBI) gene database (http://www.ncbi.nlm.nih.gov/gene/). The fold change of gene expression was obtained from the original publication if specified, or derived from the published graphs if not specified. CD36 levels were determined by flow cytometry (24). SOD1 levels were examined by western blot (21). All other identified genes were examined by real-time PCR or

A. Hcy-induced genes in human MCs (11)

Gene Name	Abbreviation	Fold change	Cells	Hcy (μM)	Treatment time (hr)	NCBI Gene ID	Reference	Gene Function
Chemokine (C-C motif) receptor 2	CCR2	2.4[a]	THP-1	1000	6-48	1231	[17]	Inflammation
Cluster of differentiation 36	CD36	1.41[a]	THP-1	200	72	948	[24]	Inflammation
Interleukin-1β	IL-1β	1.5[a]	PBMC	740[b]	72	3553	[18]	Inflammation
Interleukin-6	IL-6	1.3[a]	PBMC	740[b]	72	3569	[18]	Inflammation
Interleukin-8	IL-8	2-4	PBMC	100	96	3576	[19]	Inflammation
Interleukin-12β	IL-12β	1.9[a]	PBMC	740[b]	72	3593	[18]	Inflammation
Monocyte chemoattractant protein-1	MCP-1	2-4	PBMC	100	96	6347	[19]	Inflammation
Tumor necrosis factor-α	TNF-α	1.3[a]	PBMC	740[b]	72	7124	[18]	Inflammation
Sialic acid binding Ig-like lectin 1	SIGLEC-1	6.3[a]	PBMC	NS	NS	6614	[20]	Cell adhesion
Superoxide dismutase 1, soluble	SOD1	7.25[a]	THP-1	100	12	6647	[21]	Antioxidant
Thioredoxin	Thioredoxin	5[a]	THP-1	100	12	7295	[21]	Antioxidant

B. Cytokines-induced genes in human MCs (33)

Gene Name	Abbreviation	Fold change	Cells	NCBI Gene ID	Reference	Gene Function
TNFα (pro-inflammatory cytokine) induced genes						
Interleukin 1, alpha	IL-1α	1.3	THP-1	3552	[32]	Inflammation
Interleukin 1, beta	IL-1β	8.7	THP-1	3553	[32]	Inflammation
Interleukin 8	IL-8	1.7	THP-1	3576	[32]	Inflammation
Pro-platelet basic protein	PPBP	4.0	THP-1	5473	[32]	Inflammation
Catenin alpha-1	CTNNA1	6.0	THP-1	1495	[32]	Cell junction
Jun B proto-oncogene	JUNB	1.3	THP-1	3726	[32]	Cell signaling
PRKD3 protein kinase D3	PRKCN	2.7	THP-1	23683	[32]	Cell signaling
Transcription factor 7	TCF7	3.0	THP-1	6932	[32]	Immune response
IFNγ (pro-inflammatory cytokine) induced genes						
Chemokine (C-C motif) receptor 2	CCR2	10[a]	PBMC	1231	[33]	Inflammation
Cluster of differentiation 40	CD40	↑	PBMC	958	[36]	Inflammation
Cluster of differentiation 86	CD86	4[a]	PBMC	942	[37]	Inflammation
Platelet-activating factor receptor	PTAFR	3-4[a]	PBMC	5724	[38]	Inflammation
Complement component 2	C2	2.8[a]	PBMC	717	[39]	Complement activation
Complement factor B	CFB	6.9[a]	PBMC	629	[39]	Complement activation
Cytochrome b-245, beta polypeptide	CYBB	5-8[a]	PBMC	1536	[40]	Oxidative stress
Fc-gamma receptor I A1	FCGR1A	↑	PBMC	2209	[41]	Immune response
IL-10 (anti-inflammatory cytokine) induced genes						
15+ NAD hydroxyprostaglandin dehydrogenase	HPGD	4.2	PBMC	3248	[42]	Anti-inflammation
Cluster of differentiation 163	CD163	7.9	PBMC	9332	[42]	Anti-inflammation
Ig-like receptor subfamily A with TM domain	LILRA2	3	PBMC	11027	[42]	Anti-inflammation
Ig-like receptor subfamily A without TM domain	LILRA1	3	PBMC	11024	[42]	Anti-inflammation
IL-1 receptor antagonist	IL1RN	4.3	PBMC	3357	[42]	Anti-inflammation
S100 calcium-binding protein A9	S100A9	3.6	PBMC	6280	[42]	Anti-inflammation
Pre-B cell colony-enhancing factor	PBEF	3.1	PBMC	10135	[42]	Atherogenesis
Versican	VCAN	3.5	PBMC	1462	[42]	Cell adhesion
Protein tyrosine phosphatase, non receptor type 1	PTPN1	3.5	PBMC	5770	[42]	Cell signaling
Suppressor of cytokine signaling 3	SOCS3	3.1	PBMC	9021	[42]	Cell signaling
Glycerol kinase	GK	3.2	PBMC	2710	[42]	Glucose metabolism
Interferon induced transmembrane protein-3	IFITM3	3.1	PBMC	10410	[42]	Immune response
Interleukin 7 receptor	IL-7R	4.1	PBMC	3575	[42]	Inflammation
Signaling lymphocytic activation molecule	SLAM	4.3	PBMC	6504	[42]	Inflammation
UDP-N-acetyl-α-galactosamine	GALNT1	3.1	PBMC	2589	[42]	Protein glycosylation
Acid sphingomyelinase like phosphodiesterase	SMPDL3B	3	PBMC	27293	[42]	Myelin metabolism
ELL-related RNA polymerase II enlongation factor	ELL	3.5	PBMC	8178	[42]	Transcription regulation

Figure 2. Identification of genes induced by Hcy and cytokines in human MC. Genes induced by Hcy, pro-inflammatory cytokines and anti-inflammatory cytokine in human MC were identified by literature search using NIH/PubMed database. A. Hcy-Induced genes. 11 Hcy-induced genes were identified. B. Cytokines-induced genes. 8 TNFα induced genes, 8 IFNγ-induced genes, and 17 IL-10-induced genes were identified. [a], represents fold change quantified from original graphs; [b], represents converted unit from original data; Reference, consistent with the text and details in the "References" section; NS, not specified; MC, monocyte; TF, transcription factor; Hcy, homocysteine; TNFα, tumor necrosis factor α; IFNγ, interferon γ; IL-10, interleukin 10, PBMC, peripheral blood mononuclear cell; THP1, a human monocytic leukemia cell line.

Northern blot (17-21). Further, in order to screen genes induced by both Hcy and pro-inflammatory cytokines, we searched for genes (25-33) induced by 20 cytokines included 5 MC differentiation cytokines [granulocyte macrophage colony stimulating factor (GM-CSF), CSF-1, TNFα, IL-4 and IFNγ], 11 pro-inflammatory cytokines [macrophage inflammatory protein-1α (MIP-1α), TNFβ, IL-1β, IL-2, IL-3, IL-6, IL-7, IL-8, IL-12, IL-15 and IL-18] and 4 anti-inflammatory cytokines [transforming growth factor (TGFβ), IL-9, IL-10 and IL-11]. The overall database mining strategy is illustrated in a flowchart shown in Figure 1.

Selected TFs related to inflammation and MC differentiation (36 TFs)

Name of transcription factor	Abbreviation	Function	Reference PMID#
ETS like gene 1/serum response factor	Elk-1/SRF	Inflammation	20137095
Forkhead box O	FOXO	Inflammation	17729040
Glucocorticoid receptor	GR	Inflammation	15541765
Heat shock transcription factor	HSF	Inflammation	21723507
Nuclear respiratory factor 2&nuclear respiratory factor 1	Nrf2&Nrf1	Inflammation	9872330
Peroxisome proliferator-activated receptors	PPAR	Inflammation	11135615
TCF transcription factor/lymphoid enhancer-binding factor	TCF/LEF	Inflammation	19516021
Early growth response protein 1	EGR1	MC differentiation	17934488
ETS related gene 1	Erg -1	MC differentiation	9291089
Myelocytomatosis oncogene/MYC associated factor X	Myc/Max	MC differentiation	8224841
Activator protein 1	AP-1	Inflammation/MC differentiation	11400164/2169351
CCAAT-enhancer-binding proteins	C/EBP	Inflammation/MC differentiation	9792624/18776924
cAMP response element binding	CREB	Inflammation/MC differentiation	17975014/9030636
Hypoxia-inducible factor-1	HIF-1	Inflammation/MC differentiation	12628185/6510872
Myocyte enhancer factor-2	MEF2	Inflammation/MC differentiation	15178640/15706034
Nuclear factor κ-light-chain-enhancer of activated B cells	NFκB	Inflammation/MC differentiation	1065222/11887466
Tumor protein 53	p53	Inflammation/MC differentiation	12518062/4713961
SMAD family member 2/3/4	SMAD2/3/4	Inflammation/MC differentiation	12393416/15972691
Specificity protein 1	SP1	Inflammation/MC differentiation	20861353/12213324
Signal transducer and activator of transcription 1/1	STAT1/1	Inflammation/MC differentiation	10383940/15181831
Signal transducer and activator of transcription 1/2	STAT1/2	Inflammation/MC differentiation	18678606/19244315
Signal transducer and activator of transcription 3	STAT3	Inflammation/MC differentiation	19851315/10918585
Vitamin D receptor	VDR	Inflammation/MC differentiation	6319426/15322208
Nuclear factor of activated T-cells	NFAT	Inflammation/MC differentiation	15004161/17595377
Retinoic acid receptor	RAR	Inflammation/MC differentiation	10942397/1667479
E-twenty six	ETS	Inflammation/MC differentiation	17122446/9544570
Hematopoietic transcription factor PU.1	PU.1	Inflammation/MC differentiation	15914556/20431622
IFN consensus sequence binding protein/IFN regulatory factor 8	ICSBP/ IRF-8	Inflammation/MC differentiation	11846985/11846986
Krueppel-like factor-4	KLF4	Inflammation/MC differentiation	17339326/17762869
Maf musculoaponeurotic fibrosarcoma oncogene homolog B	MafB	Inflammation/MC differentiation	10790365/17079784
Proto-oncogene c-maf	c-Maf	Inflammation/MC differentiation	9815263/10477683
Estrogen receptor	ER	Anti-atherogenic	10615426
Androgen receptor	AR	Pro-atherogenic	11245640
E2F transcription factor 1/DP1 transcription factor	E2F/DP1	Cell proliferation	10439043
Recombination signal binding protein for immunoglobulin kappa J region	RBP-JK	Cell-cell communications	8876239
Octamer-4	Oct4	Differentiation & Development	15882627

Figure 3. TFs related to inflammation and MC differentiation. 36 TFs were selected based on their identified functions related to inflammation and MC differentiation. We used PubMed ID# to cite knowledge information regarding the function of 36 TF in inflammation and MC differentiation. MC, monocyte; TF, transcription factor.

3.2. TF selection and classification

36 TFs were selected based on their identified function implicated in inflammation and MC differentiation via literature search (Figure 3). The DNA sequences of 1,000 bp upstream of the transcription start site of the identified genes listed in Figure 2 were retrieved from the NIH/NCBI gene database and defined as putative core promoters. TF binding sites on the putative promoters were determined by using the publicly accessible TF database TESS (http://www.cbil.upenn.edu/cgi-bin/tess/tess) (34). Binding frequency denotes the number of the binding sites identified for the TF on the core promoter region. A confidence interval of binding frequency for each TF was established by evaluating the binding frequency on 4 housekeeping genes, including β-actin (ACTB), fructose-bisphosphate aldolase A (ALDOA), rho GDI 1 (ARHGDIA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (35), and 17 IL-10-induced genes (Figure 2B). These four genes are the mostly consistently expressed gene cross tissues in human and mouse as described in our recent database mining studies (35). An upper threshold of the confidence interval was set as mean+3X standard deviations (SD) of the TF binding frequencies in the promoters of 4 housekeeping genes and 17 IL-10-induced genes, and marked as the dashed lines. Binding frequency higher than the upper threshold of the confidence interval ($p < 0.01$) was defined as significant binding frequency. TFs with identified corresponding binding element were classified into three groups, class 1, 2 and 3, based on the pattern of significant binding frequency on Hcy-induced genes.

3.3. TF occurrence in the promoter of Hcy- and pro-inflammatory cytokines-induced genes

The occurrence of class 1 TFs in the promoter regions of Hcy- and pro-inflammatory cytokines TNFα and

Class 1 TF:	HSF	MEF2	NFκB	NFAT	KLF4	
Hcy induced genes						
CD36	+	+	+	+	+	
IL-6	+	+	+	+	+	
IL-12β	+	+	+	+	+	
IL-1β	+	–	+	+	+	
IL-8	+	+	+	+	–	
CCR2	+	+	–	–	+	
SOD1	+	–	+	+	–	
MCP1	+	+	–	–	–	
SIGLEC1	+	–	–	–	–	
THIOREDOXIN	+	–	–	–	–	
TNFα	–	–	–	–	–	
Ratio:	10/11	6/11	6/11	6/11	5/11	
IFNγ induced genes						
C2	+	+	+	+	+	
CD86	+	–	+	+	+	
CFB	+	–	+	+	+	
DC40	+	+	+	+	–	
FCGR1A	+	+	+	+	–	P=0.242
CCR2	+	+	–	–	+	
CYBB	+	+	–	+	–	
PTAFR	+	–	+	+	–	
Ratio:	8/8	5/8	6/8	7/8	4/8	
TNFα induced genes						
IL-1	+	–	+	+	+	P=0.032
IL-8	+	+	+	+	–	
PPBP	+	+	–	–	–	
IL-1α	+	–	–	–	–	
PRKCN	+	–	–	–	–	
CTNNA1	–	–	–	–	–	
JUNB	–	–	–	–	–	
TCF7	–	–	–	–	–	
Ratio:	5/8	2/8	2/8	2/8	1/8	

Figure 4. Class 1 TF biding site distribution in the promoter regions of Hcy- and pro-inflammatory cytokine-induced genes. The occurrence (presence of class 1 TFs in the promoter regions of Hcy- and pro-inflammatory cytokines TNFα and IFNγ-induced genes) were listed. TFs with at least one binding site on the promoter of indicated genes were recorded as occurrence positive (+), whereas, TFs that did not have binding sites on the promoter of indicated genes were recorded as occurrence negative (-). Ratio of occurrence positive on selected genes was calculated. Ratio of class 1 TFs in TNFα– and IFNγ–induced genes to Hcy–induced genes were compared and independent *t* -tests were performed to determine the *p* value of paired groups as indicated. MC, monocyte; TF, transcription factor; Hcy, homocysteine; TNFα, tumor necrosis factor α; IFNγ, interferon γ; HSF, heat shock factor; MEF2, monocyte enhancer factor-2; NFAT, nuclear factor of activated T-cells; NFκB, nuclear factor kappa light chain enhancer of activated B cells; KLF4, krueppel-like factor 4.

IFNγ-induced genes were analyzed (Figure 4). TFs with at least one binding site on the promoter of indicated genes were recorded as occurrence positive (+), whereas, TFs that did not have binding sites on the promoter of indicated genes were recorded as occurrence negative (-). Ratio of occurrence positive on selected genes was calculated. Independent *t* test was used to determine the *p* value of paired groups. A probability value $p < 0.05$ was considered to be significant.

4. RESULTS

4.1. Hcy preferentially induced pro-inflammatory genes in human MC

Via extensive literature search, we identified 11 genes whose expressions are induced by pathogenic concentrations of Hcy (≥100μM) in cultured human PBMC or THP1 cells (a human monocytic leukemia cell line) (Figure 2A). Among these 11 genes, 8 are pro-inflammatory genes, including IL-8, MC chemoattractant protein-1 (MCP-1), IL-1β, IL-6, TNFα, IL-12β, chemokine (C-C motif) receptor 2 (CCR2) and cluster of differentiation 36 (CD36). All selected genes were validated for their transcriptional regulation in their original publication. The fold change of gene expression level is indicated in Figure 2A. Interestingly, Hcy also reduced the expression of 2 anti-inflammatory TFs peroxisome proliferator-activator receptor (PPAR) α and PPARγ (data not shown).

4.2. Hcy shares features with pro-inflammatory cytokines in inducing inflammatory genes in human MC

To compare the feature of Hcy, pro- and anti-inflammatory cytokines in inducing gene expression in human MC, we further examined genes induced by pro-inflammatory cytokines TNFα and IFNγ, and anti-inflammatory cytokine IL-10 in human MC through literature search. We identified 8 genes induced by TNFα (32), 8 genes by IFNγ (33, 36-41), and 17 genes induced by IL-10 (42) (Figure 2B). All selected genes were validated for their transcriptional regulation in their original publication. The fold change of gene expression level is indicated in Figure 2B. We found that TNFα and IFNγ each induced specific inflammatory genes, respectively. Interestingly, we found that Hcy induced IFNγ responsive gene CCR2 and TNFα responsive genes IL-1β and IL-8.

Since both HHcy (7) and pro-inflammatory cytokines have been shown to promote atherogenesis (43), we examined mRNA expression induced by 20 cytokines (three groups), including 5 MC differentiation cytokines, 11 pro-inflammatory cytokines and 4 immunosuppressive cytokines. We found that 5 Hcy-induced genes (CCR2, IL-1β, IL-6, IL-8 and MCP-1) are also induced by pro-inflammatory cytokines (Figure 5), but not by anti-inflammatory cytokines.

4.3. Class 1 TFs exert high significant binding frequency on the promoter of Hcy-induced genes and are termed as putative Hcy-responsive TFs

To identify a MC relevant transcriptional profile of Hcy-induced genes, we selected 36 TFs which are implicated in inflammation and MC differentiation (Figure 3). These include 6 TFs with identified functions in facilitating MC differentiation, such as E-twenty six (ETS), hematopoietic transcription factor PU.1 (PU-1), IFN consensus sequence binding protein/IFN regulatory factor 8 (ICSBP/IRF-8), krueppel-like factor-4 (KLF4), Maf

Genes induced by both Hcy and pro-inflammatory cytokines in MC

	Gene name	Hcy	Reference
Pro-inflammatory cytokines	IL-1β	IL-6	[25]
	IL-2	IL-6 IL-8	[25,26]
	IL-7	IL-6 IL-8	[27,28]
	IL-15	IL-8 MCP-1	[29]
	IL-18	IL-1β IL-8	[30]
	GM-CSF[c]	MCP-1	[31]
	TNF-α[c]	IL-1β IL-8	[32]
	IFN-γ[c]	CCR2	[33]

Figure 5. Identification of genes induced by both Hcy and cytokines in human MC. Genes induced by Hcy and 20 cytokines including 5 MC differentiation cytokines (GM-CSF, CSF-1, TNFα, IL-4 and IFNγ), 11 pro-inflammatory cytokines (MIP-1α, TNF-β, IL-1β, IL-2, IL-3, IL-6, IL-7, IL-8, IL-12, IL-15 and IL-18) and 4 anti-inflammatory cytokines (TGFβ, IL-9, IL-10 and IL-11) were identified by literature search. Genes induced by both Hcy and cytokines are listed. [c], cytokines exerting both MC differentiation and pro-inflammatory function. Reference, consistent with the text and details in the "References" section; MC, monocyte; Hcy, homocysteine; TNFα, tumor necrosis factor α; IFNγ, interferon γ; GM-CSF, granulocyte macrophage colony stimulating factor; MIP-1α, macrophage inflammatory protein-1α; TGFβ, transforming growth factor β.

musculoaponeurotic fibrosarcoma oncogene homolog B (MafB) and proto-oncogene c-maf (c-Maf) (44-46), and several TFs involved in modulating inflammation including NF-κB, nuclear factor of activated T-cells (NFAT), forkhead box O (FOXO) and heat shock transcription factor (HSF), as detailed in Figure 3. Since most of the important TFs bind to the promoter region 1,000 bp upstream of the transcription start site to fulfill their function (47), we defined putative promoter regions as 1,000 bp upstream of transcription start site. We examined the binding frequencies of these TFs on the putative promoter regions of 5 groups of genes; (1) housekeeping genes as the controls; (2) anti-inflammatory cytokine IL-10-induced genes as negative controls (Figure 2B); (3) Hcy-induced genes (Figure 2A); (4) pro-inflammatory cytokine IFNγ-induced genes and (5) pro-inflammatory cytokine IFNγ- and TNFα-induced genes (Figure 2B). A binding frequency higher than the upper threshold of the confidence interval (mean+3xSD of binding sites of 4 housekeeping genes and 17 IL-10-induced genes) was considered as the significant binding frequency. TFs were divided into 3 classes based on the pattern of binding frequency in Hcy-induced genes (Figure 6). Class 1 TFs are defined as TFs having significant binding frequency on more than 3 Hcy-induced genes, including HSF, myocyte enhancer factor-2 (MEF2), NFAT, NF-κB, and KLF4, which are termed as Hcy-responsive TF. Class 1 Hcy-responsive TFs have similar high significant binding frequency in the promoters of all IFNγ-induced genes, but only on a few genes induced by TNFα (Figure 6A). Class 2 had 10 TFs which have significant binding frequency on less than three Hcy-induced genes, including androgen receptor, CCAAT-enhancer-binding proteins (C-EBP), ETS, early growth response protein 1 (EGR-1), glucocorticoid receptor, octamer-4 (Oct4), p53, signal transducer and activator of transcription 3(STAT3), MafB and PU.1 (Figure 6B). Class 3 contained 8 TFs, including activator protein 1(AP-1), cAMP response element binding (CREB), estrogen receptor, ETS related gene 1 (Erg-1), PPAR, retinoic acid receptor, specificity protein 1 (SP1) and TCF transcription factor/lymphoid enhancer-binding factor (TCF/LEF), which do not have significant binding frequency on Hcy-induced genes. The remaining 13 TFs, without identified binding sites in the promoters of Hcy-induced genes, were not included in the classification. Interestingly, class 1 TF binding sites are preferentially high in Hcy- and IFNγ-induced genes (Figure 6B), whereas classes 2 and 3 TF binding sites are similarly distributed in the promoter of all 5 groups.

In addition, we analyze the binding site of these 36 TFs on 5,000 bp upstream of transcription start site promoter regions of the identified genes. There is no significant difference regarding binding frequency towards the interval identified between the 5,000 bp and 1000 bp promoter (data not shown). This finding supported the strategy to focus the TF analysis on the 1,000 bp upstream of transcription start site.

4.4. Hcy-induced genes show similar TF binding profiles as IFNγ-induced genes

Because Hcy-induced genes were also induced by pro-inflammatory cytokines TNFα and IFNγ (Figure 5), we further analyzed the biding profile of class 1 TFs in the promoters of genes induced by Hcy, TNFα and IFNγ˜ As shown in Figure 4, class 1 TFs have significant binding frequency on Hcy-induced genes, except for TNFα. Among Hcy-induced genes, all 5 class 1 TFs have significant binding frequency on the promoter of CD36, IL-6 and IL-12β, 4 on IL-1β and IL-8, 3 on CCR2 and SOD1, 2 on MCP1, and 1 on other Hcy-induced genes. Similarly, class 1 TFs had significant binding frequencies in the promoters of all IFNγ-induced genes. In contrast, class 1 TFs with significant binding frequencies was only identified in 5 of 8 genes induced by TNFα. The occurrence of class 1 TF's significant binding frequencies in the promoters of Hcy-induced genes was similar to that of IFNγ -induced genes ($p=0.242$), but significantly different from that in the promoters of TNFα-induced genes ($p=0.032$) (Figure 4).

4.5. Hcy transcriptional signaling resulted in inflammation and MC differentiation

A working model of Hcy transcriptional signaling was established based on above results. As shown in Figure 7, Hcy induces pro-inflammatory gene expression (Figure 2) via class 1 TFs (Figure 6), the putative Hcy-responsive TFs. The Class 1 TF are known to mediate TNFα- (MEF2 and NF-κB), IFNγ- (KLF4) and stress (HSF and NFAT)-induced inflammation and MC differentiation (Figure 3 & 7), and has high significant binding frequency in Hcy-induced genes (Figure 6A). Hcy can directly trans-activate genes via class 1 TFs and induce inflammation and MC differentiation. The classical signaling pathways of class 1 TFs related to inflammation and MC differentiation were also listed. In addition, we propose that Hcy promote

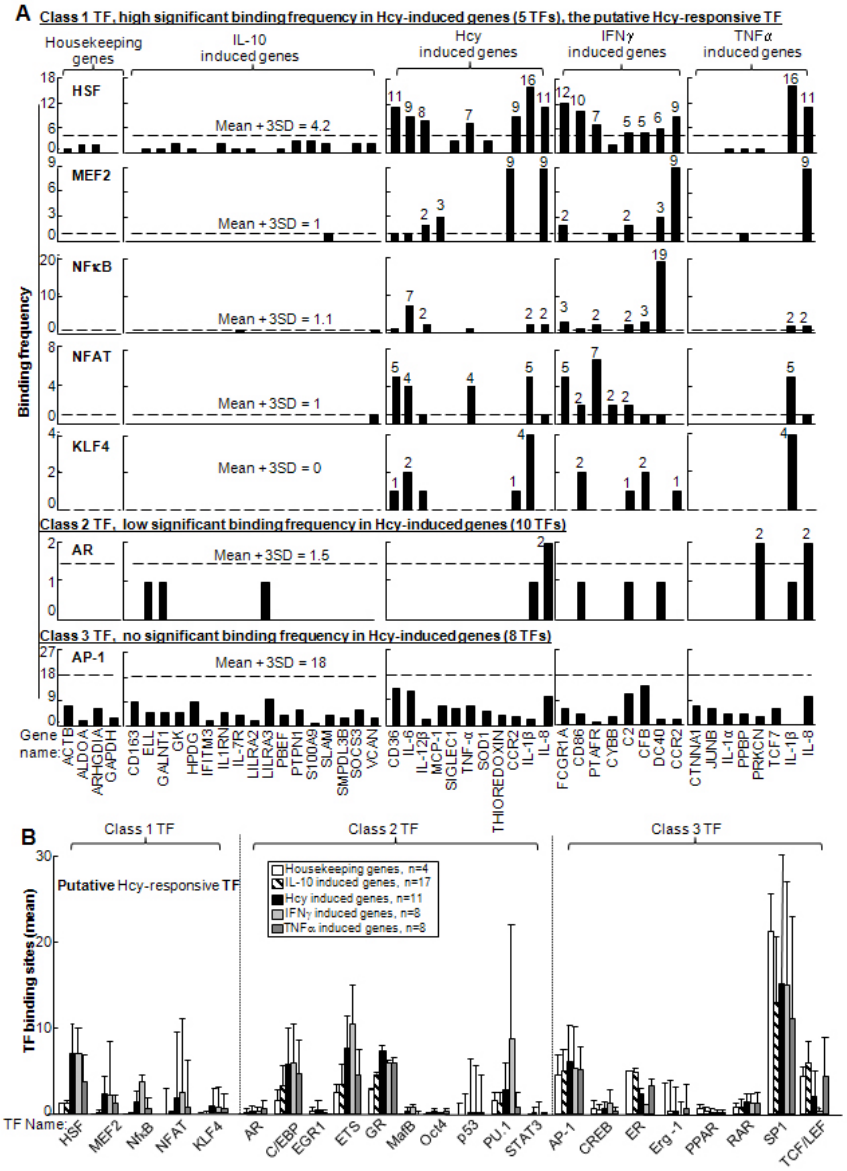

Figure 6. TF binding element analysis. Proximal promoter sequences (1,000 bp upstream of the transcription start site) of Hcy- and cytokines- induced genes and four randomly-chosen housekeeping genes were retrieved from the NIH/NCBI gene database, and analyzed for binding sites of 36 inflammation- and MC differentiation-related TFs using TESS (http://www.cbil.upenn.edu/cgi-bin/tess/tess). A. TF binding frequency. Binding frequency denotes the number of the binding sites for each TF on the promoter region. An upper threshold of the confidence interval was set as mean value+3SD of the TF binding frequencies in the promoters of 4 housekeeping genes and 17 IL-10-induced genes, and marked as the dashed lines. The binding frequencies higher than the upper threshold of the confidence interval ($p < 0.01$) were considered as significant high frequency and directly labeled with number. TFs were divided into 3 classes based on the pattern of significant binding frequency in Hcy-induced genes. Class 1 TF is defined as TFs having high significant binding frequency on more than 3 Hcy-induced genes, including HSF, MEF2, NFAT, NF-kB and KLF4, which are termed as putative Hcy-responsive TF. Class 2 has 10 TFs which have significant binding frequency on less than three Hcy-induced genes. Class 3 contained 8 TFs which do not have significant binding frequency on Hcy-induced genes. The remaining 13 TFs, without identified binding sites in the promoters of Hcy-induced genes, were not included in the classification. All class 1 TFs and one representative of class 2 and 3 TFs were shown. B. TF binding sites. Mean value and standard deviation of binding site of each TF on genes induced by IL-10-, Hcy, IFNg, TNFα were calculated. MC, monocyte; TF, transcription factor; Hcy, homocysteine; TNFα, tumor necrosis factor α; IFNg, interferon g; IL-10, interleukin 10; HSF, heat shock factor; MEF2, monocyte enhancer factor-2; NFAT, nuclear factor of activated T-cells; NF-kB, nuclear factor kappa light chain enhancer of activated B cells; KLF4, krueppel-like factor 4; AR, androgen receptor; AP-1, activator protein 1.

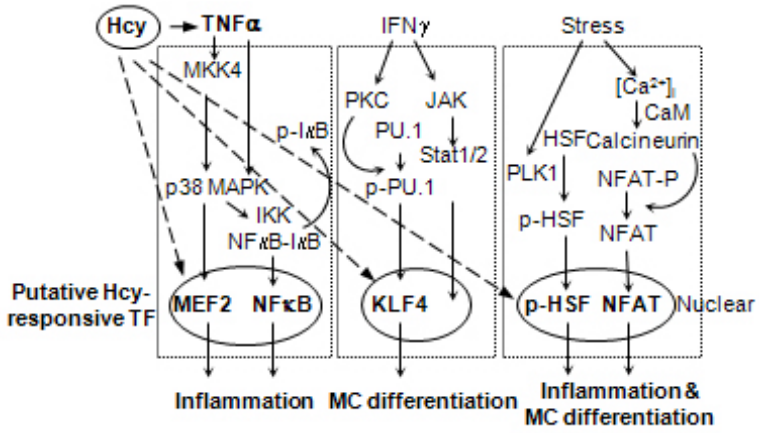

Figure 7. Hypothetical Hcy transcriptional signaling pathways mediating Hcy-induced MC differentiation and MC-derived inflammation. Five class 1 TFs and their validated signaling pathways related to inflammation and MC differentiation were summarized. MC, monocyte; TF, transcription factor; Hcy, homocysteine; TNFα, tumor necrosis factor α; IFNγ, interferon γ; HSF, heat shock factor; MEF2, monocyte enhancer factor-2; NFAT, nuclear factor of activated T-cells; NF-κB, nuclear factor kappa light chain enhancer of activated B cells; KLF4, krueppel-like factor 4; MKK4, mitogen-activated protein kinase kinase 4; p38 MAPK, p38 mitogen-activated protein kinases; IκB, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor; IKK, IκB kinase; p-IκB, phosphorylated IκB; PLK1, polo-like kinase 1; CaM, calmodulin; PKC, protein kinase C; PU.1-P, phosphorylated PU.1 transcription factor; JAK, Janus kinase; Stat, signal transducer and activator of transcription.

inflammation via TNFα induction as indicated in Figure 2A & 4, which in turn leads to MEF2 and NF-κB transactivation and inflammatory response (Figure 7) in MC.

5. DISCUSSION

It has been reported that HHcy accelerates atherogenic process and that Hcy might cause vascular inflammation by inducing MC-derived inflammatory gene expression (7-9). MCs play critical roles in the development of atherosclerosis and can transmigrate across endothelial cells into the vessel wall contributing to vessel wall inflammation. Recent studies have shown that Hcy increased gene expression in cultured human and mouse MC (17-21, 24). However, previous gene expression studies were largely based on mRNA assessment, limited by individual model system, and lacked integrative analysis and mechanistic assessment. Hcy-relevant transcriptional signaling is unknown. Therefore, it is of important significance to profile Hcy-induced gene regulation and transcriptional signaling in MC.

In this study we developed a novel dynamic model system in combining intensive literature searching, database mining of experimental genomic data, pro-inflammatory and MC differentiation TF profiling and TF screening. We identified Hcy-relevant transcriptional signaling and reported four findings: 1) Hcy (≥100μM) preferentially induced 8 pro-inflammatory gene expression (Figure 2A), 2) Hcy induced 5 pro-inflammatory cytokines (CCR2, IL-1β, IL6, IL-8 and MCP1) which can also be induced by other pro-inflammatory cytokines (Figure 5), 3) class 1 TFs (HSF, MEF2, NF-AT, NF-κB, and KLF4) have high significant binding frequency in Hcy-induced genes and are putative Hcy-responsive TFs (Figure 6), and 4) HHcy may contribute to inflammation and MC differentiation via Class 1 TF transcriptional signaling (Figure 7).

Our data support the notion that Hcy functions as a pro-inflammatory molecule and induces MC-derived inflammation and MC differentiation. This conclusion is primarily based on the observation that Hcy preferentially induced pro-inflammatory genes in human MC (Figure 2A). In addition, we found that HHcy reduced the expression of anti-inflammatory genes, macrophage migration inhibitory factor (MIF) (48), PPARγ and PPARα in human MC (49). Since PPARγ and PPARα are TFs (50). these results suggest Hcy may promote inflammatory reaction, in part, by suppressing PPARγ/α-associated anti-inflammatory signaling. Interestingly, Hcy induced antioxidant enzyme superoxide dismutase 1 (SOD1) and thioredoxin (Figure 2A). Hcy-induced SOD and thioredoxin responses might be compensatory and may be overpowered by the pro-inflammatory response. We propose that Hcy induces MC-derived inflammation via increasing pro-inflammation gene expression and suppressing anti-inflammatory gene expression.

Our study suggests that Hcy has a broader spectrum in cytokine induction than other pro-inflammatory cytokines, because five Hcy-induced genes, CCR2, IL-1β, IL-6, IL-8 and MCP-1 can also be induced by several other pro-inflammatory cytokines, but not by anti-inflammatory cytokines (Figure 5). Furthermore, we demonstrated that Hcy promotes inflammatory response not only via inducing pro-inflammatory gene expression as indicated in Figure 2A, but also through facilitating MC differentiation. As indicated in Figure 4 & 7, Hcy induces MC differentiation via trans-activating signaling similar to that of IFNγ and stress (involving KLF4, HSF and NFAT).

The Hcy-MC differentiation hypothesis is consistent with our recent findings showing that HHcy promotes the differentiation of inflammatory Ly6C^{high} MC subset, increases the accumulation of inflammatory MCs/macrophages in atherosclerotic lesions, and accelerates atherosclerosis in mice (13).

TFs are master genes controlling gene expression. It is well-accepted that a greater number of a TF binding sites in a given promoter will result in a greater likelihood for the actual binding of this specific TF (51). Most important TFs bind to the putative core promoter region (1,000 bp upstream of the transcription start site) to fulfill their functions (47). We profiled 36 TFs which were involved in inflammation and MC differentiation. We identified 5 putative Hcy-responsive TFs (HSF, MEF2, NFAT, NF-κB, and KLF4, Figure 6) and established hypothetical Hcy transcriptional signaling (Figure 7). The characterization of TF binding profile and transcriptional signaling is novel and provided integrative view of Hcy-MC response and critical insights into the identification of key mechanisms determining MC-derived inflammation.

Our strategy to identify TF binding profile and transcriptional signaling is an important advance in merging bioinformatics information and experimental science. This study, together with our previous database mining works (35, 48, 52, 53), presented novel model systems of database mining in identifying disease related signaling pathways. Our research model is featured as; *(1)* hypothesis-driven, *(2)* intensively grounded in the literature, *(3)* summarized analysis and integrative for gene and TF regulation, *(4)* database mining on the NCBI databases, *(5)* well-characterized TFs in the searchable database TESS, *(6)* statistically rigorous analysis of available public databases (52).

In summary, we developed a working model of Hcy transcriptional signaling. As shown in Figure 3. Hcy can induce gene expression via 5 Hcy-responsive TFs, the class 1 TFs, which mediate TNFα-signaling (MEF2 and NF-κB), IFNγ- and stress-signaling (KLF4, and HSF and NFAT). Hcy can directly trans-activate genes via class 1 TFs to induce the expression of genes involved in inflammation and MC differentiation.

In conclusion, our results demonstrate, for the first time, that Hcy induces pro-inflammatory gene expression via TF-dependent signaling pathways in MC, leading to MC differentiation and MC-mediated inflammation, thus contributing to vascular inflammation and atherosclerosis.

6. ACKNOWLEDGEMENTS

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