Analysis of genes and underlying mechanisms involved in foam cells formation and atherosclerosis development

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Abstract

22   **Background:** Foam cells (FCs) play crucial roles in the process of all stages of atherosclerosis.

23   Smooth muscle cells (SMCs) and macrophages are the major sources of FCs. This study aimed

24   to identify the common molecular mechanism in these two types of FCs.

25   **Methods:** GSE28829, GSE43292, GSE68021, and GSE54666 were included to identify the

26   differentially expressed genes (DEGs) associated with FCs derived from SMCs and

27   macrophages. Gene Ontology biological process (GO-BP) and Kyoto Encyclopedia of Genes and

28   Genomes (KEGG) pathway analyses were performed by using the DAVID database. The co-

29   regulated genes associated with the two origins of FCs were validated (GSE9874), and their

30   expression in vulnerable atherosclerosis plaques (GSE120521 and GSE41571) was assessed.

31   **Results:** A total of 432 genes associated with FCs derived from SMCs (SMC-FCs) and 81 genes

32   associated with FCs derived from macrophages (M-FCs) were identified, and they were mainly

33   involved in lipid metabolism, inflammation, cell cycle/apoptosis. Furthermore, three co-regulated

34   genes associated with FCs were identified: *GLRX*, *RNF13*, and *ABCA1*. These three common

35   genes showed an increased tendency in unstable or ruptured plaques, although in some cases, no

36   statistically significant difference was found.

37   **Conclusions:** DEGs related to FCs derived from SMCs and macrophages have contributed to the

38   understanding of the molecular mechanism underlying the formation of FCs and atherosclerosis.

39   *GLRX*, *RNF13*, and *ABCA1* might be potential targets for atherosclerosis treatment.

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41   **Keywords:** Foam cells, Atherosclerosis, Smooth muscle cells, Macrophages, Differentially

42   expressed genes

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61 **Introduction**

62   Atherosclerosis is a complex chronic disease characterized by the thickening of the intima

63 and the formation of atherosclerotic plaques, leading to asymmetric stenosis of the arterial

64   lumen(Wang et al. 2019a). It is the underlying pathophysiological mechanism of many cardiac-

65   cerebrovascular diseases, including coronary arterial disease, ischemic stroke, and other

66   peripheral arterial diseases, which are the leading causes of death in the world(Katakami 2018;

67   Wang et al. 2019a). The pathogenesis and molecular mechanisms involved in atherosclerosis are

68   multifactorial, including endothelial dysfunction, abnormal smooth muscle cell (SMC)

69   proliferation and migration, oxidized lipid deposition, vascular matrix changes, inflammatory

70   cell infiltration, and oxidative stress(Weber & Noels 2011; Zhou et al. 2019). In recent decades,

71   significant improvements have been made in the diagnosis, prevention, and treatment of

72   atherosclerosis. However, the comprehensive and in-depth molecular mechanisms of

73   atherosclerosis still need to be further explored(Kanter et al. 2018).

74   Intra- and extracellular lipid deposition is a crucial trigger for the development of

75   atherosclerotic lesions(Summerhill et al. 2019), while lipid-rich foam cells (FCs) are the earliest

76   manifestations of atherosclerotic lesions, and their emergence and accumulation are the original

77   causes of atherosclerotic plaques(Olivares et al. 2016). It was previously thought that all FCs in

78   human atherosclerotic lesions were derived from macrophages. However, subsequent studies of

79   human arterial plaques showed that cell types inherent to the arterial wall are also involved in the

80   formation of FCs, including SMCs, stem/progenitor cells, and even endothelial cells(Gisterå &

81   Hansson 2017; Maguire et al. 2019). SMCs and macrophages are the two main sources of FCs in

82   arterial plaques. FCs formation in atherosclerosis development is closely related to imbalances in

83   lipid metabolism, including lipid uptake and/or lipid efflux inhibition(Maguire et al. 2019;

84   Poznyak et al. 2020).

85   The purpose of "omics" research is to integrate and analyze a large amount of information

86   that represents the overall biological parameters in a particular state through advanced computer

87   technology to identify some important information related to phenotypes and/or diseases(Vernon

88   et al. 2019). A single biomarker may not be sufficient to represent the complex biological

89   processes of the disease, and omics methods can be used to capture multiple variables and

90   demonstrate the interrelationship between those variables in the disease process(Vernon et al.

91   2019). In recent years, the expression profiles of atherosclerosis have been determined by omics

92   approaches, such as microarrays and RNA-seq, and hundreds of differentially expressed genes

93   (DEGs) involved in the development of atherosclerosis have been identified(Liu et al. 2020; Tan

94   et al. 2017). Recently, many studies have attempted to explore the possible molecular

95   mechanisms of macrophage-derived FCs (M-FCs) through microarray technology and

96   bioinformatics analysis(Hägg et al. 2008; Huang et al. 2019; Reschen et al. 2015). However, few

97   studies have systematically explored the molecular mechanism of foam cells derived from

98   smooth muscle cells (SMC-FCs) through high-throughput methods. In this study, bioinformatics

99   techniques were utilized to investigate the possible mechanisms of SMC- and macrophage-

100 derived FCs to identify the common molecular mechanisms of these two different sources of

101 FCs.

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103 **Methods and materials**

104 **Data resources**

105 The GEO database (http://www.ncbi.nlm.nih.gov/geo) is an open public database established by

106   the National Center for Bioinformatics (NCBI), containing a large amount of data from

107   microarrays, gene chips, and RNA-seq(Riksen & Stienstra 2018). To obtain the genes associated

108   with the formation as well as the development of atherosclerosis, the dataset consisted of human

109   atherosclerotic plaques and normal arteries or early and advanced atherosclerotic plaques that were

110   searched in the GEO database, and two datasets (GSE28829 and GSE43292) were obtained. Then, the in-

111   vitro dataset contained ox-LDL treated smooth muscle cells (SMC) or macrophages that were screened

112   out to identify foam cell-related DEGs of the two cell types. Furthermore, because of the important role of

113   foam cells in the development of vulnerable plaques(Liu et al. 2017), the dataset composed of unstable vs.

114   stable or ruptured vs. unruptured atherosclerotic plaques were screened to explore the expression of the

115   foam cell-related genes in vulnerable plaques. The datasets of animal samples and the in-vivo human

116   datasets of serum or plasma were excluded in this study.

117   The GSE28829(Döring et al. 2012) dataset contains 13 early carotid atherosclerotic plaque

118   samples and 16 advanced atherosclerotic plaque samples. GSE43292(Ayari & Bricca 2013)

119   contains 32 normal carotid artery samples and 32 corresponding atherosclerotic plaque samples.

120   GSE68021(Damián-Zamacona et al. 2016) contains the gene expression dataset of human

121   vascular SMCs simulated with oxidized low-density lipoprotein (ox-LDL) for 0 h, 1 h, 5 h, and

122   24 h (3 technical replicates of each group). GSE54666(Reschen et al. 2015) is a dataset of in

123   vitro macrophage experiments including 6 samples of untreated macrophages and 6 samples of

124   macrophage-derived FCs stimulated with ox-LDL for 48 h. GSE9874(Hägg et al. 2008) consists

125   of the gene expression profiles of macrophages treated or untreated with ox-LDL from 15

126   healthy subjects and 15 atherosclerotic patients. GSE41571(Lee et al. 2013) contains data on 11

127   macrophage-rich regions from 5 ruptured plaques and 6 stable plaques. GSE120521(Mahmoud et

128   al. 2019) consists of an RNA-seq profile of 4 stable and 4 unstable plaque samples. The analysis

129   strategy is shown in **Figure 1**.

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131   **Identification of DEGs**

132   The downloaded gene expression profiles and their matched platform files were loaded into

133   R (version 3.6.1) software and converted into gene symbol expression profiles. The LIMMA

134   package was used to identify differentially expressed genes (DEGs) between the two

135   groups(Ritchie et al. 2015). DEGs with adjusted p values < 0.05 (adjusted by the Benjamini-

136   Hochberg method) were considered significant. Co-DEGs were found in the overlap of different

137   datasets as determined by an online web tool (http://jvenn.toulouse.inra.fr/app/example.html).

138   For the GSE68021 dataset, the DEGs of each time point (ox-LDL treated for 1 h, 5 h, 24 h)

139   compared with negative control were identified. Furthermore, the Short Time-series Expression

140   Miner (STEM)(Ernst & Bar-Joseph 2006) program was used to analyze the temporal expression

141   profiles of these DEGs. The significant clusters which manifested upregulated or downregulated

142   characteristics over time were selected for the next analysis.

143

144   **Functional enrichment analyses**

145   The purpose of gene function enrichment analysis is to determine the correlation between a

146   group of genes and functional categories through a hypergeometric test. Gene Ontology (GO) is

147   an international standardized gene functional classification system that covers three major

148   categories: biological process (BP), cellular component (CC), and molecular function (MF). The

149   Kyoto Encyclopedia of Genes and Genomes (KEGG) database is the major public pathway-

150   related database for helping researchers understand the advanced functions of genes in biological

151   systems. DAVID (https://david.ncifcrf.gov/home.jsp) is a very useful bioinformatics data

152   resource for scientists to find meaningful biological information on genes or proteins(Huang da

153   et al. 2009). Herein, GO-BP and KEGG pathway analyses were performed using the DAVID

154   database. The functional category with a p-value < 0.05(Xia et al. 2019) was considered

155   significant.

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157   **Validation of the common FCs-related genes**

158   For the validation of the common FCs-related genes, the expressions of these genes were

159   extracted out from the microarray dataset and analyzed by student's *t*-test. For the RNA-seq

160   dataset, the normalized gene expression matrix of FPKM (fragments per kilobase of exon model per

161   million reads mapped) values was downloaded and transformed into TPM (transcripts per kilobase

162   million) values for comparison(Li et al. 2010). P value < 0.05 was defined as statistical significance.

163

164   **Results**

165   **Identification of DEGs associated with atherosclerosis**

166   GSE28829 contained data on 13 early atherosclerotic plaques and 16 late atherosclerotic

167   plaque samples. Through a LIMMA analysis, a total of 2,300 DEGs were identified in the

168   advanced plaques, based on the gene expression in the early plaques (defined as atherosclerosis

169   progression-related genes), including 1,150 upregulated genes and 1,150 downregulated genes

170   (**Figure 2A**). GSE43292 contained information on 32 nonatherosclerotic plaque samples

171   (control) and 32 atherosclerotic plaque samples, and 6,378 DEGs were obtained, including 3,066

172   up- and 3,312 downregulated DEGs in atherosclerosis, based on the gene expression in the

173   control group (**Figure 2B**). After comparing the DEGs in the two datasets, 801 co-

174   downregulated and 849 co-upregulated genes were identified (**Figure 2C**, **D**). These co-

175   regulated DEGs were defined as atherosclerosis-related genes.

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177   **Identification of SMC-FCs-related genes and functional analysis**

178   GSE68021 includes the gene expression data of human vascular SMCs treated with ox-LDL

179   for 1 h, 5 h, and 24 h in vitro to simulate the status of SMC-FCs in atheroma plaques. In this

180   study, a total of 3369, 4887, and 7170 DEGs were identified in 1 h, 5 h, and 24 h compared with

181   control (Figure 3A). Moreover, these DEGs obtained from different time points were

182   investigated their expressive trends by using STEM program. As shown in Figure 3B, 13 clusters

183   with statistical significance were identified. And Cluster 1 (581 genes), 9 (601 genes), 12 (392

184   genes), 11 (413 genes), 26 (253 genes), 23 (240 genes) exhibited downregulated features over

185   time, while Cluster 42 (630 genes), 48 (570 genes), 40 (493 genes), 29 (344 genes) exhibited

186   upregulated trends over time. The expression of these genes with downregulated and upregulated

187   trends were shown in Figure 3C. Then these genes were compared with AS-related genes, and a

188   total of 432 common genes were obtained and defined as SMC-FCs-related genes (Figure 3D).

189   To clarify the biological functions of these SMC-FCs-related genes, the GO-BP and KEGG

190   pathway enrichment analyses were performed, and a total of 71 BP and 25 KEGG pathway

191   functions were enriched. As shown in Figure 3E and supplementary Table S1, the enriched

192   KEGG pathways were mainly involved in inflammation and contractive function pathways, such

193   as Regulation of actin cytoskeleton (hsa04810), Vascular smooth muscle contraction (hsa04270),

194   Chemokine signaling pathway (hsa04062), Leukocyte transendothelial migration (hsa04670).

195   Similarly, the results of GO-BP terms suggested these genes were mainly involved in the

196   contraction function, inflammation, cell cycle/apoptosis, and substance uptake and intracellular

197   transport, such as actin filament organization (GO:0007015), smooth muscle contraction

198   (GO:0006939), T cell receptor signaling pathway (GO:0050852), inflammatory response

199   (GO:0006954), apoptotic process (GO:0006915), cell cycle (GO:0007049), early endosome to

200   late endosome transport (GO:0045022), lysosome organization (GO:0007040) (Figure 3F and

201   supplementary Table S1).

202

203   **Identification of DEGs associated with macrophage-derived foam cells (M-FCs) and their**

204   **functional enrichment**

205   GSE54666 was the gene expression profiling of macrophages stimulated with ox-LDL in

206   vitro to simulate the status of M-FCs in atheroma plaques. A total of 484 differentially expressed

207   genes were obtained, including 259 upregulated genes and 225 downregulated genes (**Figure**

208   **4A**). These DEGs and previously obtained AS-related genes were integrated, and the overlapping

209   genes were defined as M-FC-related genes. As shown in **Figure 4B**, a total of 81 M-FC-related

210   genes were identified.

211   To explore the biological role of the 81 M-FCs-related genes, functional enrichment

212   analysis was performed again using the DAVID database. A total of 30 GO-BP and 1 KEGG

213   pathway were enriched. The GO-BP analysis mainly revealed lipid metabolism, foam cell

214   differentiation, and immune response, such as negative regulation of cholesterol storage

215   (GO:0010887), negative regulation of macrophage-derived foam cell differentiation

216   (GO:0010745), response to low-density lipoprotein particle (GO:0055098), and negative

217   regulation of interferon-gamma-mediated signaling pathway (GO:0060336). The enriched

218   KEGG pathway was the PPAR signaling pathway (hsa03320) (**Figure 4C** and **Supplementary**

219   **Table S2**).

220

221   **The common genes of SMC-FCs and M-FCs related DEGs**

222   To identify common molecular mechanisms in the SMC-FCs and M-FCs, the common genes

223   were screened out. As shown in **Figure 5A**, 15 common genes were identified: *CTSD, HHEX,*

224   *TNFRSF21, GLRX, EDEM2, CTSC, LAT2, SPOCD1, ABCA1, LST1, CD74, PLAUR, BRI3,*

225   *EMB,* and *RNF13*. Then, the expression trends of these 15 genes in AS tissue, SMC-FCs, and M-

226   FCs were compared to that of the respective control. As shown in **Figure 5 B-C**, all of these 15

227   genes were upregulated in the atherosclerotic group compared to the non-atherosclerotic group or

228   in advanced plaques compared with early plaques. However, only four genes (*GLRX*, *ABCA1*,

229   *HHEX*, and *RNF13*) exhibited upregulated trends in ox-LDL treated SMCs (Figure 5D), while

230   nine genes (*SPOCD1*, *PLAUR*, *CTSD*, *RNF13*, *ABCA1*, *BRI3*, *GLRX*, *TNFRSF21*, and *EDEM2*)

231   were increased in ox-LDL treated macrophages (Figure 5E). Then, the co-upregulated and co-

232   downregulated genes were screened. The Venn diagrams showed the 3 (*GLRX, RNF13,* and

233   *ABCA1*) co-upregulated genes; and no co-downregulated genes were found (**Figure 5F, G**).

234

235   **Validation of the common FCs-related genes and their expression in vulnerable plaques**

236   The expression of the 3 co-upregulated FC genes was then validated using another dataset.

237   GSE9874 contains the gene expression profile of peripheral blood monocyte-derived

238   macrophages treated or untreated with ox-LDL. As shown in Figure 6A, all of the three genes

239   were increased in the ox-LDL treated group (Foam cells group).

240   Previous studies have suggested that the formation and retention of the FCs could

241   exacerbate atherosclerosis and fuel the development of the vulnerable plaques(Bäck & Hansson

242   2015; Liu et al. 2017). Therefore, the expressions of the three FCs-related genes were

243   investigated in the vulnerable plaques. As shown in Figure 6B, only *RNF13* was statistically

244   significantly upregulated in unstable plaques, while *ABCA1* and *GLRX* showed no statistical

245   significance, though the means of *ABCA1* were larger in unstable plaques than stable plaques. In

246   ruptured plaques, *GLRX* and *RNF13* were significantly upregulated compared with stable

247   plaques, while *ABCA1* showed a slight increase with no statistical significance (Figure 6C).

248

249   **Discussion**

250   Previous studies have suggested the crucial role of foam cells (FCs) in the formation and

251   development of atherosclerosis, and the molecular mechanisms of FCs have been the focus of

252   attention in recent years(Chistiakov et al. 2017; Poznyak et al. 2020). Recently, advanced high-

253   throughput and bioinformatic technologies have been widely used for exploring the molecular

254   mechanisms and predicting biomarkers for the diagnosis, treatment, and prognosis of the

255   diseases(Dona et al. 2016). Through the gene expression microarray technology, Döring Y et al.

256   revealed the upregulation of the plasmacytoid dendritic cell markers (such as *CD123, CD83, E2-*

257   *2, CD317, CD32,* and *BDCA2*) in advanced versus early lesions(Döring et al. 2012). Ayari H et

258   al. identified two genes (*CD163* and *HO-1*), which were significantly increased in atheroma

259   plaques compared with intact arterial tissue by using GeneChip arrays(Ayari & Bricca 2013).

260   Lee K et al. identified 914 DEGs between stable and ruptured plaques by using microarray

261   technology, and highlighted the involvement of FABP4 and leptin in the progression of

262   atherosclerosis and plaque rupture(Lee et al. 2013). Mahmoud AD et al. revealed the DEGs

263   between stable and unstable plaques, which were mainly linked with the plaque instability,

264   including inflammation, matrix remodeling, and calcification(Mahmoud et al. 2019). Although

265   these studies revealed some molecular mechanisms of the atherosclerosis development in various

266   aspects, they could not reveal the cell-specific mechanisms, such as foam cells. Therefore, some

267   studies tried to investigate the molecular changes of specific cells by in-vitro experiments, such

268   as macrophages and SMCs under the stimulation of ox-LDL(Damián-Zamacona et al. 2016;

269   Hägg et al. 2008; Reschen et al. 2015). However, ox-LDL treatment alone might be much too

270   oversimplified because of the complicated environment of cells in organisms. Therefore, in this

271   study, we tried to combine the in-vitro and in-vivo experiments to relieve these influences.

272   In the present study, two datasets of gene expression in atherosclerosis tissues (GSE28829

273   and GSE43292) were analyzed, and 1650 co-regulated DEGs were identified. After screening

274   these 1650 co-DEGs with DEGs of SMC-FCs (GSE68021) and M-FCs (GSE54666), a total of

275   432 SMC-FCs-related genes and 81 M-FCs-related genes were identified. To identify the

276   common therapeutic targets of FCs, the common molecular mechanism between FCs derived

277   from SMCs and macrophages was explored. Three co-upregulated genes were identified: *GLRX,*

278   *RNF13,* and *ABCA1*. Furthermore, the expressions of these 3 genes in vulnerable atherosclerosis

279   plaques were explored, and all of them were increasingly expressed in unstable or ruptured

280   plaques compared to their expression levels in stable plaques, although some of the increased

281   levels were not statistically significant.

282   Based on murine models, it was long presumed that all FCs in human atherosclerosis were

283   derived from macrophages. However, subsequent studies demonstrated that resident cell types,

284   especially SMCs, maybe the prominent originators of FCs(Poznyak et al. 2020; Wang et al.

285   2019b). However, macrophages are still the second most prolific sources of FCs and play crucial

286   roles in the pathogenesis of atherosclerosis, in both the early and advanced stages(Owsiany et al.

287   2019). Therefore, macrophages remain the most representative model of FCs and will continue

288   to be studied.

289   The process of lipid metabolism in macrophages is divided into three main stages: lipid

290   uptake, esterification, and efflux(Maguire et al. 2019). The lipid metabolism of macrophages in

291   atheroma plaques gradually accelerates to be imbalanced, with excessive intracellular lipid

292   deposition and leading to the formation of FCs(Chistiakov et al. 2017; Maguire et al. 2019). In

293   the present study, a total of 81 M-FCs-related genes were identified, and their functions were

294   found to be mainly enriched in lipid metabolism and immune responses.

295   The final stage of cholesterol metabolism is the efflux of cholesterol, which is mediated by

296   transporters that depend on multiple key transcription factors, such as PPAR and

297   LXLR(Chistiakov et al. 2016; Chistiakov et al. 2017). These transporters mainly include ATP-

298   binding cassette transporter 1 (ABCA1), ATP-binding cassette subfamily member G1 (ABCG1),

299   and SR-B1, which play crucial roles in preventing the excessive intracellular accumulation of

300   cholesterol and the formation of FCs(DiMarco & Fernandez 2015). Similar to that of cholesterol

301   internalization, a confusing relationship characterizes these efflux transporters during the

302   progression of atherosclerosis. For example, a knockout of ABCA1 aggravated the formation of

303   FCs but did not promote atherosclerosis plaque development(Zhao et al. 2011). Moreover,

304   studies of ABCG1 and SR-B1 have also revealed controversial results, as they have a promoting

305   role in early atherogenesis but a protecting role in advanced atherosclerosis(Meurs et al. 2012;

306   Van Eck et al. 2004; Zhang et al. 2003).

307   SMCs also play crucial roles in all stages of atherosclerosis, and they are considered the

308   leading sources of FCs(Poznyak et al. 2020; Wang et al. 2019b). During the process of

309   atherosclerosis, activated macrophages may secrete many inflammatory factors or cytokines that

310   can promote the phenotypic transformation of SMCs from the resting systolic phenotype to the

311   activated synthetic or "macrophage-like" phenotype. These phenotypic changes in SMCs are

312   generally associated with the downregulated expression of contractile proteins and increased

313   proliferation(Zhang et al. 2012). In the present study, a total of 432 SMC-FC-related genes were

314   identified, and they were mainly involved in contraction function, inflammation, cell

315   cycle/apoptosis, and substance uptake and intracellular transport, reflecting the roles of SMCs in

316   atherogenesis. Moreover, compared with M-FCs, the expression of the cholesterol efflux

317   transporter ABCA1 in SMC-FCs is much lower. Furthermore, the ABCA1 level in SMCs in

318   advanced atherosclerosis was decreased compared to early atherosclerosis; however, no changes

319   in macrophages were observed(Allahverdian et al. 2014). These results provide a plausible

320   explanation for the high percentage of FCs derived from SMCs(Allahverdian et al. 2014).

321   RNF13 is an E3 ubiquitin ligase that is widely expressed in various animals and tissues.

322   Previous studies have determined the roles of RNF13 in myogenesis, neuronal development, and

323   tumorigenesis(Zhang et al. 2009; Zhang et al. 2010), in which it is mainly involved in cell

324   proliferation(Jin et al. 2011). But few studies investigated the role of RNF13 in atherosclerosis or

325   FCs. GLRX is a member of the glutaredoxin family, which highly contributes to the antioxidant

326   defense system. Overexpression of GLRX could attenuate H2O2-induced apoptosis of

327   endothelial cells(Li et al. 2017). However, GLRX inhibits endothelial cell angiogenic

328   properties(Matsui et al. 2017). Though increasing evidence has suggested the important roles of

329   oxidative stress in atherosclerosis(Sharif et al. 2020; Zhang et al. 2020), few studies have

330   revealed the role of GLRX in atherosclerosis and the formation of FCs. Therefore, the roles of

331   RNF13 and GLRX in the process of atherosclersis and FCs remain unknown, which needs

332   further exploration.

333   The present study has several limitations. Firstly, the sample size of some datasets used in

334   this study is small, which might result in some bias. For example, the GSE120521 contained only

335   4 stable and 4 unstable samples. Secondly, there may be some confounding factors, such as types

336   and morphology of atherosclerotic plaques, age, gender, and drug treatment, as well as clinical

337   complications, which might affect the interpretation of the results, and could not be identified in

338   this study. Thirdly, interexperiment variability, such as different detection platforms and

339   different resources of samples from different laboratories, may result in somewhat differences in

340   gene expressions. Moreover, different cell types, like SMCs and macrophages, might show

341   different changes of gene expression in ox-LDL treatment or pro-atheromatous environments in

342   tissues. Therefore, only three co-up regulated genes were identified in this study between the two

343   cell types of foam cells.

344

345   **Conclusion**

346   In the present study, 432 SMC-FC-related genes and 81 M-FC-related genes were

347   identified, and they were found to be mainly involved in lipid metabolism, inflammation, cell

348   cycle/apoptosis. Furthermore, 3 co-regulated genes associated with FCs were identified: *GLRX*,

349   *RNF13*, and *ABCA1*. Among these genes, the roles of *ABCA1* in the atherosclerotic process have

350   been widely described, but many contradictory results have been presented, suggesting that the

351   roles of *ABCA1* in atherosclerosis need to be further explored. Moreover, the roles of *RNF13* and

352   *GLRX* in atherogenesis remain unknown, and more experiments in the future are needed to

353   confirm their roles. These three genes have a common expression tendency in both SMCs and

354   macrophages, which may have implications for the development of possible targeted therapeutic

355   drugs in the future.

356

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359

360   **Reference**

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Figure 1

Flow chart of the analysis.

DEGs: differentially expressed genes; NC: negative control; hVSMCs: human vascular smooth muscle cells; ox-LDL: oxidized low-density lipoprotein; SMC-FCs: smooth muscle cell-derived foam cells; M-FCs: macrophage-derived foam cells; PPI: protein-protein interaction; GO: Gene Ontology; BP: biological process; and KEGG: Kyoto Encyclopedia of Genes and Genomes.
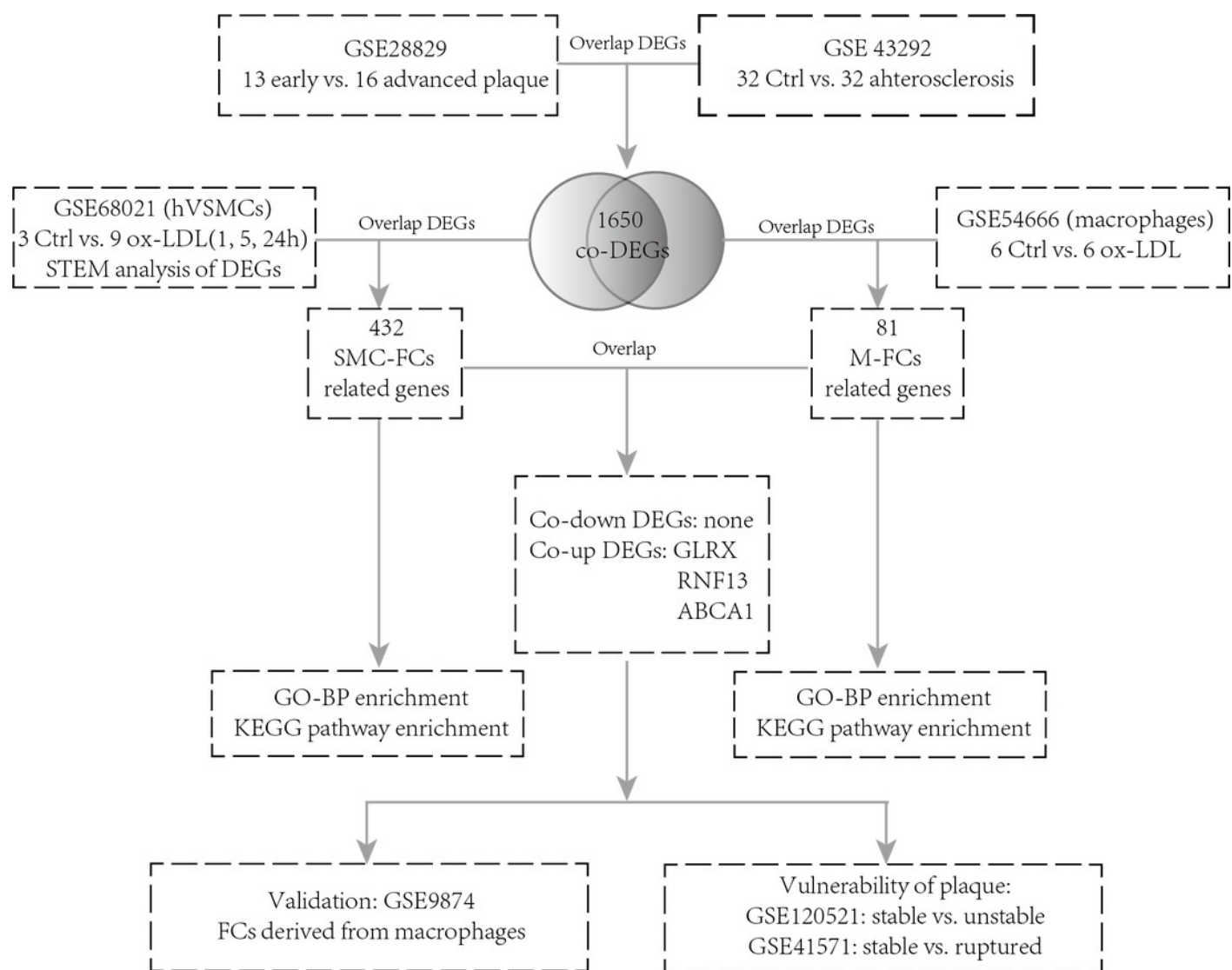
Figure 2

Identification of DEGs associated with atherosclerosis.

Hierarchical cluster heatmap of DEGs in advanced atherosclerosis compared to those in early atherosclerosis samples in the GSE28829 dataset **(A)** and those in atherosclerosis samples compared to those in non-atherosclerosis samples in the GSE43292 dataset **(B)**. Venn diagrams showing the co-upregulated DEGs **(C)** and co-downregulated DEGs **(D)** in the GSE28829 and GSE43292 datasets, which were defined as atherosclerosis-related genes.
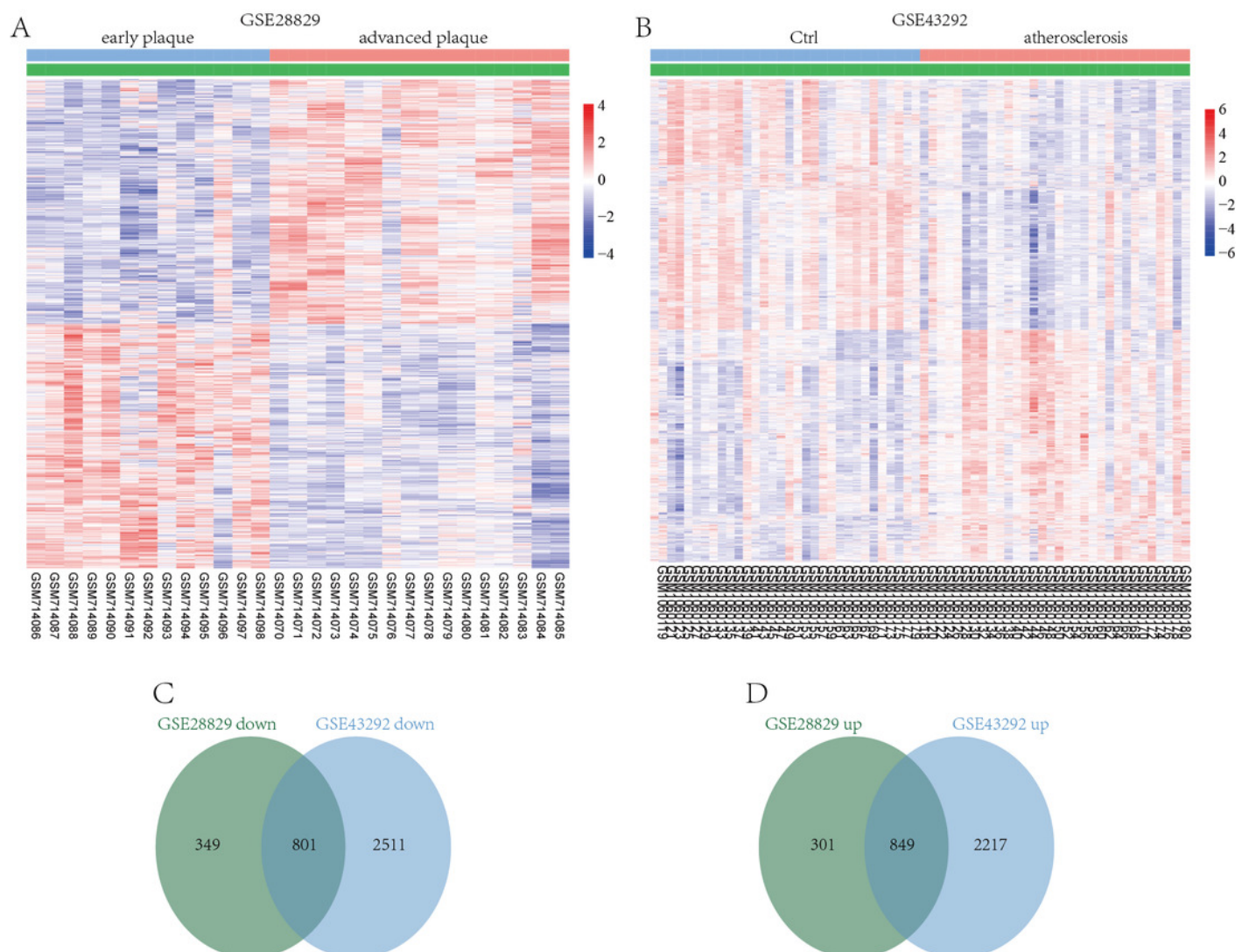
Figure 3

Identification of SMC-FCs-related genes and functional analysis.

(A) Venn diagram showing the DEGs of each time point (ox-LDL treated for 1 h, 5 h, and 24 h) compared with control. **(B)** The expression trend clusters of all DEGs obtained above by STEM analysis. **(C)** Hierarchical cluster heatmap of DEGs with downregulated and upregulated trends overtime obtained by STEM analysis. (D) Venn diagram showing the overlap of DEGs in the ox-LDL-treated SMCs (with downregulated and upregulated trends overtime obtained by STEM analysis) and atherosclerosis-related genes, which were considered SMC-FCs-related DEGs. (E) and (F) The bubble diagram of the KEGG pathway and GO-BP enrichment analyses of SMC-FCs-related DEGs. In the bubble diagram, dot sizes represent counts of enriched DEGs, and dot colors represent negative Log_{10} values (p values). SMC-FCs: smooth muscle cell derived foam cells; GO: Gene Ontology; BP: biological process; and KEGG: Kyoto Encyclopedia of Genes and Genomes; ox-LDL: oxidized low-density lipoprotein.
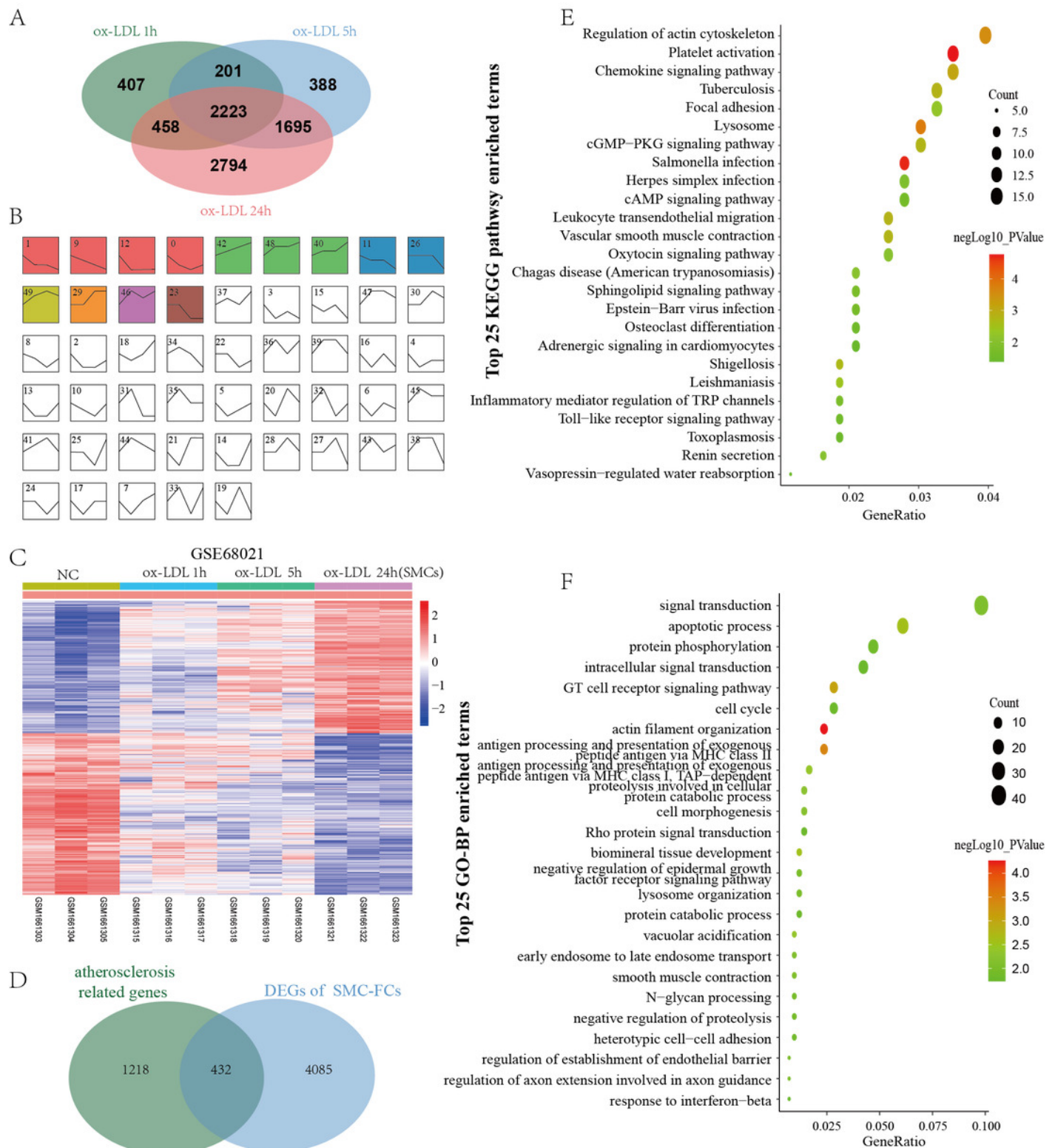
Figure 4

Identification of DEGs associated with M-FCs.

(A) Hierarchical cluster heatmap of the DEGs in the ox-LDL-stimulated macrophages compared to gene expression levels in the negative control from the GSE54666 dataset. Red represents upregulated genes, and green represents downregulated genes. **(B)** Venn diagram of the DEGs in the GSE54666 dataset compared to the AS-related genes and considered M-FC-related DEGs. **(C)** GO-BP and KEGG pathway enrichment analyses of the M-FC-related DEGs. In the bubble diagram, dot sizes represent counts of enriched DEGs, and dot colors represent negative Log_{10} values (p values). DEGs: differentially expressed genes; M-FCs: macrophage-derived foam cells; ox-LDL: oxidized low-density lipoprotein; AS: atherosclerosis; GO: Gene Ontology; BP: biological process; and KEGG: Kyoto Encyclopedia of Genes and Genomes.

A

B

C
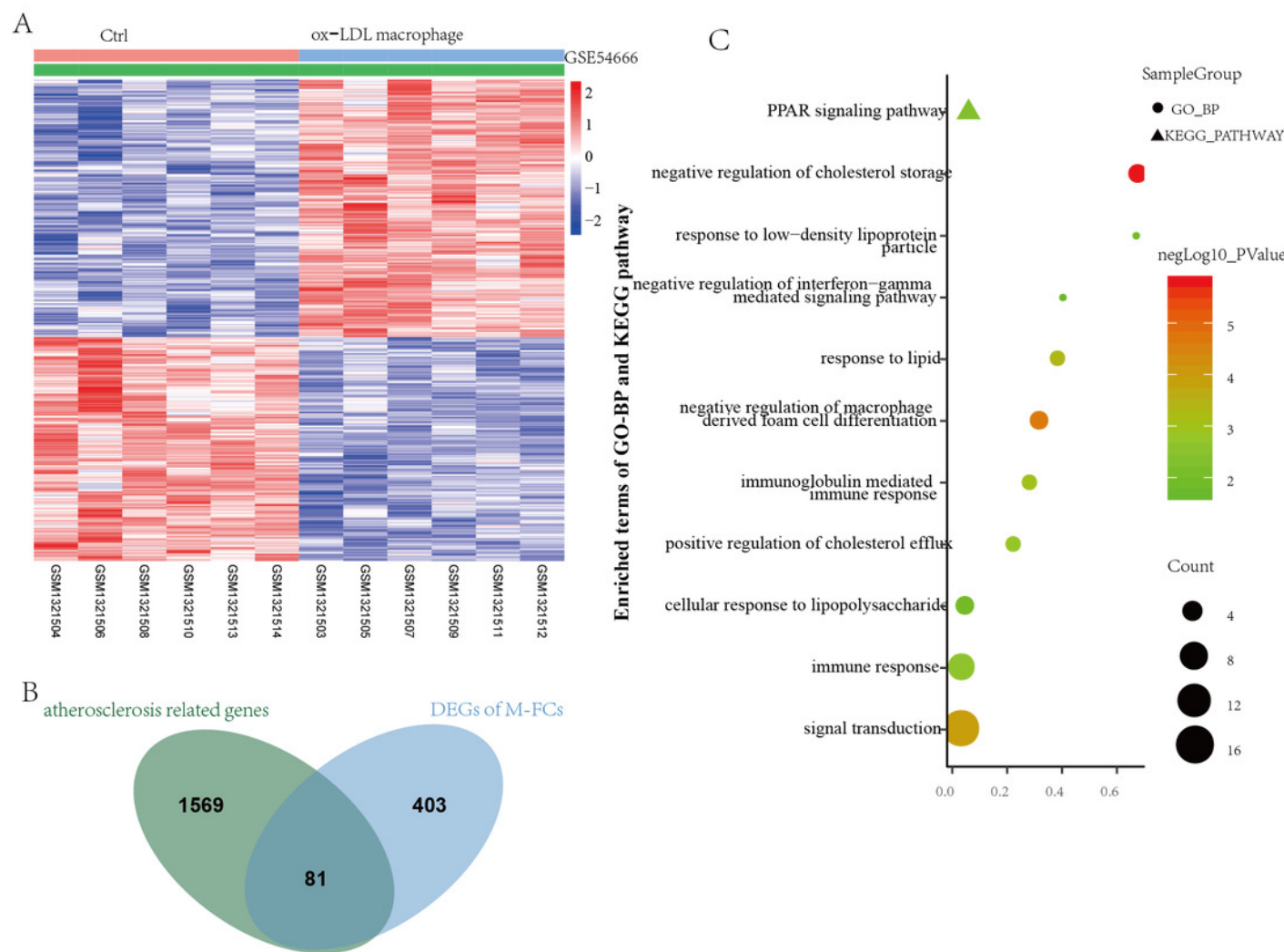
Figure 5

Identification of co-regulated genes of SMC-FCs and M-FCs.

(A) Venn diagram of common DEGs related to SMC-FCs and M-FCs. **(B to E)** Cluster heatmaps of the expression of 15 common genes in the GSE28829, GSE43292, GSE68021, and GSE54666 datasets. Red represents upregulated genes, and green represents downregulated genes. **(F)** Venn diagram showing the co-downregulated genes and **(G)** co-upregulated genes in the atherosclerosis tissue, SMC-FCs, and M-FCs. SMC-FCs: smooth muscle cell-derived foam cells; M-FCs: macrophage-derived foam cells; and DEGs: differentially expressed genes.
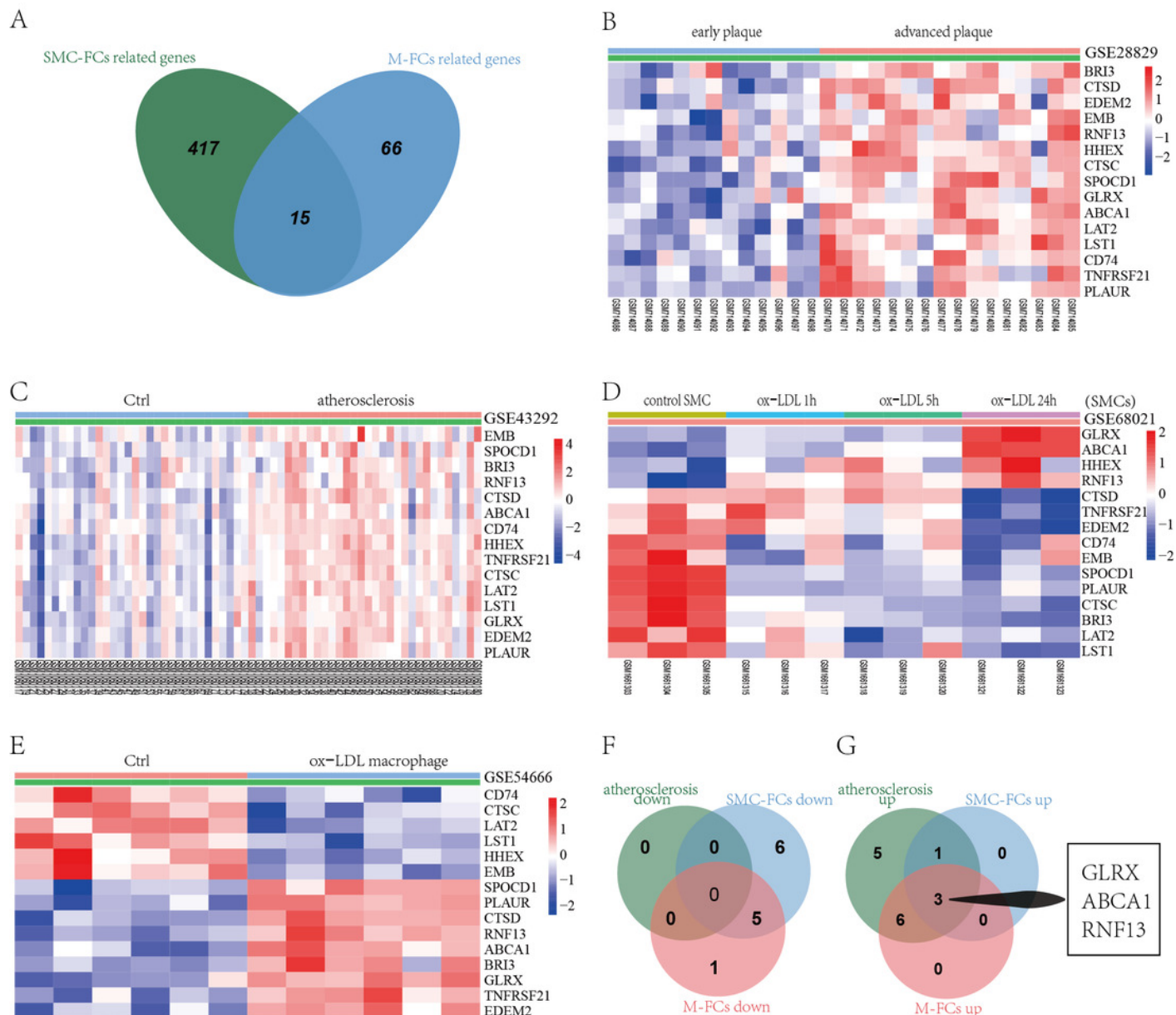
Figure 6

Validation of the common FCs-related genes and their expression levels in vulnerable atherosclerosis plaques.

Validation of the 3 co-upregulated genes: *GLRX*, *RNF13*, and *ABCA1*, in the macrophages treated with or without ox-LDL (GSE9874) (A). The expression of the 3 genes in unstable plaque samples **(B)** or ruptured plaque samples **(C)** compared to stable plaque samples in the GSE120521 or GSE41571 dataset, respectively. *p < 0.05, **p < 0.01, ***p < 0.001. FC: foam cell.