

Transcriptomic analysis reveals that bromodomain containing 9 controls signaling pathways in gastric cancer

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Research article

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Abstract

Background

According to the Cancer Genome Atlas, gastric cancers involve 30% BRD9 changes. Studying the signaling net controlled by BRD9 is important and provides useful information for the treatment of patients with gastric cancer and BRD9 alteration.

Objective

We performed this study to find the signaling pathways controlled by BRD9 in gastric cancer cells.

Methods

MGC-803 and AGS cells were selected as BRD9 overexpression and normal expression models, respectively, and added with BRD9 inhibitors BI9564 and BI7273, respectively. RNA-seq and limma R language were used to obtain differentially expressed genes (DEGs), and heatmap R language was employed for cluster analysis. Database for Annotation, Visualization and Integrated Discovery (DAVID) was applied to identify the gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichments, and STRING database was utilized to construct the protein–protein interaction (PPI) networks. Analysis was performed through Cytoscape software to determine the possible signaling pathway and target genes.

Results

Group MGC-803: 1204 and 1338 DEGs were found in MGC-803 cells added with BI9564 and BI7273, respectively, and 425 DEGs were found in the intersection of these two sets. AGS group: 974 and 1006 DEGs were found in AGS cells added with BI9564 and BI7273, respectively, and 382 DEGs were found in the intersection of these two sets. The DEG number in the intersection of groups MGC-803 and AGS was only 12, and only 3 of which showed the same regulation direction. Hence, these two types of gastric cancers are greatly altered in the signaling network. GO enrichment and KEGG signaling pathway analyses showed that in group MGC-803, BRD9 mainly controls cell adhesion molecule (CAM) pathway, and genes SPP1 and GNAO1 may play important an important role in the BRD9 controlling network. In group AGS, BRD9 mainly controls protein digestion and absorption pathway, and genes AR and GNMT2 have an important function in the BRD9 controlling network.

Conclusion

Comprehensive bioinformatics analyses were conducted to screen the DEGs and signaling pathways controlled by BRD9 in different gastric cancer cells. The findings provide a theoretical basis in curing patients with clinical gastric cancer.

Introduction

Gastric cancer is the third cause of cancer death and also is the fifth common type of malignant cancer worldwide [1]. Cancer is caused by the genetic and epigenetic alterations of genes, and the latter type heavily influences gastric cancer [2].

Epigenetic regulation is highly involved in many cellular processes, such as gene expression, DNA repair, and DNA copy [3, 4], and its abnormality is important in cancer occurrence, development, and relapse. As epigenetic regulators, “writer”, “reader”, “eraser”, and chromatin-remodeling complex perform regulation tasks in cellular mechanisms. The genes of these epigenetic regulators have changed in many cancer types. Mammalian switch/sucrose non-fermentable (SWI/SNF) chromatin-remodeling complexes have mutated in 20% of all human cancers [5], and changes in its subunits are highly frequent in almost all human cancers. Such alterations indicate late situations during cancer evolution and may promote tumor malignancy or cure resistance [6]. Readers possess particular domains, such as BRD9, a bromodomain protein and a core SWI/SNF complex subunit protein that can recognize acetylated histone tail, bind histone in nucleosomes, and form a SWI/SNF complex to relax chromatin to promote target gene transcription [7].

BRD9 overexpression is related with tumorigenesis [8], and a BRD9 inhibitor can induce cell apoptosis in triple-negative breast cancer. [9]. BRD9 is important in maintaining the survival of SMARCB1-mutant pediatric malignant rhabdoid tumors [10] and is also a critical target in acute myeloid leukemia (AML) because its inhibition can induce apoptosis. Therefore, BRD9 may promote cancer processes and change the controlling net to protect cancer cells. The Cancer Genome Atlas (TCGA) database reported 33% BRD9 alterations in all gastric cancers. The unusual changes of BRD9 in gastric cancer will induce the unusual relaxation of chromatin and the unusual expression of target genes and finally promote the malignancy of gastric cancer. Identifying the specific signal net controlled by BRD9 in different types of gastric cancer is important in developing a cure.

Gastric cancer cells MGC-803 and AGS were selected as the cellular models that were added with BRD9 inhibitors BI9546 and BI7273, respectively, and then used for genome-wide mRNA expression analysis. Differentially expressed genes (DEGs) in the test and control group samples were screened using the R software, and gene ontology (GO) pathway enrichment and KOBAS-Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were performed on the Database for Annotation, Visualization and Integrated Discovery (DAVID) (<https://david.ncifcrf.gov/>). The protein–protein interaction (PPI) networks of the DEGs were constructed from the search tool for the retrieval of interacting genes/proteins (STRING) database and analyzed by Cytoscape to determine the association of DEGs and discover the molecular interactions of BRD9 in different types of gastric cancer. The DEGs induced by BRD9 and

associated with the malignancy and development in different BRD9 types of gastric cancer were screened through genome-wide mRNA expression analysis. Many bioinformatics methods were used to analyze the DEGs and obtain useful information about BRD9 promotion of cancer malignancy and development. The valuable signaling pathways conducted by BRD9 in gastric cancers were discovered, and PPI net pathway was also analyzed. The results revealed that the changes of BRD9 in cancer cells altered the controlling net pathway in different types of gastric cells.

Materials And Methods

RNA-sequencing (RNA-seq)

RNA-seq is a new transcriptomic analysis technique that can quickly and comprehensively obtain almost all sequence and expression data in a specific cells or tissues with a certain state, including mRNA-encoding protein, various non-coding RNAs, and expression abundance of different transcripts produced by gene alternative splicing. This method can accurately analyze the important problems of life science such as gene expression difference, gene structure variation, and screening of molecular markers.

Screening of DEGs

Limma package in the Bioconductor package (<http://www.bioconductor.org/>) was used to analyze the DEGs. RNA-seq data were analyzed by limma R language. P-value <0.05 and log fold change (FC) >1.5 genes were identified as DEGs.

KEGG pathway and GO enrichment analyses of DEGs

DAVID is an important foundation to abstract bioinformation from RNA-seq data and therefore was used to analyze DEGs through GO annotations and KEGG pathway analysis and further understand the alteration of signaling pathway in gastric cancer cells after the addition of BRD9 inhibitors.

PPI network integration

The STRING database (<http://string-db.org>) identifies the interactions between proteins, and the Cytoscape software can graphically display, analyze, and edit networks. Each node displayed in Cytoscape is a gene or protein, and the connections between nodes represent the interaction of these gene or protein. DEG interaction relation data were obtained from STRING and downloaded as input data for Cytoscape software input data. Low interaction relation genes were deleted, and strong interaction relation genes were highlighted.

Results

BRD9 alteration in various types of gastric cancer

TCGA data showed that BRD9 is changed to some extent in various types of gastric cancer. Figure 1A shows that BRD9 exhibits multiple alterations, such as low mRNA, high mRNA, deep deletion, and amplification in mucinous stomach adenocarcinoma; low mRNA, high mRNA, and mutation in tubular stomach adenocarcinoma; and low mRNA, high mRNA, and deep deletion in diffuse-type stomach adenocarcinoma. Through the data processing module of analyzing cancer copy number variation, GISTIC analysis revealed that BRD9 shows deep deletion, shallow deletion, diploid, gain, amplification, and other changes in gastric cancer (Fig. 1B). TCGA database concluded that BRD9 expression is changed in 143 (30%) of 478 patients with gastric cancer (Fig. 1C). On basis of these analysis, the BRD9 alteration data of tumor cells in TCGA were employed to screen BRD9 high expression (MGC-803 cells) and normal expression (AGS cells) gastric cancer cells as cell models to discuss the influence of BRD9 on gastric cancer.

RNA-seq data and identification of DEGs in gastric cancer cells

RNA-seq was used to determine gene alteration in MGC-803 cells and AGS cells after the addition of BRD9 inhibitors, namely, BI9564 and BI7273 and to identify the DEGs in gastric cancer cells. Information on the groups is as follows:

Group MGC-803: MGC-803 cells (control group), MGC-803 cells added with BI9564 (experimental group 1, T1), and MGC-803 cells added with BI7273 (experimental group 2, T2).

Group AGS: AGS cells (control group), AGS cells added with BI9564 (experimental group 1, T1), and AGS cells added with BI7273 (experimental group 2, T2).

Results for group MGC-803: 1204 DEGs were obtained when group MGC-803 T1 dataset was screened by the limma package (corrected P-value < 0.05, logFC > 1.5). Among these DEGs, 564 were upregulated, and 640 were downregulated. Meanwhile, 1338 DEGs were obtained for group MGC-803 T2 dataset. Among which, 754 were upregulated, and 584 were downregulated. The number of overlap DEGs for group MGC-803 T1 dataset and group MGC-803 T2 dataset was 425, of which 248 were upregulated, and 177 were downregulated (Fig. 2A).

Results for group AGS: 974 DEGs were obtained for group AGS T1 dataset, of which 539 were upregulated, and 435 were downregulated genes. Meanwhile, 1006 DEGs were obtained for group AGS T2 dataset. Among which, 536 were upregulated, and 470 were downregulated. The number of overlap DEGs for group AGS T1 dataset and group AGS T2 dataset was 382, of which 245 were upregulated, and 137 were downregulated (Fig. 2B).

Heatmap R package was used for cluster analyses to draw a heatmap of DEG expression levels as shown in Figs. 2C and 2D. Group information is shown in horizontal axis, and vertical axis represents the genes. Owing to space limitation, the gene names on the right were selectively displayed. Figures 2C and D show significant difference in DEG expression between the experimental and control groups.

Analysis of DEGs in gastric cancer cells added with BRD9 inhibitor

The intersection of 425 DEGs in group MGC-803 and 382 DEGs in group AGS were analyzed by heatmap R package. As shown in Fig. 3A, only 12 DEGs in the intersection. Through the comparison of these 12 genes (ARMC8, AVEN, MTRR2, MYH16, NPAS4, PCDH17, PLGLB2, RFPL3S, RNA28S5, SAMD9L, SHBG, and TMEM178B), we found that only three genes (MYH16, SAMD9L, and TMEM178B) had the same regulatory direction. Therefore, we think that for high BRD9 expression cell model-MGC-803 cells the controlled genes and pathways are quite different from those of AGS cells with normal BRD9 expression. Therefore, we can discuss the regulatory pathways of BRD9 in gastric cancer in two cases: one is gastric cancer (MGC-803 cells) with high BRD9 expression, the other is gastric cancer cells (AGS cells) with normal BRD9 expression.

GO and KEGG pathway enrichment analyses of DEGs in MGC-803 cells

Comprehensive bioinformatics analysis of RNA-seq data was performed using DAVID online analysis tool to obtain the biological annotation and regulated signal pathway of the DEGs controlled by BRD9 in MGC-803 cells. Figure 4 shows that the GO analysis of DEGs was divided into three functional groups, namely, molecular function, biological processes, and cell composition. In the molecular function group, the DEGs were mainly concentrated in secretion by cell, chloride transport, ion transport, intracellular signal transduction, and cell adhesion. In the cellular component group, the DEGs were mainly enriched in integral component of plasma membrane golgi lumen, integral component of membrane, and plasma membrane. In the molecular function group, the DEGs were mainly enriched in calcium ion binding, basic amino acid transmembrane transporter activity, and arginine binding. These GO analysis results indicate that most DEGs are substantially concentrated in cell secretion function, transport function, adhesion ability, signal transduction, and membrane formation. Meanwhile, KEGG pathway analysis indicated that the DEGs are mainly enriched in cell adhesion molecules, oxytocin signaling pathway, gastric acid secretion, calcium signaling pathway, and estrogen signaling pathway.

Analysis of DEGs controlled by BRD9 in group MGC-803 by using a PPI network

The DEG expression products in MGC-803 cells were constructed using the STRING database (<http://string-db.org>) to construct PPI networks with a total of 425 DEGs. The constructed data were inputted into Cytoscape software. Nodes that are isolated and with minimal connections with other nodes were deleted, and those with many connections with surrounding genes were highlighted. Visual analysis was also conducted. After the removal of isolated and partially connected nodes, a complex network of DEGs was constructed and is shown in Fig. 5. Among the 86 genes showing significant interaction, only three named PECAM1, SPP1, and GNB3 had the strongest interaction and were marked with red box. ANO1, ALPL, NOS3, VWF, GNAO1, ADRB1, ADCY1, CCL28, and GRM4 genes are the second most closely related genes and were marked with yellow triangle. PPI network indicated that these genes might play a key role in the MGC-803 cells controlled by BRD9.

GO and KEGG pathway enrichment analyses of DEGs in AGS cells

Comprehensive bioinformatics analysis of RNA-seq data was performed using DAVID online analysis tool to obtain the biological annotation and regulated signal pathway of the DEGs controlled by BRD9 in AGS cells, and the results are shown in Fig. 5. In the molecular function group, the DEGs were mainly concentrated in collagen catabolic process, receptor internalization, cell surface receptor signaling pathway, calcium ion transmembrane transport, positive gene regulation, and signal transduction. In the cellular component group, the DEGs were mainly enriched in integral component of plasma membrane, plasma membrane, extracellular region, integral component of membrane, and extracellular space, endosome. In the molecular function group, the DEGs were mainly enriched in extracellular matrix structural constituent, calcium ion binding, carboxypeptidase activity, interleukin-8 receptor activity, calcitonin receptor activity, interleukin-8 binding, and fibronectin binding. These GO analysis results indicate that most DEGs are remarkably concentrated in collagen catabolism, the functional pathway of receptor on cell surface, and the function of calcium ion transmembrane transport. Meanwhile, KEGG pathway analysis indicated that the DEGs are mainly enriched in protein digestion and absorption, vitamin digestion and absorption, and calcium signaling pathway.

Analysis of the DEGs controlled by BRD9 in group AGS by using a PPI network

The DEG expression products in AGS cells were constructed using the STRING database (<http://string-db.org>) to establish a PPI network with a total of 382 DEGs. The constructed data were inputted into Cytoscape software. Nodes that were isolated and had minimal connections with other nodes were deleted, and those with many connections with surrounding genes were highlighted. Visual analysis was also performed. After the removal of isolated and partially connected nodes, a complex network of DEGs was constructed and is shown in Fig. 7. Among the 70 genes with significant interaction, only two, namely, AR and GNGT2 had the strongest interaction and were marked with red hexagon. APOB, PRKG1, PRKGA, 7H, INSL3, RAMP1, CRHR2, HRH2, DRD5, CALCRL, CXCR1, CXCR2, CXCL10, CXCL11, GALR3, and SSTR5 genes are the second most closely related genes and were marked with yellow box. PPI network indicated that these genes might play a key role in the AGS cells controlled by BRD9.

Discussion

Gastric cancer is the most common malignant tumor of digestive system and is one of the top five with highest incidence and mortality rate among malignant tumors [11]. Gene mutation and epigenetic changes mainly induce gastric cancer. In many types of cancers, epigenetic regulations are involved in gene expression, DNA repair, and DNA replication [3, 4]. BRD9 is a subunit of SWI/SNF complex, the reader of histone acetyllysine, and can guide SWI/SNF complex to bind in histone, promote chromatin loosening, and upregulate gene expression. Therefore, abnormal BRD9 expression induces abnormal oncogene expression. Studying the signal pathway regulated by BRD9 in gastric cancer will provide a theoretical basis for the clinical precise treatment of gastric cancer patients with abnormally expressed BRD9.

High throughput gene sequencing technology can sequence hundreds of thousands to millions of DNA at one time and thus can comprehensively analyze the transcriptome and genome of cell samples. RNA-seq can determine the DEGs between the experimental and control groups, identify the signal pathways causing the differences, and provide theoretical basis for clinical treatment. In this research, BRD9 inhibitors were used to detect the regulatory signal pathways regulated by BRD9 in gastric cancer. The results showed that the regulatory network pathway controlled by BRD9 in MGC-803 cells differs from that in AGS cells. Therefore, classification by stages and types of gastric cancer is necessary for the treatment. Given the variations of signal regulatory network in different types of gastric cancer, identifying the specific regulatory signal network controlled by BRD9 in gastric cancer cells is helpful for the treatment of gastric cancer in patients with high BRD9 expression.

Two different types of gastric cancer cell models, namely, high (MGC-803 cell) and normal (AGS cell) BRD9 expression cell models were established to explore the signal pathway regulated by BRD9 in gastric cancer cells. RNA-seq using limma R package was conducted after the BRD9 inhibitors were added. A total of 1204 and 1338 DEGs were found in MGC-803 cells after the addition of BI9564 and BI7273, respectively. The number of intersecting DEGs for the above two conditions is 425 in MGC-803 group. In addition, 974 and 1006 DEGs were found in AGS cells after the addition of BI9564 and BI7273, respectively. The number of intersecting DEGs for the above two conditions is 382 in AGS group, and that between MGC-803 and AGS groups is 12. Heatmap results showed that only 3 out of the 12 DEGs number have the same regulation direction. Therefore, the cancer cells with different BRD9 expression levels have different regulation networks. Hence, the signaling pathway in gastric cancer cells controlled by BRD9 was discussed in two conditions, one is MGC-803 cells with high BRD9 expression, and the other is AGS cells with normal BRD9 expression (Fig. 8).

GO and KEGG enrichment analyses revealed that in the MGC-803 group, BRD9 mainly regulates cell adhesion pathway, which may be related to cell migration and invasion. In the MGC-803 group, the platelet and endothelial cell adhesion molecule 1 (PECAM-1), secretory phosphoprotein 1 (SPP1), and G protein subunit beta 3 (GNB3) genes were located at the core of PPI network. SPP1 is related to the occurrence and development of gastric cancer and is mediated by mir-340 to regulate gastric cancer progression through the PI3K/Akt signal pathway. Silencing SPP1 can inhibit the occurrence of gastric cancer in vivo, thus reducing the proliferation, invasion, and migration of gastric cancer cells [12]. Compared with that in normal ovarian tissues, SPP1 expression is higher in epithelial ovarian cancer. Its silencing can inhibit cell proliferation, invasion, and migration by regulating the Akt signaling pathway [13]. These results indicate that SPP1 inhibition can obstruct p-Akt and inactivate the PI3K/Akt signaling pathway. In addition to SPP1, anoctamin 1 (ANO1) overexpression is closely related to the occurrence of gastric cancer and therefore may be a potential target for the treatment of this disease [14–15]. G protein subunit alpha o1 (GNAO1) is highly expressed in gastric cancer, and its inhibited expression can promote the apoptosis and inhibit the migration of gastric cancer cells [16]. C-C motif chemokine ligand 28 (CCL28) is a mucosa associated epithelial chemokine that is abnormally expressed in breast cancer, lung adenocarcinoma, rectal cancer, and colon cancer and is negatively correlated with tumor occurrence [17–18]. Platelet endothelial cell adhesion molecule 1 (PECAM-1) is a type I

transmembrane cell adhesion and signal receptor that has a molecular weight of 130 kDa, a tissue-limited expression pattern. It is expressed on endothelial cells and hematopoietic cells and regulates angiogenesis, vascular permeability, and cell reactivity [18]. PECAM-1 plays an important role in interference with tumor clearance [19–22], and Terashima et al. found that it is substantially related to peritoneal recurrence in patients with stage II and III gastric cancer. This molecule is involved in several processes related to the growth and spread of primary cancer by encoding proteins, including angiogenesis, vascular permeability, and extracellular leukocyte transport. Moreover, mouse experiments showed that anti-PECAM-1 antibody effectively inhibits tumor metastasis in late stage shift [23–24]. GNB3 mainly encodes the gene of G-protein β 3 subunit and is related to hypertension, obesity, and atherosclerosis. In hypertension, GNB3 stimulates vasoactivity and vascular proliferation [25]. C825T polymorphism in GNB3 gene is associated with obesity [26]. Epidemiological evidence and related animal studies showed that having a high salt diet easily leads and promotes the occurrence of gastric cancer [27–28]. The C825T polymorphism of GNB3 gene is related to hypertension, salt sensitivity, many diseases [29–30], and diffused gastric cancer in TT genotype [31]. Therefore, BRD9 may affect the development of gastric cancer by regulating the intracellular network pathway of MGC-803 through key genes such as GNAO1, SPP1, and CCL28.

GO and KEGG enrichment analyses revealed that in the AGS group, BRD9 mainly regulates antigen processing and presentation pathway, cytotoxic pathway mediated by natural killer cells, cAMP signaling pathway, and cGMP PKG signaling pathway. The former two are mainly related to immunity, and the latter two are related to nucleic acid cascade reaction (Fig. 8).

In the AGS group, androgen receptor (AR) and G protein subunit gamma transducin 2 (GNGT2) genes are located at the core of PPI network. As an androgen receptor, AR is a member of nuclear receptor superfamily and participates in cell function as a ligand-activating transcription factor. Abnormal AR expression leads to oral squamous cell carcinoma, bladder cancer, and gastric cancer [32–34]. AR is a target gene of curing prostate cancer, breast cancer and other tumors [35–37]. Xia et al. [36] found that ar-v12 is highly expressed in gastric cancer cells and related to gastric cancer metastasis. High ar-v12 level can promote the migration and invasion of gastric cancer cells by directly regulating myosin light chain kinase (MYLK) expression. Wang et al. [39] also showed that AR plays an important role in gastric cancer occurrence, and its knockout weakens the colony formation, invasion, and migration of gastric cancer cells. High AR expression promotes the migration and invasion of gastric cancer, resulting in poor prognosis. These results confirm the carcinogenic role of AR in gastric cancer progression. GNGT2 is a member of the G protein γ family and can mediate β -inhibitory protein 1-induced Akt phosphorylation and NF- κ B activation [40–42]. Proinflammatory cytokines can activate the NF- κ B pathway, and the increase in NF- κ B activity inhibits cell apoptosis and promotes tumor occurrence, invasion, and metastasis [43]. Wang et al. [44] found that after esophagectomy and gastric pedicle transplantation, the decrease in the survival rate of patients with esophageal cancer was accompanied by high GNGT2 expression. Hence, the expression level of GNGT 2 gene is negatively correlated with survival rate. This differential expression of GNGT2 may be related to tumor occurrence and development. Its upregulation may

activate NF- κ B pathway, enhance the anti-apoptosis ability of gastric cancer cells, and eventually lead to gastric cancer.

In conclusion, comprehensive bioinformatics was used to study the signaling pathway of BRD9 in gastric cancer cells and revealed great differences in signal transduction between gastric cancer cells with high and normal expression BRD9 expression. Therefore, the different signal pathways must be analyzed according to the different types of gastric cancer in clinical practice. The result may serve as a theoretical basis for clinical treatment and provide reasonable guidance for clinical medication through the classified treatment of patients.

Declarations

Author contributions

Yuan Wang and Xi-Yong Yu conceived and designed the study. Yuan Wang, Chen Wang, Chu-Wen Li, Yu-Ting Mo and Wen Yi Tan performed analysis data. Yuan Wang, Chen Wang wrote the manuscript. All authors reviewed and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest

All authors declare that they have no conflict of interests.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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BRD9 expression in gastric cancer A: Alterations of BRD9 in different types of gastric cancers; B: Analysis of BRD9 copy number alterations from GISTIC; and C: Analysis of BRD9 gene changes in 478 patients with gastric cancer.

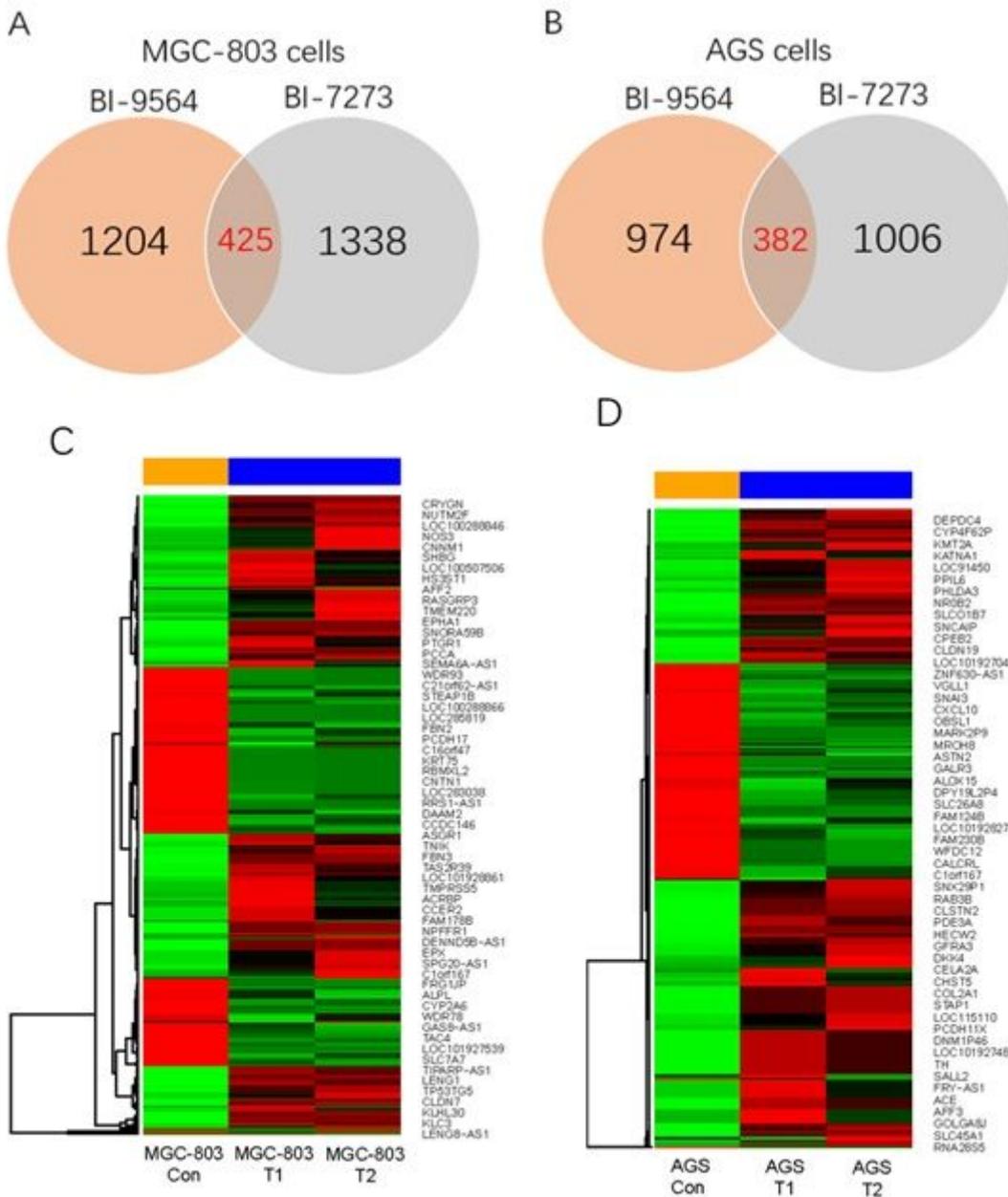


Figure 2

Differentially expressed genes and their intersections and their heatmaps A: Differentially expressed genes and their intersections in MGC-803 cells. B: Differentially expressed genes and their intersections in AGS cells. C: Heatmaps of differentially expressed genes in group MGC-803.

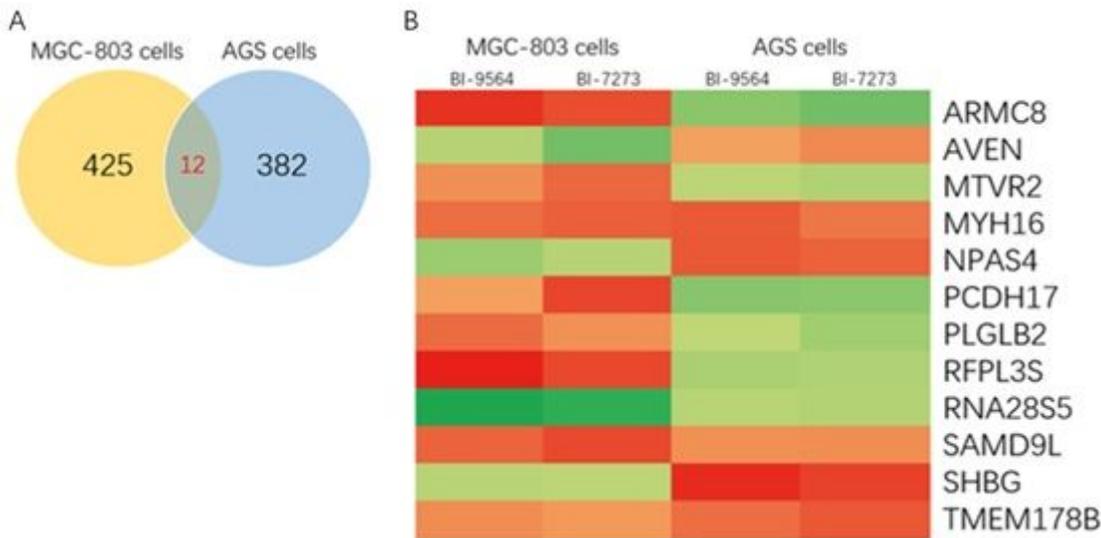


Figure 3

Differentially expressed genes and their intersections and thermogram A: Differentially expressed genes intersection between MGC-803 cells and AGS cells added with BRD9 inhibitors B: Thermogram of differentially expressed genes in MGC-803 cells and AGS cells added with BRD9 inhibitors.

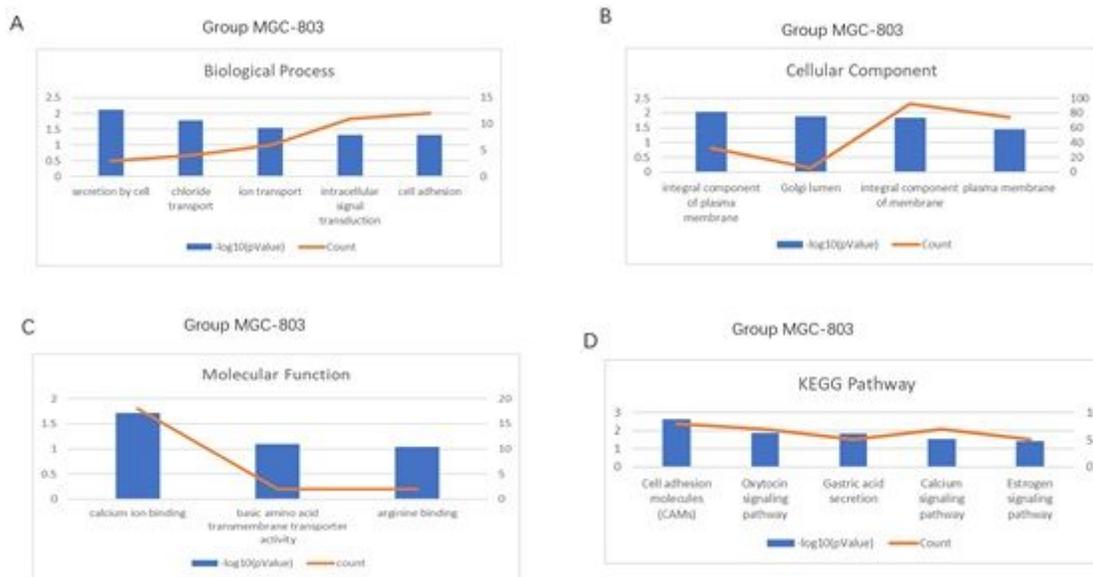


Figure 4

GO enrichment analysis and KEGG analysis of DEGs controlled by BRD9 in MGC-803 cells A: biological process analysis of group MGC-803, B: cellular component analysis of group MGC-803, C: molecular function analysis of group MGC-803, and D: KEGG analysis of group MGC-803.

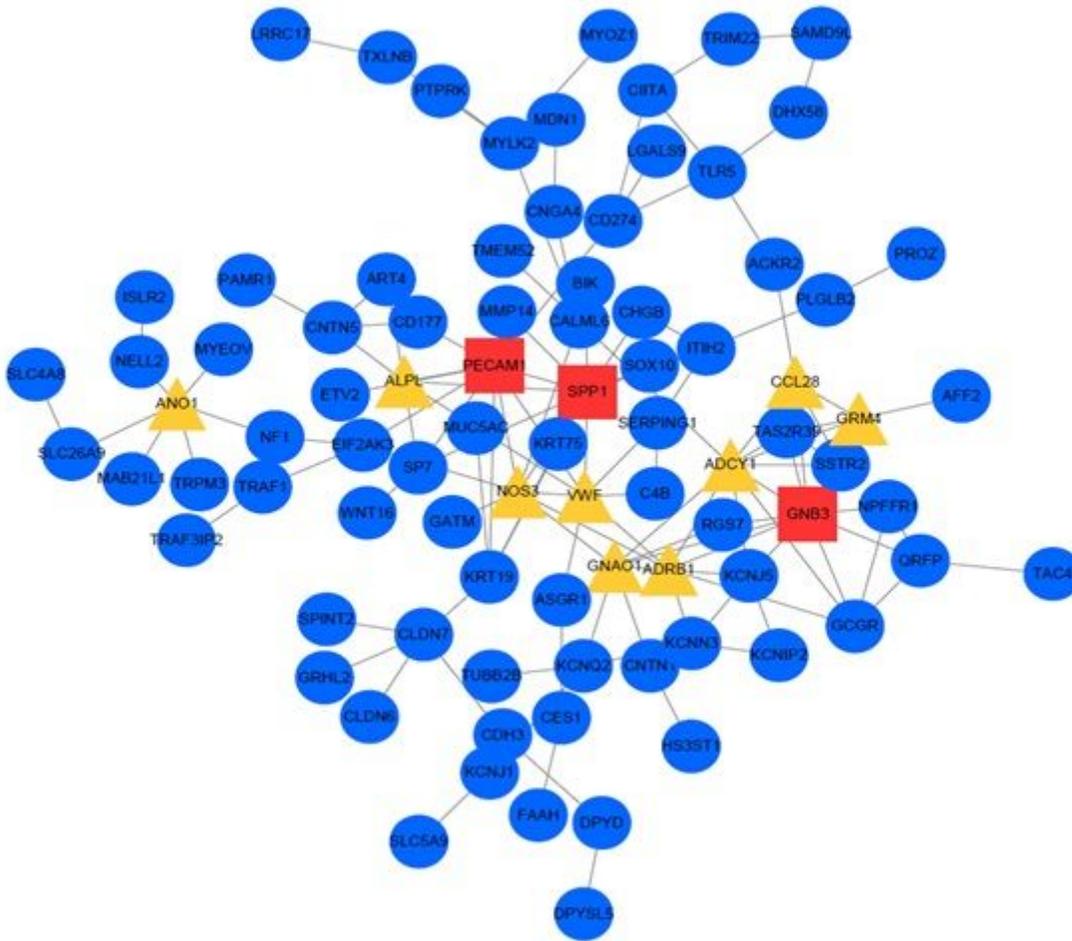


Figure 5

Protein-protein interaction network of differentially expressed genes in group MGC-803

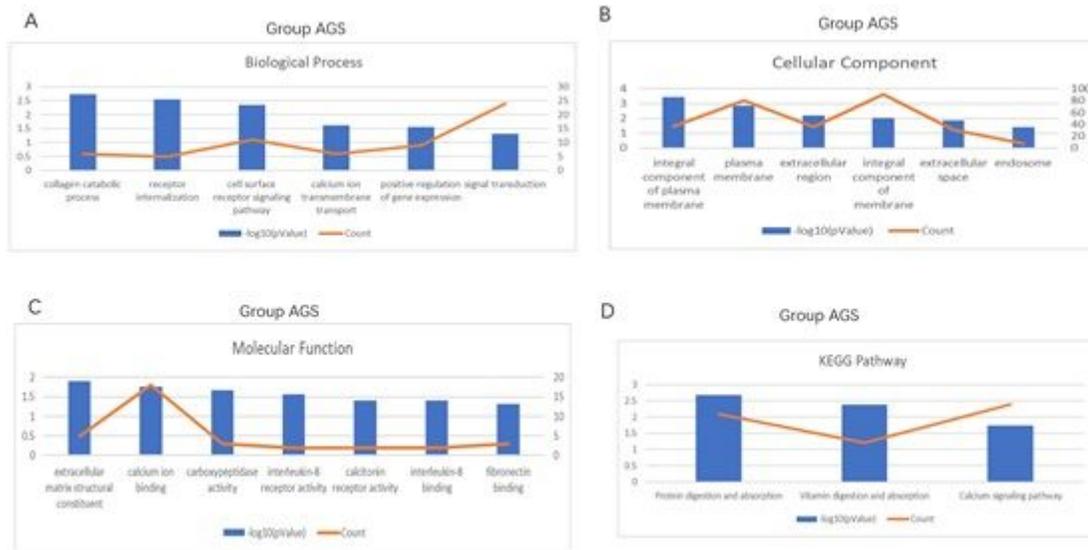


Figure 6

GO enrichment analysis and KEGG analysis of DEGs in AGS controlled by BRD9 A: biological process analysis of group AGS, B: cellular component analysis of group AGS, C: molecular function analysis of

group AGS, and D: KEGG analysis of group AGS.

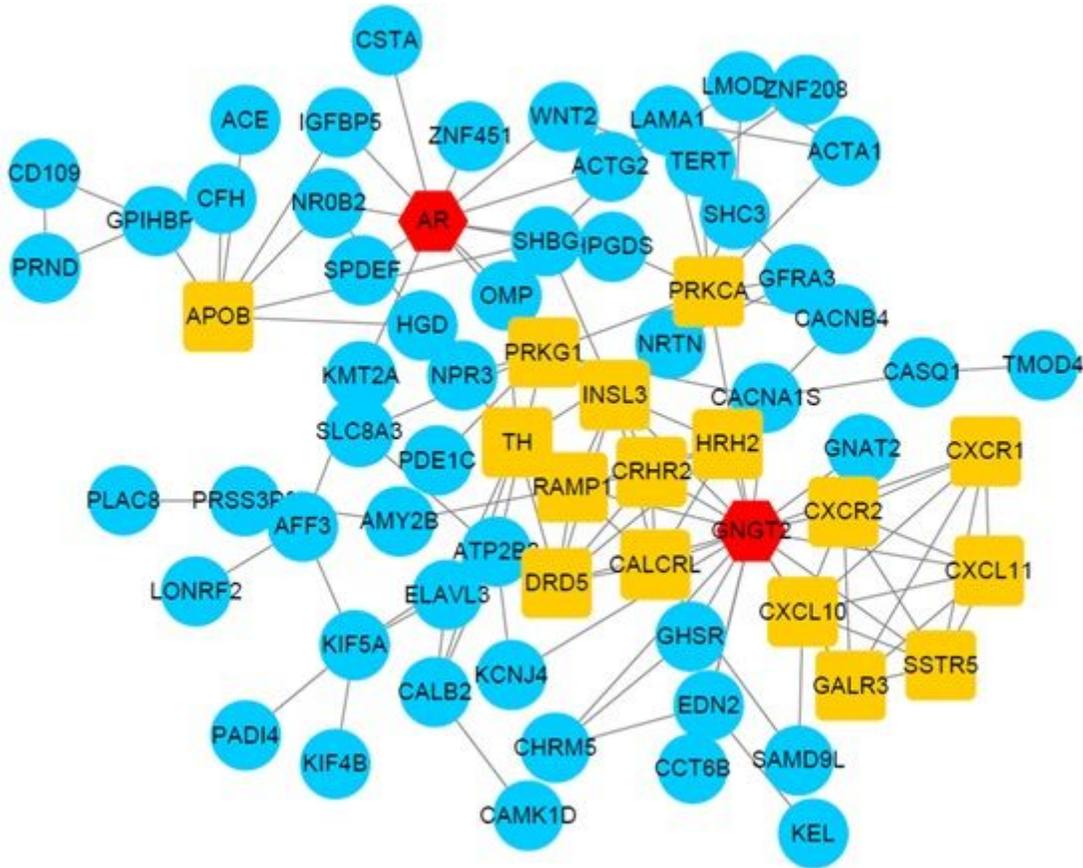


Figure 7

Protein–protein interaction network of differentially expressed genes for group AGS

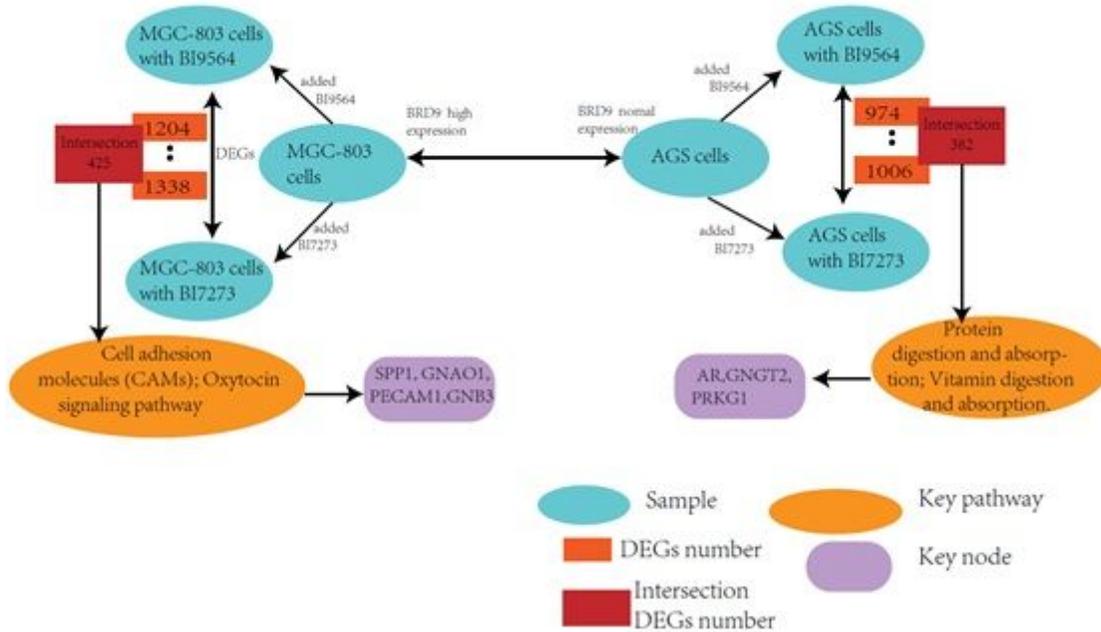


Figure 8

