Bioinformatics analysis of four treatments based on murine hindlimb ischemic model

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Abstract: Peripheral arterial disease (PAD) is a major manifestation of peripheral atherosclerosis. Murine hind limb ischemic (HLI) model is widely used to study the appropriate treatment to treat PAD. Currently, there are multiple strategies developed for vascular regeneration, validated through HLI model. However, limited publications have offered the in-depth analysis of the therapeutic effects among all these treatments. Here, we handpicked four different therapies (nano-therapy, gene knockout therapy, drug therapy and adenovirus therapy) for the treatments of hindlimb ischemic disease and compared their RNA-Seq data to elaborate the common activation genes and pathways in vascular regeneration. We found that different treatments can activate similar pathways related to angiogenesis, further promote related biological processes, and achieve the purpose of angiogenesis.

1. Introduction

Limb ischemia is a common clinical disease with two major clinical manifestations: acute and chronic ischemia. Acute hindlimb ischemia is caused mainly due to the interruption of blood flow to the limb generated by embolism or thrombotic vascular occlusion in situ, which leads to insufficient blood supply to the distal limb [1]. In severe cases, it can lead to limb loss and even threaten to the lives of patients, so it is critical to adopt the appropriate treatments with timely intervention [2]. For the possible treatment of acute hindlimb ischemia, murine hindlimb ischemic model has been developed [3]. The model is mainly based on the artery ligation of the femoral artery in the leg, which causes ischemia in the distal limb of the mouse [4]. Researchers have subsequently used different approaches to treat the disease and developed several potential treatment strategies. However, limited studies have been performed to compare the therapeutic effects and the triggered cascades of signal pathways from these rescue strategies. (Figure1a-b).

Nano therapy, as a new type of treatment, it has been widely studied in recent years because of its good targeting properties in treatment. This research was performed by injecting the apoA-I modified nanoparticles into the mouse leg ischemia model constructed site, the apoA-I is the predominant high-density lipoproteins (HDL) ApoA-I is the predominant structural component of HDL, and there is substantial evidence that plasma levels of HDL-C are inversely associated with the risk of cardiovascular disease (CVD) (apoA-I mimetics). Therefore, apoA-I has great potential in the treatment of vascular diseases. Studies have shown that apoA-I nano therapy saved ischemic limbs and to promote vascular and muscle regeneration in mice [5]. Our analysis used the RNA-seq data of endothelial cells and macrophages in the treatment and control groups after 7 days of nanoparticle treatment. The drug treatment was to treat leg ischemia in mice with an orally available soluble guanylate cyclase activator called praliciguat. Studies have shown that treatment with this drug promotes blood flow recovery and increases in arterial diameter in ischemic muscle in mice [6]. In this paper, we analysed the genomic sequencing data of the muscle of the treatment group and the control group after 28 days of drug treatment.

Another treatment modality is using gene knockout therapy. Studies have shown that regulatory T cells (Tregs) are critical to blood vessel formation [7, 8]. Among them, the functional activity of Treg cells affects the revascularization in a model of hindlimb ischemia, and it is mainly involved in this process through the cytokine IL-10 [9]. In this study, T-cell activity was regulated mainly by

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suppressing the expression of the gene G protein-coupled receptor 174 (GPR174) [10]. The results showed that GPR174 knockdown Tregs enhanced endothelial cell function and reduced pro-inflammatory macrophage migration and endothelial cell apoptosis, thereby achieving the effect of promoting blood flow recovery in the mouse limb ischemia model. We analysed using RNA-seq data from the leg muscles of knockout and wild-type mice taken seven days after surgery in this study

The adenovirus treatment was further tested for its efficacy in angiogenesis and inflammatory response by intramuscular injection of E-selectin/AAV2/2 (adeno-associated virus serotype 2/2) into mice that had been developed leg ischemia models [11]. E-selectin, a cell adhesion molecule, is essential for wound healing and neovascularization of ischemic area [12, 13]. The results showed that the legs of the mice treated with adenovirus injections had little necrosis and that blood flow to the leg vessels was largely restored. We used the expression data of 48 genes related to angiogenesis and inflammation analysed by RT-qPCR array analysis in this study.

In this paper, we mainly analyze the similarities and differences of the activated genes and pathways of four commonly used therapies (i.e., nano-therapy, gene knockout therapy, drug therapy and adenovirus therapy), to identify some common regenerative mechanisms of angiogenesis promoted by these different therapeutic methods from the perspective of bioinformatics (Figure1a-b). We download the sequencing data of the above four treatments from the website GEO Datasets, and compare and analyze the four groups of data from multiple perspectives (Figure. 1). This paper focuses on the similarities and differences of the activation of angiogenesis in the four treatments, as well as the differences of inflammatory response caused by different treatments in immunity. Through the comparative analysis of differential genes and activated pathways, we discovered the angiogenesis pathways that need to be activated by different approaches to treat leg ischemia from the perspective of bioinformatics, which provided new ideas for clinical treatment and drug research.

2. Results and analysis

2.1. Differentially expressed Gene analysis and Gene Ontology (GO) analysis

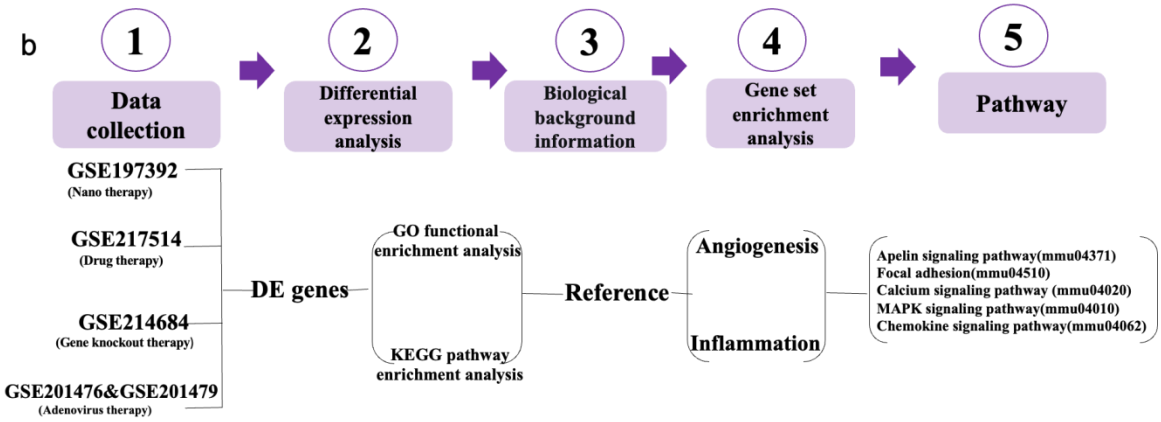
We mainly focused on biological processes related to angiogenesis, so we first performed GO enrichment analysis for each group of data, focusing on the biological process (BP). Here we used DESeq R package[14] on drug therapy (GSE217514), gene knockout therapy (GSE214684), nano therapy (GSE197392) RNA-seq datasets.

Gene Ontology (GO) is a classification system of gene functions. Reduce and Visualize Gene Ontology (REViGO) can eliminate possible redundancy arising from GO term enrichment. Here, GO enrichment analysis of differential genes was performed using the cluster Profiler R package and similarity clustering and visualization of GO terms were performed by REViGO. We found similarities in GO terms among these four treatments. The results were as follows (Figure 1 c-f). The main terms of drug therapy (GSE217514) were muscle tissue development (GO:0060537), endothelial cell(EC) migration (GO:0043542), and cellular response to vascular endothelial growth factor stimulus (GO:0035924). In which ECs migrate along a concentration gradient of endothelial growth factor (VEGF) [15], thus better promoting vascular regeneration [16]. This may be the reason for the recovery of ischemic tissue by this therapy. The main terms of nanotherapy (GSE197392) were VEGF signaling pathway (GO:0038084) and regulation of smooth muscle cell(SMC) proliferation (GO:0048660). And the fibroblasts(FBs), SMCs, and ECs are involved in the formation of blood vessels [17]. The main terms of gene knockout therapy (GSE214684) were muscle tissue development (GO:0060537), EC differentiation (GO:0045446) and regulation of angiogenesis (GO:0045765). The main terms of adenovirus therapy (GSE201476&GSE201479) were vascular EC proliferation (GO:0101023) and positive regulation of receptor signaling pathway via JAK-STAT(GO:0046427). JAK/STAT pathway is involved in cell proliferation and differentiation, organ development and immune homeostasis [18].

Therefore, we can summary that the pathways activated by the four treatments were mainly involved in muscle development, angiogenesis and anti-inflammation.

a

GSE number	Theapy approach		control	treatment
GSE197392	Nano-therapy	Endothelial cells(EC)	Control(4×)	T2DM(4×)
		Macrophage(Mac)	Control(4×)	T2DM(4×)
GSE217514	Drug therapy		pla(4×)	pra(4×)
GSE214684	Gene knockout therapy		WT(4×)	gpr(4×)
GSE201476	Adenovirus therapy	vascular regeneration	LacZ / AAV2 / 2	E-selectin/ AAV2 / 2
GSE201479		inflammatory	LacZ / AAV2 / 2	E-selectin/ AAV2 / 2



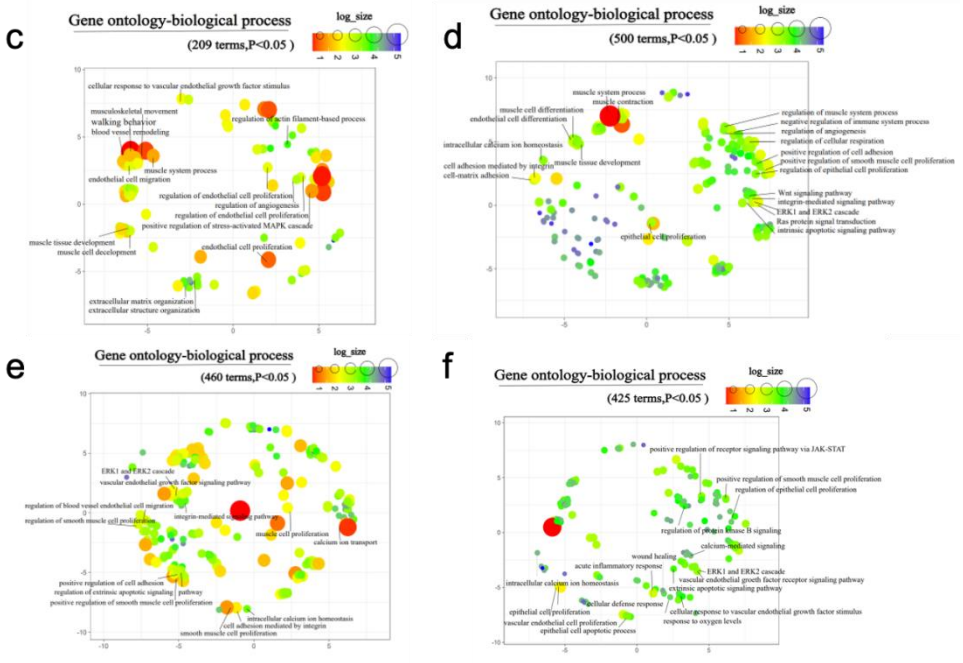


Figure 1. The general scheme of the article. a: Data classification and GSE number; b: Process of experimental analysis. c: Visualization of all significant GO terms of DEG (fold change >1 or < -1, padj < 0.05) between pla and pra group for drug therapy (GSE217514). d: Visualization of all significant GO terms of DEG (fold change >1 or < -1, p adj < 0.05) between WT and gpr group for gene knockout therapy (GSE214684). e: Visualization of all significant GO terms of DEG (fold change >1 or < -1, p adj < 0.05) between Control and T2DM group for nano therapy (GSE197392). f: Visualization of all significant GO terms for Adenovirus therapy (GSE201476&GSE201479). The GO terms for BP were organized in a semantic similarity-based scatter plot using REViGO. REViGO combines redundant terms into a representative term based on a simple clustering algorithm that relies on a semantic similarity metric.

2.2. KEGG (Kyoto Encyclopedia of Genes and Genomes) enrichment analysis

Kyoto Encyclopedia of Genes and Genomes (KEGG) is a knowledge base for systematic analysis of gene functions, linking genomic information with functional information [19]. And the KEGG

Pathway database integrates small molecule interaction networks, such as metabolism, membrane trafficking, signal transduction and cell cycle. Here, we used cluster Profiler R package for KEGG enrichment analysis of DEG. From TOP 50 enrichment results, we found Calcium signalling pathway (mmu04020), ECM-receptor interaction(mmu04512), Focal adhesion (mmu04510), Apelin signalling pathway (mmu04371) and MAPK signalling pathway (mmu04010) were mostly enriched in drug therapy(Figure 2a). Among them, Apelin (AP Jendogenousligand) is the endogenous receptor of angiotensin receptor-like 1 (APJ). It can regulate vasodilation or contraction [20]. Focal adhesion kinase (FAK), a cytoplasmic tyrosine kinase, not only is a central mediator of integrin transduction but also involved in the signalling of other cell surface receptors. FAK and its downstream signalling pathways can regulate cell migration and angiogenesis [21]. Calcium is a versatile signalling molecule that plays a central role in many vascular functions [22]. Mitogen-activated protein kinase (MAPK) pathway is a common signal transduction pathway that transmits extracellular signals to downstream effector molecules and participates in the regulation of a variety of cellular physiological processes, including cell proliferation, differentiation, stress, inflammation and apoptosis [23]. These significantly activated correspondent pathways and together promote vascular regeneration.

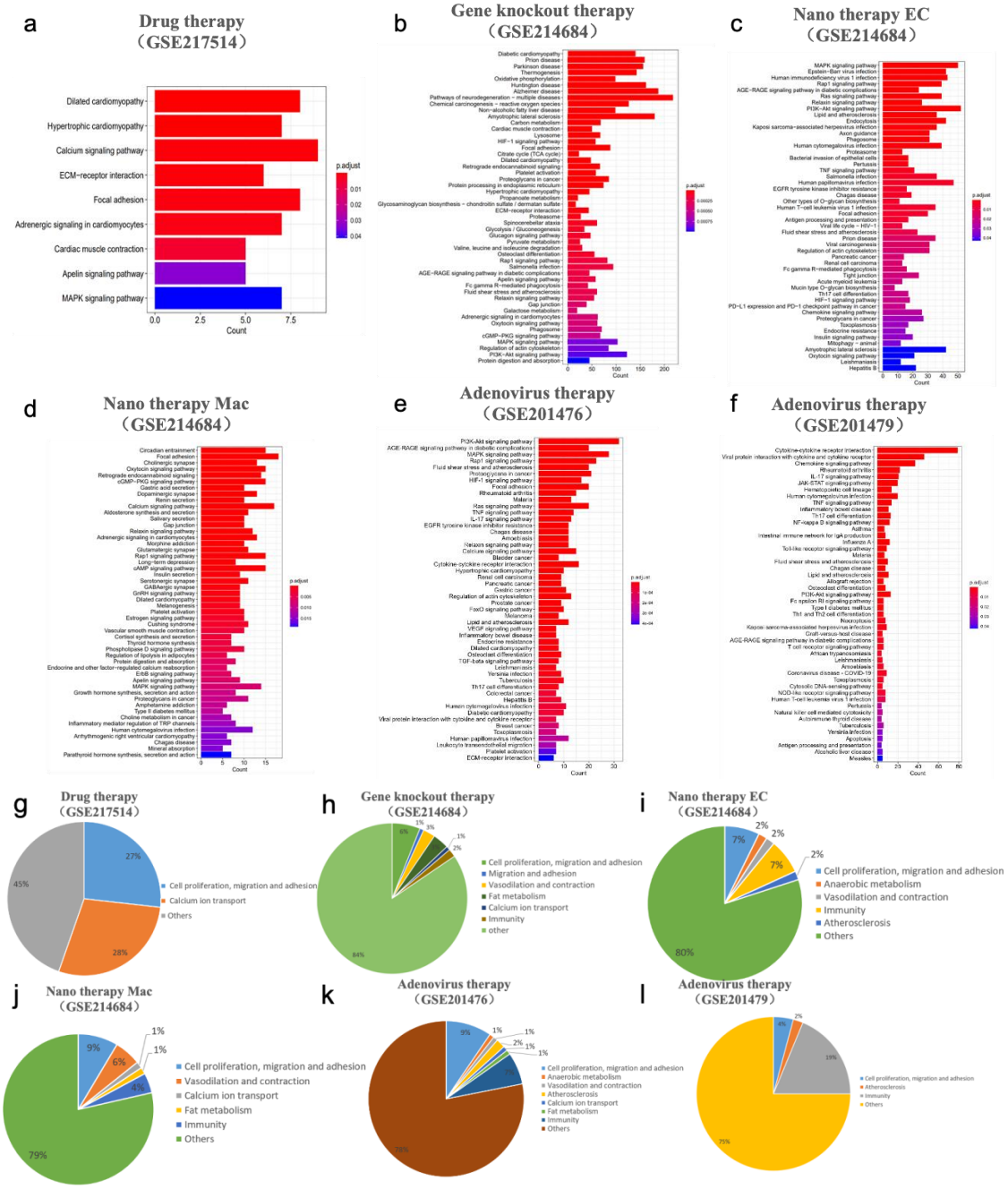
Then we can see HIF-1 signalling pathway (mmu04066), Fluid shear stress and atherosclerosis (mmu05418) and PI3K-Akt signalling pathway (mmu04151) were enriched in gene knockout therapy(Figure 2b). Hypoxia-inducible factor-1(HIF-1) can leads to the transcriptional induction of a series of genes that participate in angiogenesis, iron metabolism, glucose metabolism and cell proliferation when cells are starved of oxygen [24]. Atherosclerosis is a common cause of cardiovascular disease. Studies have found that endothelial dysfunction is a necessary condition for atherosclerosis [25]. The PI3K-Akt signalling pathway was identified as a key factor in the process, leading to VEGF-induced EC survival and playing a critical role in vascular regeneration [26].

We found TNF signalling pathway (mmu04668), cGMP-PKG signalling pathway (mmu04022) and Inflammatory mediator regulation of TRP channels (mmu04750) were enriched in Nano therapy(Figure 2c-d). cGMP is a second messenger that produces its effect by interacting with intracellular receptor proteins. cGMP-dependent protein kinase (PKG) are receptors present in SMCs. The interaction between cGMP and PKG can regulate the contractile activity of SMCs [27]. The TRPM2 channel is one of the core participants in the immune inflammatory response in cardiovascular diseases and thus represents a promising therapeutic target [28].

In adenovirus therapy, VEGF signalling pathway (mmu04370), Chemokine signalling pathway (mmu04062), JAK-STAT signalling pathway (mmu04630), NF-kappa B signalling pathway (mmu04064) and Toll-like receptor signalling pathway (mmu04620) were enriched(Figure 2e-f). Among them, the JAK-STAT signalling pathway plays a key role in the vascular regeneration of this treatment, which is composed of a series of transcription factors involved in biological processes such as inflammation, immunity, cell proliferation, differentiation and survival [29].

According to this biological background knowledge, we made a classification: cell proliferation, migration and adhesion, vasodilation and contraction, calcium ion transport, fat metabolism, anaerobic metabolism, immunity, atherosclerosis and other. We calculated their percentages in different treatment methods (Figure 2g-l). In drug therapy (GSE217514) (Figure 2g), cell proliferation, migration and adhesion is 27%, calcium ion transport is 28%, and others are 45%. In gene knockout therapy (GSE214684) (Figure 2h), cell proliferation, migration and adhesion is 6%, anaerobic metabolism is 1%, vascular relaxation and contraction is 3%, lipid metabolism is 3%, calcium ion transport is 1%, immunity is 2%, and others are 84%. In nanotherapy-EC(GSE197392) (Figure2i), cell proliferation, migration and adhesion is 7%, anaerobic metabolism is 2%, vasodilation and contraction is 2%, immunity is 7%, and others are 80%. In nanotherapy-Mac (GSE197392) (Figure 2j), cell proliferation, migration and adhesion is 38%, vascular relaxation and contraction is 25%, calcium ion transport is 6%, blood flow regulation is 6%, fat metabolism is 38% and immunity is 19%. In adenovirus therapy-angiogenesis (GSE201476) (Figure 2k), cell proliferation, migration and adhesion is 9%, anaerobic metabolism is 1%, vasodilation and contraction is 1%, vascular sclerosis is 2%, calcium ion transport is 1%, fat metabolism is 1% and others are 78%. In adenovirus therapy-inflammation (GSE201479) (Figure 2l), cell proliferation, migration and adhesion is 4%,

vascular sclerosis is 2%, immunity is 19% and others are 75%. According to the above statistical results, it can be seen that although all four therapeutic methods can achieve blood flow restoration, their mechanisms of action are different. Drug therapy may mainly promote calcium-related material transport and membrane transport processes; gene knockout therapy, Nano therapy and adenovirus therapy may mainly focus on the involvement of cell-related biological processes, such as promotion of cell proliferation, migration and adhesion. In addition, we analysed the pathways enriched by different treatment approaches, and the results of the Venn diagram showed that we found four commonly activated pathways, namely Calcium signalling pathway, Apelin signalling pathway, MAPK signalling pathway and Focal adhesion pathway (Figure 2m), and we will further analyze the activation of each pathway in the following.



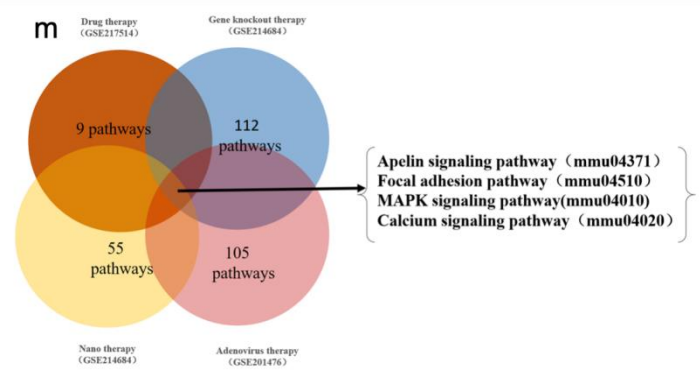


Figure 2. KEGG enrichment analysis of differential expression gene (DEG). a-f. Visualization of TOP50 significant KEGG pathways of DEG for drug therapy (GSE217514) (a), gene knockout therapy (GSE214684) (b), Nano therapy (c-d) and adenovirus therapy (e-f); g-l. The pie chart showing the percentage of pathways activated by drug therapy (GSE217514) (g), gene knockout therapy (GSE214684) (h), Nano therapy(i-j) and Adenovirus therapy(k-l). m: Venn diagram of the enrichment pathway in four treatment modalities.

2.3. Calcium signalling pathway, Apelin signalling pathway, MAPK signalling pathway and Focal adhesion pathway analysis

Next, we analysed the four similar pathways activated by different therapy. These pathways are associated with angiogenesis. Since only 48 genes related to vascular and inflammation were selectively detected by adenovirus therapy, the pathway maps were enriched for fewer genes, so we mainly compared the similarity of the pathway maps of the other three therapies.

Apelin signaling pathway in angiogenesis mainly influences the process of angiogenesis by regulating the migration and proliferation ability of ECs [30] (Figure 3a). In the Apelin signaling pathway (mmu04371), the three therapies induced the biological process of vasoconstriction through the activation of genes Adcy6, Aplnr, Myl4, and Klf2, as shown in the red box in the access diagram (Figure 3b-c). Transcription factor Klf2 can participate endothelial homeostasis, Vaso regulation and vascular growth/remodeling [31]. And Aplnr is a receptor which is widely expressed in blood vessels, heart, and cardiovascular regulatory regions of the brain [32]. The three therapies induced the biological process of cardiovascular development through the activation of genes Gnb1, Aplnr and Mef2b, as shown in the blue box in the access diagram (Figure 3b,3d).

MAPK signalling pathway is an important pathway regulating vascular regeneration, which can directly affect the production of VEGF and have an impact on the activity of ECs [33] (Figure 3e). In the MAPK signalling pathway(mmu04010), the three therapies all activate Csf1r, Mapk1, Fos, Nlk and other genes to cause the biological process of cell proliferation and activate the downstream Wnt signalling pathway (Figure 3f-h). Wnt signalling is critical to developmental processes, including cell proliferation, differentiation, and organization [34].

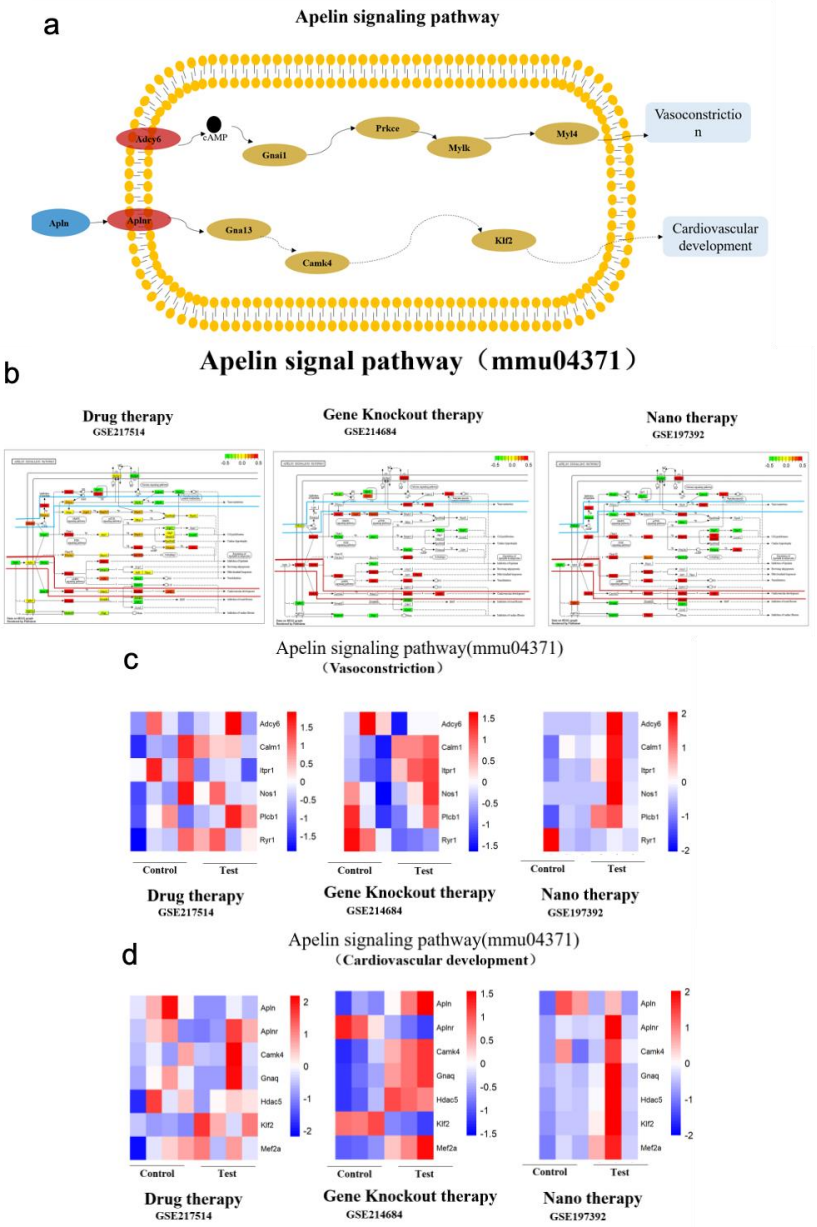
Calcium signalling affects vascular function primarily by modulating arterial tone, including the interaction of transmembrane proteins in calcium channels, so that blood vessels can sense and respond to physiological stimuli, such as changes in intravascular pressure [35] (Figure 4a). We found that in the calcium signalling pathway (mmu04020), drug therapy, gene knockout therapy and Nano therapy activated the downstream MAPK signalling pathway through Adora2a, Pln, Calm1, Ppp3ca (Figure 4b-c).

The regulation of vascular function by the Focal adhesion pathway is mainly mediated by VE-cadherin, a component of inter-EC adhesion junctions, which plays a key role in maintaining vascular integrity [36] (Figure 4d). In Focal adhesion pathway(mmu04510), three treatments induced downstream cell survival by activating Itga2b, Itgb1, Egfr and Pak genes (Figure 4b).

2.4. Immune-related signal pathway analysis

Based on the differential genes enriched in the four treatment modalities, the immune response triggered by drug treatment was small and did not enrich for many differential genes related to

immunity. In contrast, RNA-Seq performed on cells used for nanotherapy also detected fewer immune-related genes. Thus, we mainly analyzed the immune genes that were commonly enriched in the knockout treatment and adenovirus treatment. Comparative analysis of knockout therapy versus adenoviral therapy revealed that both of them activated chemokines signaling pathways in terms of the inflammatory response of concern [37] (Figure 5a). Chemokines are a family of low-molecular-weight secretory proteins that attract immune cells such as leukocytes to sites of inflammation. In addition, chemokine signaling can also guide the migration of neurons, nerve sheath cells and germ cells during embryonic development, and regulate the patterning and remodeling of the vascular system [38]. The heat map showed the genes that are still significantly expressed in the chemokine pathway in both knockout and adenoviral treatment (Figure 5b). Pathway map showed that adenovirus treatment may activate Chemokine signaling pathway by up-regulating Cxcl1 gene. Gene knockout therapy down-regulates the expression of Cxcr4, Akt1 and other genes, which may inhibit this pathway (Figure 5c). In addition, we found that chemokine signaling pathways can be associated with angiogenesis-related pathways, and further activate JAK-STAT, ECM-receptor and Wnt signaling pathways, thus affecting cell migration, differentiation and proliferation, and ultimately achieving the effect of promoting angiogenesis (Figure 5d).



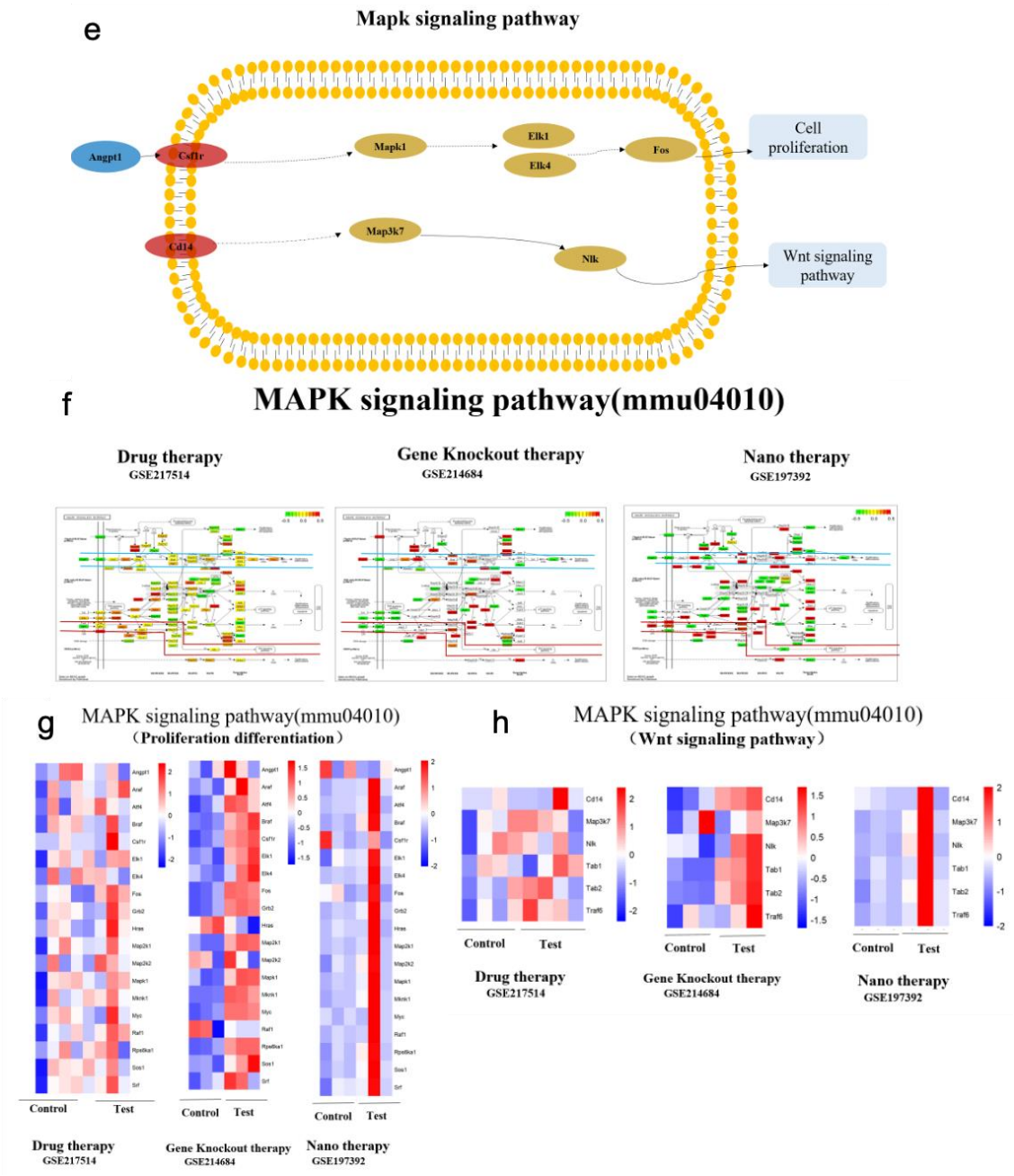


Figure 3. The Heatmaps and pathway map of Apelin signalling pathway(mmu04371) (a-d), MAPK signalling pathway(mmu04010) pathway in drug therapy (GSE217514), gene knockout therapy (GSE214684) and Nano therapy (GSE214684) (e-h).

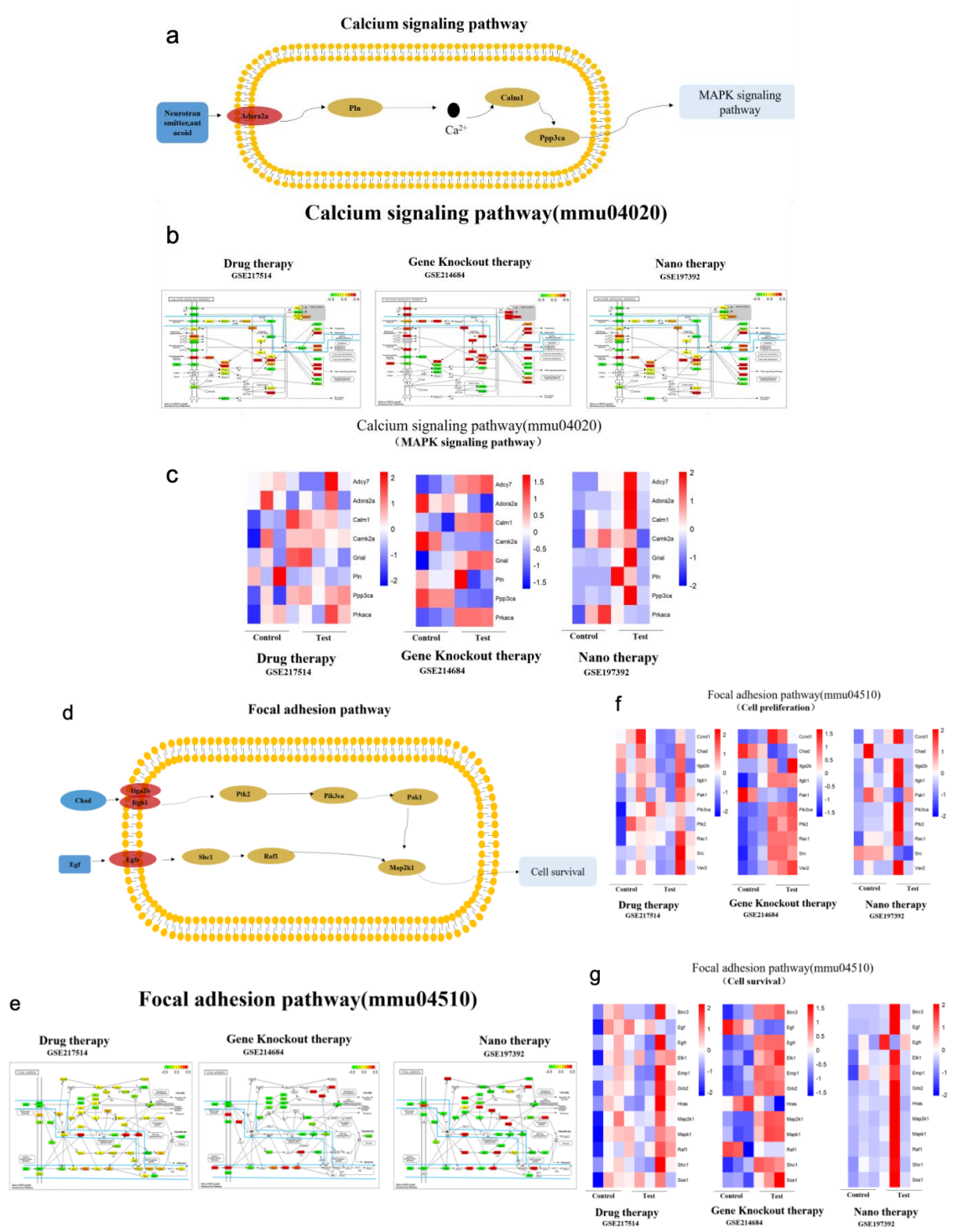


Figure 4. The Heatmaps and pathway map of Calcium signalling pathway (mmu04020) (a-c), Focal adhesion(mmu04510) (d-g) in drug therapy (GSE217514), gene knockout therapy (GSE214684) and Nano therapy (GSE214684) (e-h).

3. Discussion and conclusion

At present, the complications of many diseases can cause limb ischemia, and many research attempted to use different methods to improve the vascular remodelling of ischemic limbs. In this paper, we compare the differences of genes and pathways activated by four ways from the perspective of bioinformatics. Through the GO enrichment and KEGG enrichment analysis of the differentially expressed genes from each treatment modalities, we found that there were four commonly activated pathways in the vascular regeneration related pathways, which were Apelin signalling pathway, Focal

adhesion pathway, MAPK signalling pathway and Calcium signalling pathway (Figure 2m). By comparing the pathway maps of Apelin signalling pathway, we found that both Mylk and Myl4 genes were activated in gene knockout and nanotherapy, which are important proteins regulating cardiomyocyte activity [39], thus leading to the activation of the vasoconstriction biological process (Figure 3b). Moreover, in this pathway, all four treatment approaches significantly activate the cell proliferation process, which may be one of the factors for their all ability to restore hindlimb ischemia. We observed that the genes related to ECM-receptor interaction, cytokine-cytokine receptor interaction process were activated in all four treatment approaches by comparing the pathway maps of Focal adhesion pathway, among them, the extracellular matrix (ECM) was composed of different of complex structures that determine the function and structure of each organ and provide scaffolds for material exchange, mechanical support and adhesion migration of cells, which play an important role in vascular remodelling [40]. This is one of the factors that enable each treatment modality to save the ischemic limb. By comparing the pathway maps of the Calcium signalling pathway, we found that more genes are activated by this pathway in drug therapy, gene knockout and nanotherapy, and since calcium signalling is closely related to many biological processes, it provokes a series of cascade responses, such as the activation of MAPK signalling pathway and the activation of metabolism-related biological processes. Although the cellular material used for adenovirus was sequenced with a smaller number of activated genes, activation of the vascular-associated growth factor Egf and its receptor was still observed (Figure 3e-h). In addition, each treatment activates the MAPK signalling pathway, the activation of this pathway is essential for vascular remodelling, and MAPK regulates various pathophysiological processes such as cellular inflammation, differentiation, proliferation, oxidative stress and apoptosis [41, 42]. It can regulate oxidative stress and apoptosis in endothelial cells [43], which can affect angiogenic activity in hindlimb ischemic disease. Therefore, through our study, we found that different treatments have some common activated signaling pathways, which interact with each other to activate the classical vascular regeneration related pathways, such as PI3K-Akt and Wnt signalling pathways, and then regulate the biological processes of vascular related cells, and ultimately achieve better efficacy in the mouse leg ischemia model (Figure 5d).

In summary, our study analysed the common features of different treatment modalities in achieving the promotion of vascular regeneration and salvaging the hind limb ischemic model from a bioinformatics perspective. We found some critical pathways that need to be activated in the process of vascular remodelling, explained the reasons for the efficacy of different treatments, and provided theoretical basis and research direction for the follow-up clinical treatment of the disease.

4. Materials and Methods

4.1. Data collection

We searched datasets on hindlimb ischemia from National Library of Medicine (NCBI) web site (https://www.ncbi.nlm.nih.gov/). Four RNA-seq datasets about nano therapy, drug therapy, gene knockout therapy, and adenovirus therapy for mouse hindlimb ischemia model were obtained. These datasets stored in the GEO DataSet under the data numbers GSE217514, GSE214684, GSE197392, GSE201476, and GSE201479, respectively. We collected and downloaded the supplementary file for each GEO dataset.

4.2. Data pre-processing

The supplementary file of each data set was organized into a matrix containing the gene count of control group and experimental group. Using the Bioconductor package in RStudio Desktop(https://posit.co/download/rstudio-desktop/) and R version 4.2.1(https://www.r-project.org/) for differential expression analysis and gene set enrichment analysis [44].

4.3. Differential expression analysis

The gene count matrix containing experimental and control groups was loaded into RStudio. The R package “DESeq2” was used for the following differential expression analyses: Control vs T2DM

(GSE197392), pla vs pra (GSE217514) and WT vs gpr (GSE214684) [[14]]. We set a filtering condition(|log2FoldChange|>=1&padj<=0.05) to obtained differentially expressed genes (DEG) for pla-vs-pra(drug treatment)(Figure 2c), WT-vs-gpr(gene knockout treatment)(Figure 2d), Control-vs-T2DM(nano therapy)(Figure 2e), and adenovirus therapy(Figure 2f). In addition, the DEG dataset was further divided into up-regulated (log2FoldChange>=1) and down-regulation (log2FoldChange<=1) gene list.

4.4. Gene set enrichment analysis

ClusterProfiler R package was used to perform GO functional enrichment analysis and KEGG pathway enrichment analysis. Reduce and Visualize Gene Ontology (REViGO) software helped to remove redundant GO terms and to perform GO similarity terms clustering [45].

4.5. Biological background information analysis

Searching a number of references on pubmed (https://pubmed.ncbi.nlm.nih.gov/15603830/) to obtain biological background information about GO terms and KEGG pathways we have enriched.

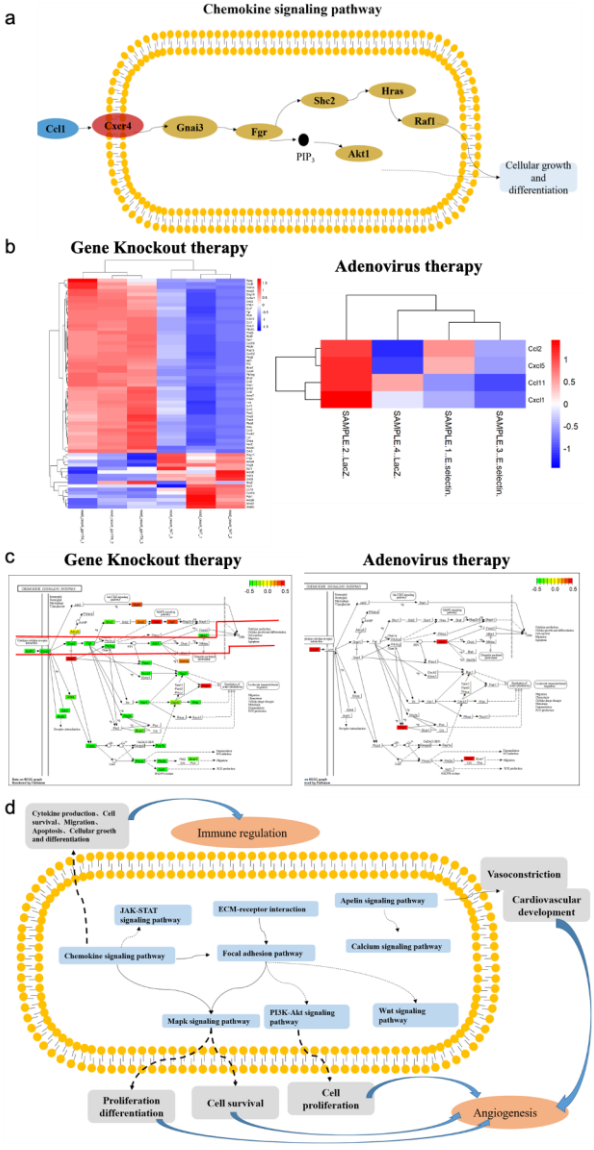


Figure 5. a: Chemokines signalling pathways conduction diagram. b: The Heatmaps of chemokines signalling pathways in gene knockout therapy (GSE214684) and adenovirus therapy (GSE2014765). c: The pathway maps of chemokines signalling pathways in gene knockout therapy (GSE214684) and adenovirus therapy (GSE2014765). d: Schematic diagram of signal pathway interaction.

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