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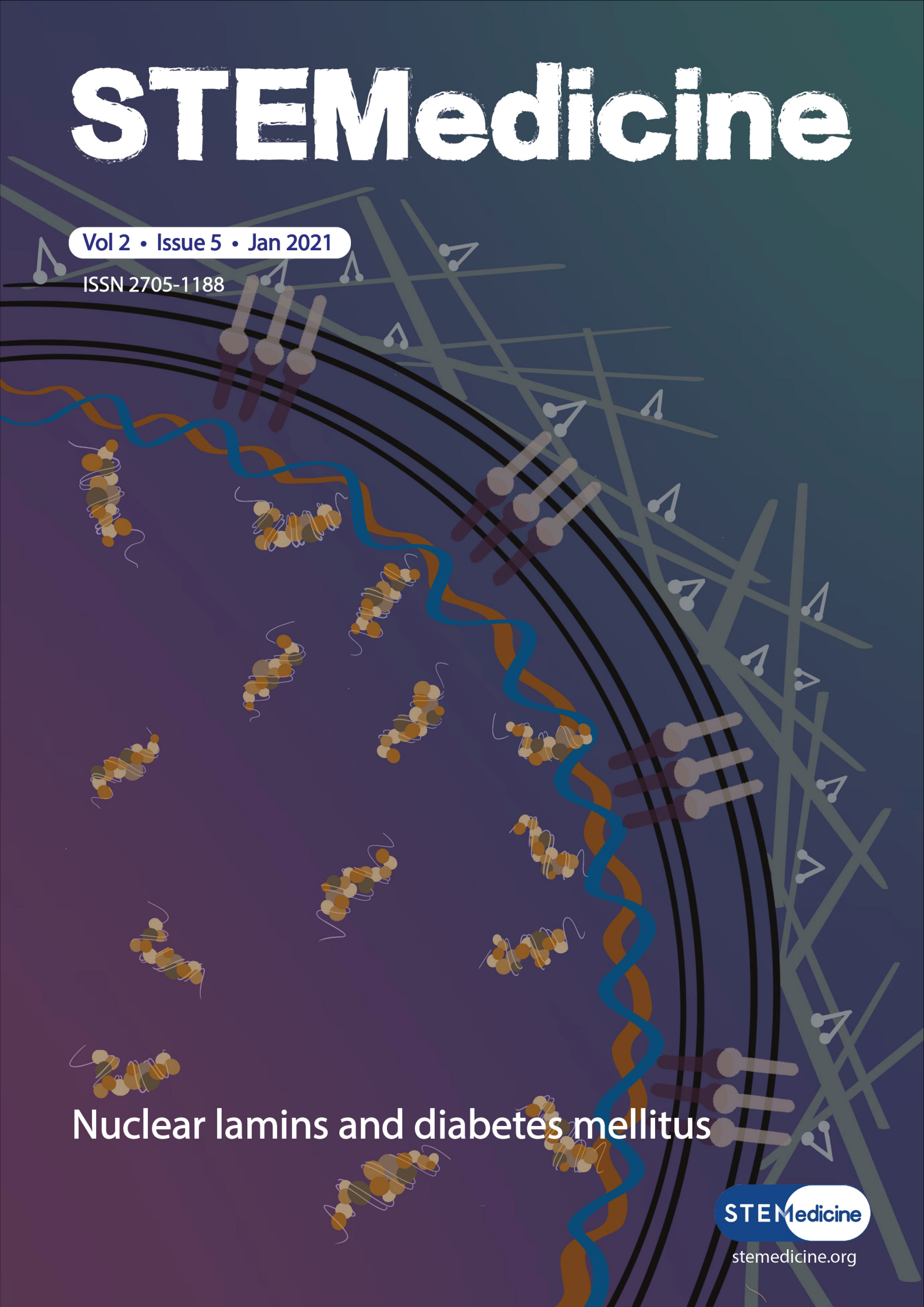

Nuclear lamins and diabetes mellitus

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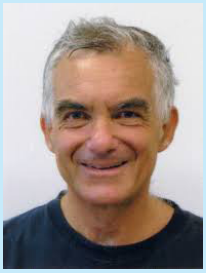
Reflections on STEMedicine and path forward to 2021



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Being a scientist with years of experiences, I've had a vision of a platform for both the scientific and medical communities to share their novel discoveries and technological advances. Now, at the beginning of year 2021, as I look back to my first year as the Editor-in-Chief of STEMedicine, I would like to express my gratitude to all the authors, reviewers and our editorial team for their support and invaluable efforts in bringing this vision on a path to reality.

STEMedicine is an international, peer-reviewed, open access journal and published its inaugural issue with seven articles in January 2020. In its first year, a total of four quarterly issues have been successfully published on time, containing 22 research and review articles, all of which peer reviewed. As I read these articles, I am proud and amazed of their innovative approach and high-quality. There are papers from various fields, including cancer, stem cell therapy, neurobiology, inflammatory diseases, etc demonstrating the wide range of science covered by STEMedicine. This breadth of scientific and medical topics enables STEMedicine to connect with researchers from different yet related disciplines.

Besides the broad scope of our articles, STEMedicine also strives to cover the most current research trend. For instance, as the COVID-19 pandemic has just begun to rage throughout the world, two articles on the coronavirus SARS-CoV-2 were published in the 2nd issue of STEMedicine in April (1, 2), summarizing the latest understanding of the virus and discussing potential therapy to date. In addition, soon after the news report in Singapore of a British boy's recovery from leukaemia following the experimental chimeric antigen receptor T (CAR-T) treatment, an article was also published in the third issue covering the progress of CAR-T therapy (3). For this I am greatly thankful for the authors and our editors, especially as the new editorial team is still in the early phase of the learning curve, whose combined efforts have allowed the speedy publication of these timely and excellent articles.

By the end of 2020, STEMedicine has been indexed by Crossref, Directory of Open Access Journals (DOAJ) and Google Scholar. The editorial team and I are confident to make the journal better and bring STEMedicine into the view of an even broader readership in the year of 2021.

Sincerely,

Editor-in-Chief
Vincent Torre

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REVIEW ARTICLE

Nuclear lamins and diabetes mellitus

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ABSTRACT

In metazoans, a thin filamentous network referred to as the nuclear lamina plays an essential role in providing mechanical support to the nucleus. The major constituent of the nuclear lamina is type V intermediate filament proteins that are collectively referred to as lamins. A variety of diseases collectively termed laminopathies have been linked to mutations in genes encoding nuclear envelope proteins, in particular lamins, such as X-linked Emery Dreifus muscular dystrophy, dilated cardiomyopathy, Dunnigan type familial partial lipodystrophy and Hutchinson-Gilford progeria syndrome. Apart from laminopathies, genome-wide association studies have also been implicated nuclear lamins in the pathophysiology of type 2 diabetes mellitus, although little information in terms of the function of lamins in its pathogenesis. Our current review attempts to summarize risk factors of diabetes mellitus that could be attributable to lamin mutations and indirectly linked to lamin-associated factors identified in the last two decades.

Keywords: Lamins · Diabetes mellitus · Nuclear lamina · Laminopathy · Dunnigan type familial partial lipodystrophy

The nucleus and nuclear lamins

Eukaryotic cells are defined by the presence of a nucleus enclosed by a pair of lipid bilayers, the outer nuclear membrane (ONM) and the inner nuclear membrane (INM), that together comprise the nuclear envelope (NE) (1). In metazoans, a thin filamentous network lining the nuclear face of the INM, which is referred to as the nuclear lamina, plays an essential role in providing mechanical support both to the NE itself and to the nucleus as a whole. In addition, the lamina provides anchoring sites at the nuclear periphery for higher order chromatin domains. In the way, the nuclear lamina represents a key element in the maintenance of global nuclear architecture (2). The major constituent of the nuclear lamina are type V intermediate filament (IF) proteins that are collectively referred to as lamins. Like all IF proteins, lamins will spontaneously self-associate to form filaments. The lamin filament network displays multiple interactions with integral proteins of the INM as well as with chromatin proteins (3). In addition, the lamina shares associations with nuclear pore complexes. These massive macromolecular structures are situated at annular junctions between the INM and ONM and regulate the exchange of macromolecules between the nucleus and cytoplasm. Besides having a critical structural role in the overall architecture of the nucleus, the nuclear lamins are pivotal for other aspects of normal nuclear function. In addition to regulating chromatin, particularly heterochromatin distribution, the lamins have been implicated in aspects of DNA replication and DNA damage repair (4-7).

The family of lamin proteins fall into two broad classes based on sequences, A-type and B-type. In humans, the two major A-type lamins, A and C, are encoded by the LMNA gene and are derived by alternative splicing of the same primary transcript. Two minor isoforms AΔ10 and testis-specific C2, are similarly encoded by LMNA. The major B-type lamins, B1 and B2, are encoded by the LMNB1 and LMNB2 genes, respectively. Lamin B3, a minor testis-specific B-type lamin isoform is an alternatively spliced product of LMNB2. A type lamins have roughly neutral isoelectric points and are found in the majority of adult cell types. However, they are absent from early embryonic cells as well as from some stem cell niches. In contrast, the B type lamins, which have acidic

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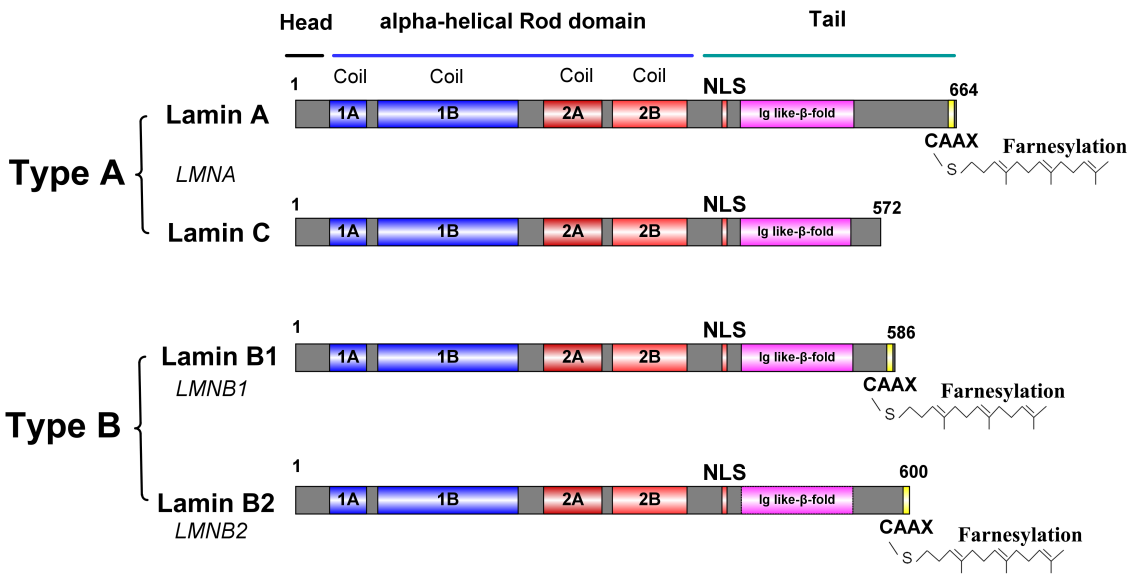

Figure 1. Schematic illustration of type A and B lamins. NLS, nuclear localization sequence.

isoelectric points, are, as a class, constitutively expressed, with lamin B1 and/or lamin B2 found in all nucleated cell types, both embryonic and adult (8).

All lamin proteins share the characteristic IF protein structure: a central α-helical coiled-coil "rod domain", a non-α-helical ''head'' at the N-terminus, and a globular "tail" at the C terminus (**Figure 1**). Unique to lamins, the core of this tail domain is organized as an immunoglobulin fold. Between the Ig-like β-fold domain and the central rod domain is a basic SV40-like nuclear localization sequence (NLS) that is essential for correct trafficking of newly synthesized lamins across the NE via NPCs. All mammalian lamins, with the exception lamin C, contain a C-terminal Ras-like CAAX motif that featuring a cysteine residue (C) followed by two aliphatic amino acid residues (AA), and terminating with any other amino acid (X, but usually methionine or leucine). The CAAX motif represents a site of prenylation (farnesylation in the case of the lamins). This takes very soon after release of the newly synthesised protein from the ribosome and is catalyzed by a farnesyl transferase that attaches the 15-carbon farnesyl lipid group to the CAAX cysteine via a thioether bond. Farnesylation is followed immediately by proteolysis of the AAX tripeptide. In the case of the B-type lamins this is accomplished by Rce1 (Ras converting enzyme 1) whereas lamin A utilizes ZmpSte24, a membrane associated protease. Removal of the AAX tripeptide exposes the farnesylated cysteine as the new C-terminus. This is then carboxymethylated by isoprenylcysteine carboxymethyl transferase (ICMT). Carboxymethylation represents the completion of C-terminal processing of the B-type lamins. However, in the case of lamin A an additional proteolysis step mediated by ZmpSte24 removes the C-terminal 14 amino acids to generate the mature protein. This final proteolysis step occurs within about an hour of synthesis but after pre-lamin A has been incorporated into the nuclear lamina. In this way, while B-type lamins remain permanently farnesylated, lamin A retains this modification only transiently. This divergence in C-terminal processing has marked consequences in terms of the behaviour of A-type versus B-type lamins. This is most notable during mitosis during which time the NE, including the lamina, is broken down so that the condensed chromosomes can engage with the mitotic spindle, a cytoplasmic structure in higher cells. While A-type lamins become dispersed as soluble proteins throughout the mitotic cytosol, the B-type lamins remain membrane-associated. As will be seen below, the effects of differential farnesylation on lamin function have important consequences in terms of our understanding of diseases associated with lamin defects (9, 10).

Lamins and associated diseases

A variety of diseases have now been linked to mutations in genes encoding NE proteins. The first of these to be described was X-linked Emery Dreifus muscular dystrophy (EDMD), which is caused by defects in an INM protein known as Emerin. EDMD is characterized by early contractures in the Achilles tendons and at the elbows, muscle wasting and cardiac conduction system defects associated with dilated cardiomyopathy. Later an autosomal dominant form of EDMD was mapped to mutations within LMNA. Studies employing a Lmna deficient mouse strain, which develops a syndrome that bears a striking resemblance to human EDMD, revealed that Lamin A and C can associate with Emerin and that these two lamins are required for Emerin localization to the INM, at least in fibroblasts and muscle cells. Multiple other diseases are also caused by mutations in LMNA. These include dilated cardiomyopathy (DCM), limb girdle muscular dystrophy, Dunnigan type familial partial lipodystrophy (FPLD), a peripheral neuropathy (Charcot Marie Tooth disease, CMT2) and Hutchinson-Gilford progeria syndrome (HGPS), a premature aging disorder.

HGPS, an extremely rare disease, is most frequently caused a 50-residue deletion within the lamin A tail which eliminates the ZmpSte24 cleavage site. This results in the accumulation of a truncated form of lamin A which is permanently farnesylated. Loss of ZmpSte24 itself results in Restrictive Dermopathy, a rare recessive perinatal lethal disorder. An intriguing aspect to this is that lamin A is the only known substrate of this protease.

The range of diseases caused by LMNA mutations has been a matter of considerable scrutiny over the issues. An issue here is the fact that LMNA is expressed in the vast majority of adult cell types. However, different LMNA mutations affect different tissues, ranging from striated muscle to adipose and neuronal tissues. In addition, metabolic perturbations may also be observed to a greater or lesser extent. One explanation for this curious situation is that lamin defects may compromise the structural integrity of the cell nucleus leading to DNA damage and cell death. This is very likely the case in mechanically stressed tissues such as striated muscle. However, it cannot account for diseases such lipodystrophy. FPLD in particular is caused by mutations in the Lamin A/C tail domain in the vicinity of Arginine 482. These mutations have seemingly little or no effect on striated muscle. Consequently, it has been suggested that FPLD is caused by lamin-dependent transcriptional perturbations. An attractive possibility is that certain lamin mutations affect interactions with other components that are themselves expressed in a tissue-specific manner. It is most likely that all of these effects contribute to LMNA linked diseases with their differential impacts varying according to tissue type.

The majority of disease-causing mutations in genes encoding NE proteins are found within LMNA. As alluded to above several hundred such deleterious LMNA mutations have been identified. Other disorders, including X-linked EDMD and adult onset autosomal dominant leukodystrophy occur as a result of mutations in EMD, the gene encoding emerin, and LMNB1 respectively, the latter involving a gene duplication. Similarly, mutations in LEMD3, encoding an INM protein, MAN1, are associated with Buschke-Ollendorff syndrome. Mutations in LBR, encoding another INM protein, lamin B receptor, variously cause Pelger-Huet anomaly and Greenberg Skeletal Dysplasia. These major lamin-associated disorders are collectively termed “laminopathies” and have been described in detail in an excellent review by Worman (11). Our current discussion focuses on a much more widely spread disease particularly in developed countries - diabetes mellitus (DM) and the possible role of A-type lamins.

Diabetes mellitus (DM)

DM typically involves inadequate insulin activity and/or secretion, which often leads to chronic hyperglycemia and impaired metabolism of lipids, proteins, and carbohydrates (12). There has been a continuing rise of DM in developed countries, including both Singapore and the United States. Two major types of diabetes have been described, namely insulin-dependent diabetes mellitus (type 1 DM, T1DM) as well as non-insulin-dependent diabetes mellitus (type 2 DM, T2DM), the latter of which is the more common, accounting for 90%- 95% of all DM cases (13). Furthermore, incidences of T2DM has been estimated to increase to approximately 439 million by 2030 (14), thereby posing a serious global public health problem.

It has been widely established that T1DM and T2DM are characterized by the aberrant functions or even failures of pancreatic β-cells. Hyperglycemia in T2DM is caused by the gradual aggravation of insulin resistance at peripheral sites such as in the adipose or muscle tissues, in addition to pancreatic β-cell dysfunction. Hence, it is of great value to investigate the intracellular events that take place within the pancreatic β-cells. Understanding of such events will facilitate the identification of potential drug targets for managing and/or preventing DM.

Although the pathophysiology of T2DM has not been fully elucidated, genetic risk factors are widely considered to be a major component of the disease. Genome-wide association studies (GWAS), which analyze the correlations between common diseases and single-nucleotide polymorphisms (SNPs) through comparisons of the genetic features of patients and healthy subjects, have identified a number of susceptibility loci of T2DM (15). Some of these polymorphisms map to chromosome location 1q21.2 that harbours the LMNA gene.

Apart from the GWAS, there has been little information in terms of the function of lamins in the pathogenesis of DM. This review attempts to summarize risk factors of T2DM that could be attributable to lamin mutations and indirectly linked to lamin-associated factors identified in the last two decades.

LMNA mutations associated with DM

Dunnigan-type familial partial lipodystrophy (FPLD)

Mutations in LMNA gene were first identified to be the cause of Dunnigan-type familial partial lipodystrophy (FPLD) through an inspired piece of intuitive detective work (16). FPLD patients typically present symptoms that include subcutaneous fat loss, glucose intolerance, hypertension and insulin resistance, along with other characteristic changes associated with metabolic syndrome that eventually lead to DM (**Figure 2**). LMNA was one of more than 100 positional candidates implicated in FPLD. However, Bonne and colleagues realised that the site-specific pattern of fat loss in FPLD was quite similar to the pattern of muscle wasting in EDMD, which had only recently been linked to LMNA mutations (17). Sequence analyses of the LMNA locus in FPLD patients revealed that mutations in exon 8, typically in the codon for R482 in the Ig-fold region of the tail domain (and in rare cases, codons 465, 486, 582 or 584) were responsible for FPLD (18-21).

Since the original linkage of FPLD to LMNA mutations within the Ig fold, there has been speculation that these might adversely affect the stability of lamin A and C multimers, thereby compromising the integrity of the nuclear lamina as a whole. This could then impact adipocyte function as a result of abnormal interactions between lamins and transcription factors, nuclear

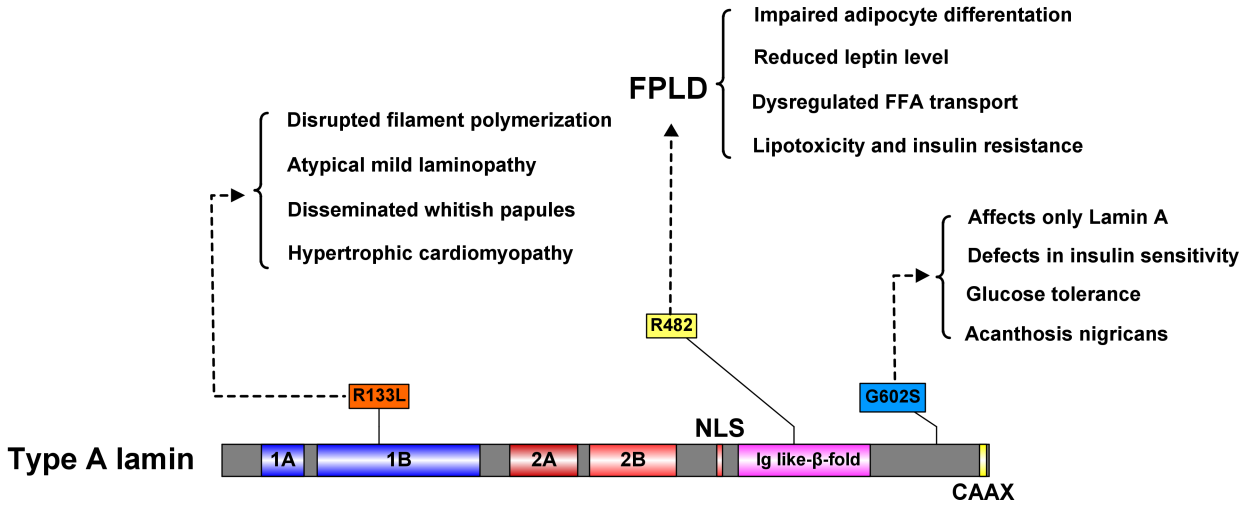

Figure 2. LMNA mutations associated with diabetes mellitus (DM). R482 mutation leads to Dunnigan-type familial partial lipodystrophy (FPLD), showing symptoms such as subcutaneous fat loss, glucose intolerance, hypertension, and insulin resistance that eventually lead to DM. R133L mutation leads to atypical mild laminopathy, showing lipodystrophy and insulin resistance that are characteristic of type A insulin resistance syndrome. G602S mutation affects only lamin A protein and leads to insulin sensitivity, glucose tolerance and acanthosis nigricans, and is significantly associated with T2DM risk.

membrane proteins, chromatin, and/or other cellular proteins (22). The mutant LMNA has also been suggested to impair adipocyte differentiation, pre-adipocyte proliferation and modulation of apoptosis, all of which could lead to decreased adipose tissue mass (23). On the other hand, the plasma level of leptin was significantly lower in FPLD individuals with the LMNA R482 mutations (24). Leptin is a peptide hormone that depresses hunger and is therefore an important metabolic regulator. Deficiency of leptin is associated with insulin resistance (25). As peripheral adipocytes are the primary producer of circulating leptin, it is reasonable to conclude that decreased adipose tissue mass (a consequence of the LMNA mutation) may lead to lowered leptin production or secretion. From a functional perspective, leptin aids the preferential delivery of free fatty acids (FFA) to adipocytes, versus liver and skeletal muscle (26). Therefore the leptin deficiency in FPLD may augment the possible FFA exposure to non-adipocytes, causing lipotoxicity and insulin resistance (27, 28).

R133L LMNA mutation leading to atypical mild laminopathy

FPLD was initially regarded as the only form of laminopathy-related DM. However, subsequent investigations demonstrated that DM and insulin resistance could be an important hallmark of more complex laminopathy phenotypes. Caux et al. described a case study of an atypical milder form of laminopathy with features that overlapped with FPLD (29). The patient was initially diagnosed with hepatic steatosis, hypertriglyceridemia and insulin-resistant DM. As the patient also suffered lipodystrophy as well as insulin resistance that was characteristic of type A insulin resistance syndrome, usually caused by mutations of the insulin gene, molecular assays on insulin receptors and seipin were first conducted, only to find no defect in any of these genes.

A later screen for mutations in the LMNA gene revealed that a heterozygous R133L LMNA mutation was responsible for the symptoms (**Figure 2**). Other symptoms typically associated with LMNA gene mutations were later identified, including disseminated whitish papules and hypertrophic cardiomyopathy with valvular involvement. Arginine 133 locates in a charged peptide segment that is highly conserved in vertebrate lamin A/C. The change from arginine (positively charged) to leucine (hydrophobic) within the lamin A/C coiled-coil dimerization domain would potentially compromise the polymerization of lamins and subsequent assembly of lamin filaments. Consistent with this speculation, nuclear abnormalities were indeed reported in cultures of primary fibroblasts from this patient.

G602S LMNA mutation

In another case study of a non-obese patient without lipodystrophy, the type A insulin resistance syndrome was linked to a newly-identified heterozygous G602S mutation in LMNA gene (30). Different from the earlier mentioned R133L mutation that impairs both lamins A and C, G602 resides in exon 11 therefore this missense mutation affects only the lamin A protein. Nuclear shape in the patient fibroblasts also appeared abnormal, not dissimilar to that observed in the patient with R133L mutation. Most significantly, this G602S LMNA mutation is accompanied by defects in insulin sensitivity, glucose tolerance, and acanthosis nigricans, therefore indicating that LMNA mutations can impair the insulin signalling, even without signs of clinical lipodystrophy.

In addition to this case study, a stronger link between G602S mutation and T2DM was recently established in a large scale, ethnically diverse population study involving

over 60,000 unrelated individuals in the ExAC cohort (31, 32). LMNA variants were independently analysed via the T2D Knowledge Portal, and the results revealed that G602S was significantly associated with T2DM and could serve as a risk factor for DM among African Americans.

Lamin A/C expression and T2DM

Besides lamin A, the other product of the LMNA gene, lamin C has also been implicated in the pathological processes of DM. Using a mouse model that expresses exclusively lamin C, Toledo et al. reported that when challenged with DM-inducing conditions, these mice were able to normalize their fasting glycemia through enhancing both the regeneration of β-cells and the secretion of insulin, while the mice expressing both lamins A and C exhibited disrupted glucose homeostasis (33). The capacity to regenerate β-cells in the lamin C only expressing/lamin A deficient mice suggested an alternative therapeutic potential for DM treatment through regulating LMNA splicing, because an insufficient supply of functional insulin-producing β-cells is a clinical feature commonly found in T1DM and T2DM.

There is, however, one caveat in the above study: it remains unclear that whether the lack of lamin A expression indeed causes the observed β-cell regeneration phenotype or the sole lamin C expression (or an imbalance of the two) is actually responsible. This possibility is certainly echoed by three independently earlier reports that, in adipose tissue samples collected from obese subjects and T2DM patients, expression of lamin A/C is upregulated (34-36).

Later on, Kim et al. have brought the discovery one step forward, and found that the upregulated lamin A/C mainly occurs in adipose tissue macrophages (ATMs), and in particular, the epididymal white adipose tissue (eWAT), where it triggers enhanced production of pro-inflammatory cytokines through activating NF-κB (37). Given the fact that eWAT contains more inflammatory cells and higher expression levels of proinflammatory genes (38-41), it can be speculated that in the ATM the elevated lamin A/C increases expression levels of pro-inflammatory cytokines and that it is these that are mechanistically linked to insulin resistance (41-44). This hypothesis is certainly supported by observations in the same study, that deficiency in myeloid cell-specific lamin A/C attenuated obesity-associated inflammation in adipose tissues and resistance to insulin (37). These findings collectively indicate that in ATMs, lamin A/C acts as a novel regulator in obesity-elicited insulin resistance and inflammatory responses in adipose tissues.

Lamin-associated factors linked to DM

Apart from being implicated in the upstream events that affect insulin production/resistance, nuclear lamins are also implicated in the transcriptional modification of downstream insulin-response machinery through its associated factors (**Figure 3**).

An exemplary case, illustrated by the work of Pak and colleagues, is Caveolin-2 (45, 46). Caveolins are a group of lipid raft-associated integral membrane proteins that participate in diverse cell signal transduction and mechanotransduction processes (47). It has been reported that upon insulin stimulation, Caveolin-2 is transported to the INM, where it initiates direct interaction with Type A lamins (45) and subsequently undergoes phosphorylation at Tyr-19 and mono-oligomerization (48). Interestingly, the maintenance of pY19-Caveolin-2 oligomer is dependent on A-type lamins (46). By interacting with lamin A or C, pY19-Caveolin-2 decreases histone H3 lysine 9 trimethylation (H3K9me3), thereby opening the lamin A/C sequestered (transcription-inactivated) chromatin domains containing the promoters of insulin-response genes such as Elk-1 and JunB, as well as signal transducer and activator of transcription 3 (STAT3) (49, 50). Besides releasing transcriptionally repressed chromatin domains, the lamin A/C associated pY19-Caveolin-2 also actively promotes transcription of the insulin-response genes by recruiting RNA polymerase II to promoter regions through increased acetylations at histone H3 lysines 9, 18 and 27 (45). In this way, Caveolin-2 acts as an A-type lamin dependent epigenetic activator of insulin-response genes.

In addition to modulating transcriptional activity through histone modification, lamin A/C is also able to regulate the retinoblastoma protein (RB) and E2-factor (E2F) family of transcription factors (51). The regulation of RB, a nuclear lamina-associated transcription factor, is carried out by lamina-associated-polypeptide-2α (LAP2α) and A-type lamins, where lamin A/C acts to promote subnuclear localization of RB, as well as to stabilize RB against proteasome-mediated degradation (52). In pancreas-selective conditional knockout mice, lamin A/C deficiency destabilizes RB to activate E2F, leading to ER stress, loss of acinar cell markers, enhanced autophagy, proliferation, as well as apoptosis, eventually leading to chronic pancreatitis (51). As the RB-E2F pathway is critical for lipogenesis in liver, pancreas, and adipocytes (53-55), the regulation of RB stability by A-type lamins proposed by Elenbaas et al. is also of particular relevance to FPLD. Taken together, all of these reports imply that A-type lamins have a hitherto under-appreciated role in the regulation of energy metabolism in diverse cell types.

Perspective

T2DM, as well as its complications, has posed as an increasingly severe health issue worldwide especially in developed economies. Studying the underlying causes, in particular genetic factors, is crucial in the prevention and treatment against T2DM. In the current review, we have discussed one of such risk factors of diabetes mellitus: the nuclear lamins. In addition to laminopathies, which are mainly caused by mutations in lamins and associated proteins, lamin mutations also contributes, directly or indirectly, to diabetic symptoms. Understanding the correlation between lamin mutations with T2DM could make the clinical management of patients affected by both more effective.

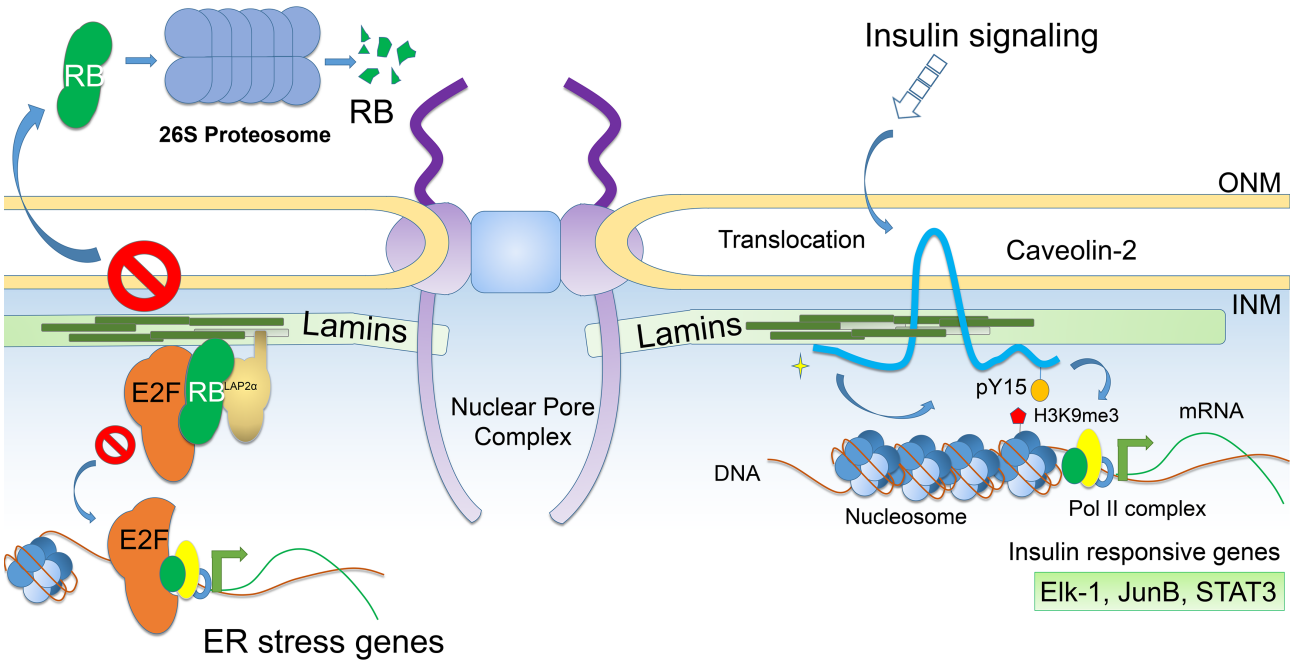

Figure 3. Lamin-associated factors linked to diabetes mellitus (DM). Apart from being implicated in the upstream events that affects insulin production/resistance, nuclear lamins are also suggested in the transcriptional modification of downstream insulin-response and ER stress response machinery through its associated factors.

Acknowledgement
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Conflict of interest
The authors declare that there is no conflict of interests regarding the publication of this paper.

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REVIEW ARTICLE

Mesenchymal stem cell spheroids: potential cell resource for cell therapy

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ABSTRACT

Mesenchymal stromal/stem cells (MSCs) have been applied in an increasing number of clinical trials in recent years. MSCs have many advantages in cell therapy for regenerative medicine for their extensive sources, low immunogenicity, self-renewal ability and multilineage differentiation potential. However, the clinical application of MSCs is still confronted by many challenges including low survival ability *in vivo*, the loss of main original characteristics due to two-dimensional (2D) culture for fast expansion, and the lack of technologies for mass production of high-quality MSCs. Three-dimensional (3D) culture has been widely regarded as a more preferable and closer physiological microenvironment for cell survival, growth and behavior, because 3D culture can artificially create an environment allowing cells to interact with their surroundings in complete three dimensions. Recently, many different 3D spheroid culture methods have been developed to optimize the biological characteristics of MSCs to meet the demand of regenerative medicine. In this review, we comprehensively discuss the merits and demerits of different spheroid formation methods, expound the mechanisms of spheroid formation and its microenvironment, and illustrate their optimized biological functions and the pre-clinical applications in tissue injury and regeneration. In the end, we prospect the trends of this research field and propose the key problems needed to be solved in the future.

Keywords: Mesenchymal stromal/stem cells · Three-dimensional culture · Spheroids · Stem cell optimization · Regenerative medicine

Introduction

Mesenchymal stromal/stem cells (MSCs) are non-hematopoietic multipotent stem cells originated from the mesoderm. Since MSCs were initially isolated from bone marrow by Friedenstein *et al*. in 1970 (1), nowadays, they have been easily separated from a variety of adult or embryonic tissues including bone marrow, adipose tissue, umbilical cord, amnion, placenta, umbilical cord blood and dental pulp, and can be expanded successfully *in vitro* (2-7).

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MSCs are characterized by their abilities of self-renewal, multilineage differentiation, extensive proliferation and paracrine. Moreover, MSCs lack immunogenicity and have the homing ability to migrate to sites of tissue injury in response to inflammatory factors by intravenous administration (8). They modulate the immune response and inhibit the inflammation by secreting multiple bioactive factors to create a niche which promotes the recovery of injured cells and angiogenesis (9, 10). In order to easily compare and contrast the increasing data among different studies, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy proposed minimal criteria to define human MSCs (hMSCs) in 2006 (11). MSCs would keep adherence to plastic under standard culture conditions with the capacity

to differentiate into osteoblasts, chondrocytes and adipocytes *in vitro*, and express specific surface markers CD73, CD90 and CD105, while lack the expression of several hematopoietic and endothelial markers such as CD45, CD34, CD11b or CD14, CD79α or CD19 and HLA-DR surface molecules (11). Unlike embryonic stem cells and induced pluripotent stem cells, MSCs have a wide range of sources without concerns of ethic and teratoma formation, so they are considered promising materials for cell therapy in clinic (8, 12, 13).

The first clinical trial of MSCs occurred in 1995. MSCs were applied as cellular pharmaceutical preparation for 15 patients with hematologic malignancies by auto-transplantation after adherent culture *in vitro*, and no adverse reactions were observed in application (14). Since then, the research and clinical applications of MSCs have been booming for their properties of self-renewal and multi-lineage differentiation potential (3, 15-19). It is noteworthy that as a potential treatment, MSCs were used to prevent and reverse the cytokine storm caused by severe COVID-19 pneumonia for their comprehensive powerful functions in immunomodulatory and tissue injury repair (20, 21). As of August 2020, around the world, more than 1172 clinical trials using MSCs were registered on ClinicalTrials.gov. The related diseases involve myocardial infarction, graft versus host disease, diabetes, liver cirrhosis, spinal cord injury, osteoarthritis, Crobn's disease, multiple sclerosis, brain injury and COVID-19 pneumonia.

Generally, to acquire enough MSCs for therapy in clinic, MSCs are isolated from human tissues and 2D cultured in monolayer for fast expansion. However, numerous investigations demonstrated that the stemness of MSCs was gradually weakened with increased number of cell passage. The causative cell aging, reduced paracrine capacity as well as low survival rate after transplantation led to bad therapeutic effects (22, 23). Therefore, the 2D-cultured MSCs need to be optimized to improve cell quality before clinical application. In 2009, Lee *et al*. found that the intravenous hMSCs did not secrete the anti-inflammatory protein TSG-6 until cells were embolized in the lung and this improved myocardial infarction in mice (24), suggesting that 3D MSCs aggregates benefit the implementation of MSC function. From that time on, 3D-cultured MSCs have been considered effective therapeutic agent in *in vitro* experiments and pre-clinical therapeutics by scientists and clinicians (6, 25-27). So far, it has been widely recognized that long-terms of 2D culture can change the original characteristics and traits of MSCs, while 3D culture can better preserve MSC properties due to creating a more natural physiological microenvironment (28-30).

Cell 3D culture methods can be classified as scaffold-based and scaffold-free culture systems (31). For scaffold-based 3D culture, cells are embedded in hydrogels or cultured in the presence of fibers or sponge-like structures to provide support, nutrition and extracellular matrix (ECM). Scaffold-free 3D culture allows the cells to self-assemble into spheroids to mimic the microenvironments *in vivo* by their own ECM. Compared with scaffold-based culture, scaffold-free 3D culture is more widely used in cell therapy research for its unique merits. 3D spheroid culture can endow MSCs the ability to tolerate the stresses *in vivo*, such as inflammatory microenvironment, hypoxia, nutrient depletion, which improves the survival of MSCs *in vivo* and enhances their beneficial function on damaged tissues. Moreover, the spheroids are generally formed with consistent size and shape without the need of dissolving the special scaffold that may cause immune response problems, which benefits high-throughput screening and optimization of MSCs as functional units for the demand of tissue engineering. Optimization of cell-based therapy by spheroid culture of MSCs has been widely recognized because it can maintain stemness, improve the ability of anti-apoptosis and homing post-transplantation, enhance anti-inflammatory and immunomodulatory effects, and increase paracrine capacity compared to traditional 2D-cultured MSCs (13, 32, 33).

In this review, we will discuss the biological features and the medical applications of scaffold-free MSC spheroids, summarize different culture methods for MSC spheroid formation, and evaluate their advantages and disadvantages for application. We will interpret the internal interactions and microenvironment of MSCs that may contribute to aggregations, and illustrate the biological optimization of MSCs for their functions in spheroid cultures and its therapeutic effect in pre-clinical animal models. In the end, we will prospect the trends of this research field and the therapeutic challenges in the future.

The mechanism of MSC spheroid formation *in vitro*

In vitro, although the exact mechanism needs to be further elucidated, it has been accepted that the process of multicellular spheroid formation is through three steps, including loose cell aggregation via integrin-ECM binding, cadherin accumulation, and spheroid compaction (30). After self-assembly of MSCs via integrin-ECM binding or homophilic cadherin-cadherin interactions, cells will be reorganized to form aggregates for minimizing the free energy (32). Calcium-dependent interactions between cells and E-cadherin are key inducers to mediate MSC 3D-spheroid formation (6). From the morphologic view, high-cadherin-expression MSCs interact inside with round shape, and high-integrin-expression MSCs fully stretch outside with a spindle shape by intracellular tension of the aggregates, indicating the existence of mechanical polarization (32, 34, 35). Endogenous ECM enhances the formation of MSC aggregates (36), and actin mainly mediates the viscoelastic behavior to promote fusion and compaction (35). In addition, the volume of cells in MSC spheroids is obviously reduced by 75-90% compared to that of traditional 2D-cultured MSCs (26, 37).

The methods for MSC spheroid culture

Spheroid culture artificially creates an environment permitting cells to interact with their surroundings in complete three dimensions, which may better preserve

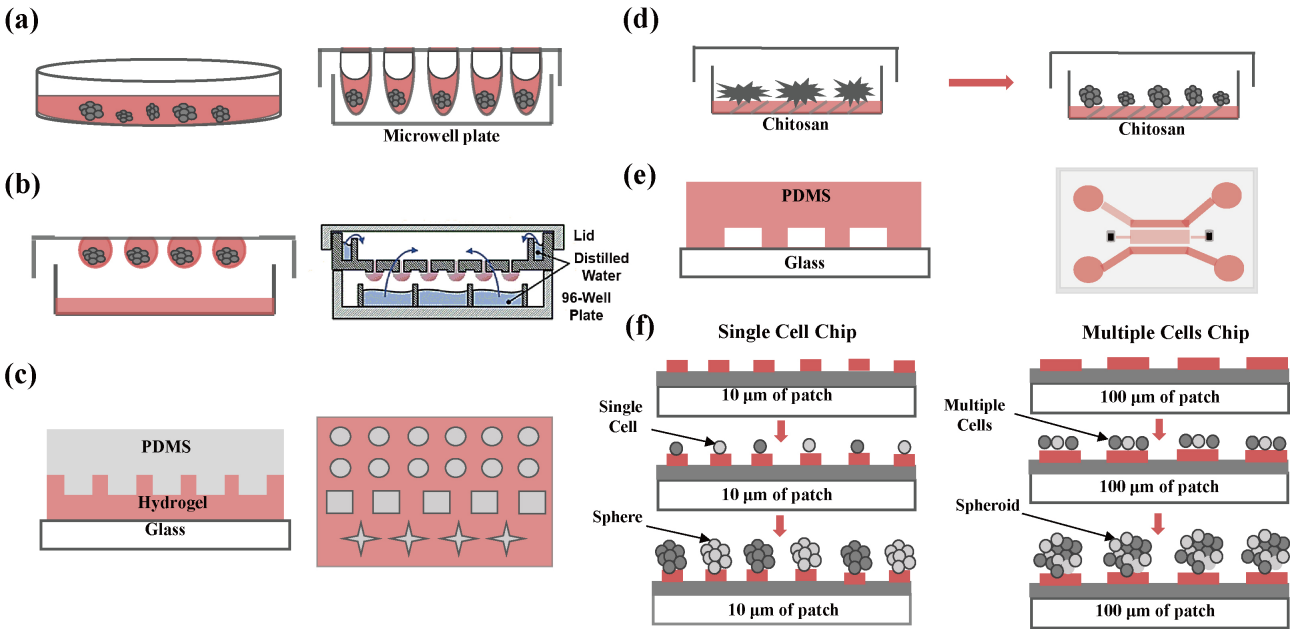

Figure 1. Methods used to generate MSC spheroids. (a) Overlay/Non-adhesive surface culture and ultra-low attachment microwell plates. (b) Gravity-induced spheroids by hanging drop culture and modified strategy (46). (c) Micropatterned substrates by micro-molding techniques using polydimethylsiloxane (PDMS). (d) Chitosan-mediated spheroid formation after transient adherence. (e) Microfluidic system (47). (f) Single cell derived sphere formation (45).

MSC properties due to mimicking a natural physiological microenvironment. Therefore, it is widely regarded as a preferable way to optimize MSCs (38-40). So far, many different methods have been developed to form MSC spheroids (**Figure 1**), such as non-adhesive surface (6), hanging drop (41), spinner flask (42), magnetic levitation (43) and chitosan-mediated (44). Especially, a novel method regarding single cell derived sphere (SCDS) formation on cell chip based on self-renewal screening dogma was developed recently, which exhibited its unique merits and shed new light in this research field (45). Although the characterization and properties of spheroids produced can be changed with different fabrications as well as different tissue sources, they all demonstrate their advantages to correspond to different treatment models.

Overlay/non-adhesive surface culture is commonly used for cell spheroid formation (**Figure 1a**). Suspended cells are seeded on plates coated with non-adhesive substrates, e.g. agarose gel/film (48), polyacrylamide gels (49), poly-HEMA (50) or in ultra-low attachment culture dishes (6, 51). It is easy to operate but forming non-uniform spheroid size and shape. Lee et al. reported that different sized human umbilical cord blood-derived MSC (hUCB-MSC) spheroids were fabricated through suspension culture and obtained the spheroids by filtration using 100 μm and 40 μm mesh strainer. After transplantation to rat myocardial infarction (MI) model, 3D-bullet of hUCB-MSCs had better therapeutic effect than 2D-cultured cells (6). Now, commercial microwell plate with ultra-low attachment has been developed to control the numbers and size of cell aggregates (35, 52). However, all these methods still cannot be used for mass production.

Hanging drop cell culture method is the most widely used technique for spheroid formation (**Figure 1b**). Cells were plated in hanging drops in 35-40 μL of suspension containing 10,000-25,000 cells/drop for 3-4 days of culture to spontaneously form cell aggregates (26, 53). MSC spheroids generated by this method was used to enhance cartilage repair in a monosodium iodoacetate-induced osteoarthritis rat model (7) and to alleviate the hepatic injury resulted from ischemia-reperfusion (54). Another improved 384 hanging drop array for high-throughput 3D spheroid culture was developed for drug testing by robot. In this method, drops with cells are added to the hydrophilic plate surface that can be quickly attracted to form a hanging drop and confined within the plateau (46). These kinds of methods can produce uniform sized spheroids due to the controllable number of cells for spheroid formation, whereas it is difficult to obtain high yield of spheroids.

Micropatterned substrates are technologies for spheroid generation usually based on photolithography (**Figure 1c**). Polydimethylsiloxane (PDMS) stamps are used to mold a layer of polymer (e.g., HA/PEG/polystyrene) and fabricate controlled microstructures (25, 55, 56). The micropatterned plates were used to generate MSC spheroids exogenously expressed with brain-derived neurotrophic factor (BDNF). These spheroids significantly improved recovery of hindlimb motor function in spinal cord injury (SCI) (57). Micropatterns can be manufactured in various sizes and shapes to meet different experimental requirements. Aggregates can be achieved and evaluated with high throughput but it also needs specialized facilities.

Chitosan-mediated spheroid formation is a new popular substrate-mediated method to generate 3D spheroids (**Figure 1d**). As a natural alkaline polysaccharide, chitosan is considered as a biocompatible and chemically

modified polymer containing free amino groups. For this method, acetic acid solution contain 1% chitosan was coated on coverslip glass to form chitosan membranes. Hyaluronan, a kind of acid mucopolysaccharide, is added on chitosan membranes for increasing biological characteristics of spheroids. With positive charge, chitosan can easily form a complex with the strong negatively charged hyaluronan (58). Human adipose tissue derived MSC spheroids generated from the chitosan-based substrates could maintain the stemness and enhance the ability of cartilage differentiation (58), and showed higher cellular retention ratio than dissociated 2D cells after injected into muscle of nude mice (44). Formation of MSC spheroids on chitosan substrates is a little different from other common non-adhesive bio-membranes. Cells adhere transitorily on the chitosan membranes by their degree of deacetylation and then gradually aggregate to form spheroids. However, the sizes of spheroids cannot be even and this method cannot produce spheroids on a large scale.

Microfluidic system with microfluidic chips as the reaction devices can produce 3D cell spheroids by microwell (59) or microchannel (60) (**Figure 1e**). It was reported that the MSC spheroids could rapidly be formed in the controllable microenvironment with enhanced ability of osteogenic differentiation after encapsulated into hydrogel (60). In a 3D microfluidic system, glial cell-derived neurotrophic factor overexpressing hMSCs and human neural stem cells were co-cultured, leading to reduced glial differentiation and obviously enhanced neuronal differentiation (47). For this type of method, the most commonly used processing material is PDMS for its good biocompatibility and breathability. Although 3D culture on the chip can simulate the dynamic microenvironment in vivo with reduced reagent consumption, with small volumes, it is difficult to collect spheroids for subsequent analysis and treatment. Moreover, it needs complex chip design and operational control (61).

SCDS formation via cell array chip was recently developed using umbilical cord mesenchymal stem cells (UCMSCs) by Suo research team (45). This novel method is totally dependent on single cell 3D proliferation to form spheres, which is different from the above-mentioned 3D culture methods for multiple cell derived spheroid (MCDS) formations that are dependent on cells aggregation (**Figure 1f**). For SCDS formation, the cell chips are stamped with 10 μm of diameter of polyethylenimine patches and 50 μm of gap between two neighboring patches. One single cell is just allowed to attach on one patch. Therefore, single MSCs can be patterned on a chip by 2D and cultured to form SCDS by 3D. Since SCDS formation is based on the screening or training of cell self-renewal ability defined by single cell proliferation, it possesses some unique advantages in many aspects, such as stemness maintenance, cell survival, stress resistance, angiogenesis and homing, which greatly meets clinical requirements. In previous research, SCDS cultured MSCs displayed great therapeutic potential on acute liver failure in mice (45). Otherwise, this method can produce SCDS with small and uniformed size which is conductive to direct intravenous injection administration without further cell dissociation and avoids the heterogeneity problem of MSCs to a greater extent, therefore has potential for standardized production in large scale in the future. Remarkably, SCDS culture is completely superior to 2D culture and MCDS culture in all the above properties for MSC optimization (45). The study suggested that the microenvironment for SCDS formation can stimulate the self-renewal potential of MSCs and then optimizes MSCs. Although MCDS is generated by the aggregation of multiple cells, it may also provide a microenvironment in spheroids to promote SCDS formation and expansion, and then to enhance MSC stemness properties as well. However, the relatively enclosed environment generated by interactions among SCDSs may weaken the viability and stemness of MSCs in the entire MCDS, which suggests that the smaller size of MCDS may lead to better effect of MSC optimization. Despite many advantages of SCDS, there are still some mechanisms need to be further explored, for example, how SCDS culture impacts on MSC inflammatory response, paracrine capacities, immunomodulatory capacities, and cellular metabolism. In addition, more efforts need to be made to solve the problems regarding massive production.

Spheroid culture creates a natural physiological microenvironment

In multicellular organisms, every cell is surrounded by other cells and ECM to form a complex cellular society through cell communication, adhesion, junction and cell interaction. Cell social connections influence the behavior and fate of cells, as well as morphological structure and function. Moreover, physical effects of the *in vivo* microenvironment are important for stem cells and matrix elasticity to direct MSC lineage specification (49). In traditional 2D cell culture, cells are stretched and grown on flat plastic petri dishes, whereas in spheroid culture system, cells are aggregated into spheroids spontaneously or by force. So, for 2D cells, little areas are exposed to other cells and nearly half surface areas are exposed to stiff plastic culture dish (range of stiffness is 1-10 GPa), the other half are exposed to fluid medium (62, 63). However, for 3D spheroid cultured cells, almost all surface areas are covered via cell-cell contact and cell-ECM contact, which is closer to physiological microenvironment. Moreover, complex proteins such as collagen and elastin participate in the formation of ECM structure, contributing to the elasticity of spheroids. It was reported that typical elastic modulus of single cells in spheroids is approximately 103 Pa (64), which is far softer than 2D culture condition. Cell communication and signal transduction in spheroids are also closer to in vivo condition than in 2D culture.

Microenvironment inside spheroid affects the cell viability

3D-cultured cell spheroids have the potential to simulate the microenvironment and physiological activities *in vivo*. Cell spheroid has been used as a physiological model to

study the occurrence of solid tumors and the differentiation of stem cells (30). Many mathematical and experimental models have been developed to simulate the transport of oxygen, carbon dioxide, nutrients and metabolic waste inside cell spheroids (65-67). When the diameter of cell spheroid is greater than 500 μm, there will be a three-layer structure with different proliferation states, including the marginal zone with proliferative and metabolic activity, the intermediate layer keeping quiescence and the necrotic core zone (30). Due to limited diffusion capacity, metabolic waste accumulated in the central layer of the multicellular spheroid may cause cell necrosis. Although the diameter of 100-150 μm is considered the stable size that is adopted for simulating tumor spheroids based on the consideration of oxygen and nutrition limitation (68), it may not be appropriate for MSCs since MSCs typically reside in niches with much lower physiological oxygen tensions than cancer cells (66, 69). One recent study showed that 353 ± 18 μm diameter of MSC aggregates composed of up to 60,000 MSCs did not exhibit an obvious hypoxic core. The oxygen tension values from the outside edge of spheroids to the center varied less than 10%, but cellular metabolism was decreased with increasing cell numbers and spheroid size. This may result from the decreased packing density and ECM deposition with increasing spheroid size. Moreover, caspase 3/7 activity was increased with spheroid size especially over 500 μm in diameter, but Annexin V was not detected in these spheroids (66). These results indicated that the enhanced function of MSC spheroids is not oxygen mediated, which contradicts many hypotheses that spheroid formation potentiates cell function by generating a hypoxic core within spheroids with large diameters. In addition, it is also consistent with the SCDS study that suggests that MCDS formed by aggregation of fewer cells and expansion to smaller size (no more than 50 μm) may maintain more stemness owing to the better microenvironment for cell metabolism and self-renewal (45).

The mechanism by which spheroid culture better preserves MSC phenotype and innate properties

Spheroids can maintain the stemness of MSCs and endow them strong multipotency. Spheroid-cultured MSCs exhibited higher colony-forming efficiency than 2D-cultured MSCs (26, 44, 70). The expression of stem cell markers and genes related to self-renewal, such as Nanog, Sox2, Oct4, Klf4, SSEA-4 and β-catenin, are significantly increased in MSC spheroid cultures (44, 45, 70-72). Ling et al. reported that 3D spheroid culture of MSCs showed elevated expression levels of histone H3K9 acetylation in the promoter regions of Oct4, Nanog and Sox2. Moreover, the expression of microRNAs involved in stem cell potency was changed in accordance with the expression of these stemness genes (70). Release of actin cytoskeleton tension promotes Nanog expression, together with Suv39h1 (H3K9 methyltransferase) and H3K9me3 expression down-regulation in 3D spheroid formation (53). Compared with monolayer culture, the spheroid culture significantly upregulated the expression of Runx2 and osteocalcin genes in MSCs after osteogenic induction (44, 72, 73). It was reported that the differentiation capabilities of MSCs were significantly enhanced after spheroid formation, including increased trans-differentiation efficiency into neurons and hepatocyte-like cells by detecting the increased expression of Nestin and Albumin (44).

Spheroid culture is effective in improving the paracrine activity of MSCs (4). Spheroid-cultured MSCs secreted larger amounts of angiogenesis-inducing and immunosuppressive factors than 2D-cultured MSCs, such as VEGF, bFGF, angiogenin, IL-6, IL-8, IL-18, IL24, PGE2, CCL2, CCL5, TSG-6 and STC-1 (4, 26, 41, 73, 74), which contributes to MSC application in cell therapy.

In addition, spheroid culture promotes the survival and migration ability of MSCs, which is important for MSCs to home the lesion and function effectively in vivo (35, 75). Formation of MSC spheroids restores the functional expression of CXCR4 that is well known as an alpha-chemokine receptor specific for SDF-1α, a crucial mediator of cell migration (35, 76). The study found that more extracellular molecules were produced by spheroids than by 2D-cultured MSCs, such as laminin and fibronectin that contributes to cell migration and survival (44). When MSCs aggregate into spheroids, the expression of ECM proteins is increased and the E-cadherin mediated cell-cell adhesion is enhanced, which activates the pEPK and PI3K pathways, regulates higher levels of SOD2 to decrease oxidative stress and then facilitates cell survival (7, 33, 42). Several studies demonstrated that MSC spheroids improved their potential by expressing some genes at higher levels, for instance, CXCR4 that increases the ability of homing to the inflammatory sites and adherence to endothelial cells, IL-24 that inhibits tumor growth and TSG-6 that has anti-inflammatory activity (26, 76). Moreover, under hypoxic environment, spheroid culture enhanced expression of SDF-1α and HIF-1α, thereby enhancing MSC stress resistance, survival, homing and angiogenesis *in vivo* (42, 45).

Generally, adult stem cells are normally quiescent in the tissue, but can be activated for growth, migration and differentiation under changed microenvironment such as injury (77, 78). The niche *in vivo* helps to keep MSCs in quiescence, which benefits the maintenance of stem properties such as self-renewal (79). Due to better mimicking the properties of MSC niche, spheroid culture can promote the MSCs to enter G0 phase, which contributes to the recovery of the original characteristics of MSCs such as mesenchymal trait, stemness, self-renewal and migration ability that have been lost during long-term of 2D culture (45). Quiescence also endows MSCs the ability to adapt to hostile environment such as hypoxia, nutrition starvation and ECM-detachment, eventually enhancing MSC survival. The AMP-activated protein kinase (AMPK)/ mammalian target of rapamycin (mTORC) signaling pathway may be involved in spheroid culture of MSCs (45). AMPK is a crucial metabolic regulator to maintain cellular energy homeostasis and thereby benefits cell survival under stresses. The role of mTORC1 is to activate translation of proteins and then contributes to cell proliferation (80). Spheroid culture

significantly increased the activity of AMPK and decreased the mTORC activity. This may explain why spheroid culture can enhance the quiescence of MSCs. Recently, evidence has increasingly shown that mTORC1 is a crucial factor to induce and maintain the cell senescent state (81, 82). The decreased mTORC1 activity may delay the senescence occurrence in MSCs.

Application of MSC spheroids in pre-clinical studies

Spheroids have greater advantages in survival, factor secretion, maintenance of stemness, migration, anti-aging, anti-inflammatory and angiogenesis than normal adherent-cultured cells, indicating their good prospects of clinical application in tissue wound-healing and regeneration for inflammatory diseases and common diseases including bone and cartilage injury (72, 83), tissue inflammation (26, 45, 57, 74), cardiovascular infarction (6) and ischemic injury (33, 42, 54). For bone and cartilage injury, one study reported that the transplantation of 3D spheroids of mouse bone marrow derived MSCs rapidly promoted new bone regeneration in aged mice with a calvarial defect through activation of the Wnt/β-catenin and Smad signal pathways (72). In this study, 3D spheroid MSCs expressed higher levels of stemness markers and upregulated expression of osteogenesis-related molecules than 2D monolayer MSCs in vitro. It was also reported that the implantation of synovium MSC spheroids at relatively low density improved chondrogenesis and subsequent cartilage formation. Aggregates of MSCs up-regulated the expression of chondrogenic genes (SOX9, COL2A1 and ACAN) and exhibited better cartilage repair than 2D culture (83, 84). For cardiovascular infarction and ischemic injury, it was reported that the transplantation of UCMSC spheroids was superior to cells from conventional 2D culture in therapeutic efficacy for rat MI model. As a result, the fibrosis area was smaller, infarct wall was thicker, capillary density was significantly higher and contractility was improved (6). Another study reported that spheroid cultured human adipose-derived MSCs significantly reduced the rate of limb loss in ischemic tissue owing to the enhanced angiogenesis by higher level of angiogenic growth factor secretion than 2D-cultured cells (42). This is consistent with other studies in which the spheroid culture of MSCs showed better ability of angiogenesis than 2D-cultured MSCs (33, 45). For other injuries, it was found that UCMSC spheroids were better than 2D-cultured UCMSCs for therapy of acute liver failure in mouse model due to the reduced expression of tumor necrosis factor-α (TNF-α) and alpha smooth muscle actin (α-SMA) (45, 71). In a study regarding SCI in mice, BDNF-transfected MSC spheroids significantly enhanced the motor function recovery with reduced damage to spinal neurons, while conventional 2D-cultured MSCs failed to exert therapeutic effects (57). In summary, it is of more importance to apply spheroid cultured MSCs for regenerative medical studies at present.

Limitation and difficulties of MSC spheroid application in clinic

Although most of the studies support that MSCs can be optimized by 3D spheroid culture, under some situations, spheroids may be not better than 2D-cultured cells for application (85-89). These studies indicate that MSC spheroids need to be used in suitable conditions, and in-depth understanding about key molecules responsible for the cell fate control of MSCs during spheroid formation is needed. In addition, spheroid culture promotes the MSCs to enter G0 phase, which inhibits the expansion of cells although quiescence contributes to the maintenance of cell stemness. Therefore, more reasonable methods need to be developed to optimize the MSCs. Recently, Yan *et al*. reported a new method for efficient large-scale hMSC expansion with low indications of senescence phenotype and stable characteristics for high cell recovery rate (90). However, this method cannot realize the optimization of MSCs to enhance MSC properties as spheroid formation did.

Although the existing methods of spheroid formation can optimize MSCs, they generally cannot avoid cell dissociation, uneven quality and non-uniform size, which prevents high-quality optimization and standardized production of MSCs, and impedes the application of MSC spheroids in clinic. The method of MSC SCDS formation based on self-renew screening has tried to overcome the above shortcomings, but more efforts need to be made to realize the cell expansion. Furthermore, once the clinical applications are involved, standardized, large-scale and uniform MSC spheroid culture is required, and the corresponding detection methods for spheroid identification, quality inspection and evaluation are also essential. However, these problems have not been fully resolved so far.

Conclusions and perspectives

Spheroid cultured MSCs have shown the abilities of anti-apoptosis, enhanced stemness, increased sections, anti-inflammatory and immunomodulatory properties as well as induced angiogenesis in vitro and *in vivo*. These features indicate their unique therapeutic potential in tissue damage, regenerative medicine and inflammatory diseases, however, a wealth of problems need to be solved in the future. First, the in-depth molecular mechanisms by which crucial regulators, signaling pathways and biological processes are involved in MSC survival, senescence, stemness, homing, factor secretion and differentiation should be uncovered, as well as the relations between spheroid structure and cell function. Actually, gene expression differences between MSC spheroids and 2D cultures have been widely detected in various spheroid investigations, but little has been done for further deep explorations. Second, optimized MSC spheroids for clinical applications should meet the demands of uniform size, reproducibility, low cell heterogeneity, stable properties, easy operation for preparation, and large-scale and standardized production. Third, the optimal dosing strategy and administration route of 3D MSC spheroids need to be investigated in pre-clinical research. Fourth, more clinical trials need to be

performed to verify the good therapeutic potential of MSC spheroids. Given the big size of spheroids preventing them from vein administration, the therapeutic advantages may be first fully exerted in skin tissue repair of clinical wounds, ulcers, burns, scars, surgical defects and deformities, in which the MSC spheroids can be applied without relying on vein administration.

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Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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REVIEW ARTICLE

The composition of chrysanthemum extracts and their pharmacological functions

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ABSTRACT

Chrysanthemum is a widely planted cash crop worldwide, due to its high ornamental and medicinal values. In addition, as a frequently used material in Traditional Chinese Medicine, studies have revealed that chrysanthemum extracts are complicated mixtures of flavonoids, volatile oils, organic acids, polysaccharides and other minor components, which possess pharmacological effects against many pathological conditions such as toxin-induced cell loss, bacterial infection-induced inflammation, reactive oxygen species-induced oxidative stress and tumor formation induced by various stimuli. In this mini review, we collected and summarized the current understanding on the composition and pharmacology of chrysanthemum extracts originally published in both Chinese and English, and aimed to broaden the readership, in order to build up the basis for eliciting more valuable investigations in this topic.

Keywords: Chrysanthemum extracts · Flavonoids · Volatile oils · Organic acids · Polysaccharides

1. Introduction

Being a leading valued flower worldwide, chrysanthemum is planted and studied due to its attractive appearance of colors and shapes (1), dynamic plant architectures, a variety of flowering times and post-harvest quality (2), and potential medicinal and therapeutic applications of its extracts (3). In addition, chrysanthemum has long been treated as an important medicinal substance with a wide usage in Traditional Chinese Medicine (TCM) (4-8). As documented in the classical literatures of TCM, which could date back to 1,000 B.C., the dried head-shaped flower sequence of chrysanthemum, with the Latin name of *Dendranthema morifolium (Ramat.) Tzvel*. possessed "sweet and bitter taste" ("taste" is usually referred to as the pharmacological characteristics of a certain medicine in TCM), and has the effects of dispersing "liver wind" (a TCM term indicating a range of health hazards in the liver that occur suddenly), balancing "liver and eyesight" (according to the theory of TCM, liver is highly correlated with eyes), clearing "heat" and detoxifying (9). Modern pharmacological studies have also suggested that chrysanthemum extracts possess cell protection (10, 11), anti-colitis, anti-tumor (12-14), anti-oxidation (15) and anti-bacterial effects (16-19), as summarized in **Figure 1**. Moreover, the extracts of chrysanthemum have been shown to be capable of preventing lipid accumulation in the liver (20-22).

Modern Chinese literatures have demonstrated that the usefulness of chrysanthemum tea in alleviating symptoms such as headache, dizziness, thoracic fever, coronary heart disease and hypertension. According to the various origins and processing methods of the medicine, four varieties namely "Hang Ju", "Gong Ju", "Chu Ju" and "Bo Ju" have been suggested in the First Section of the Chinese Pharmacopoeia 2005 Edition. Additionally, other varieties of chrysanthemum are also seen in the market. Although some minor ingredients among varieties could be distinct, most of them include flavonoids (including apigenin, luteolin and quercetin), volatile oils (including mono- and sesquiterpene, their oxygen-containing derivatives and aromatic compounds), organic acids (such as chlorogenic

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acids), polysaccharides (composed of galactose, glucose, mannose and pectinose), and other minor components (vitamin C, selenium, etc.) (23, 24). For a better illustration, the general composition and possible pharmacological effects of chrysanthemum extracts are summarized in **Table 1**. In this mini review, we collect and summarize the general components of chrysanthemum extracts and their pharmacological effects reported in Chinese literatures, as well as recent findings in English literatures, with the aim to build up the basis for more valuable investigations and avoid unnecessary redundant attempts for the separation of their composition and illustration of their pharmacological effects.

2. Flavonoids in chrysanthemum extracts and their potential pharmacological effects

2.1 Types of flavonoids

The flavonoids contained in chrysanthemums are mainly apigenin, luteolin, quercetin and their derivatives, such as flavonoid glycoside, flavonols and flavonol glycosides. Among these flavonoids, luteolin and quercetin are the most intensively studied, as summarized in **Table 2**.

2.2 Pharmacological effects of flavonoids

It has been reported that chrysanthemum total flavonoids possess inhibitory effect on oxidation induced by intensive lipid uptake and accumulation in a dose-dependent manner (25). This effect could be explained by the reduction of multiple phenol groups in flavonoids. Free radicals produced by the oxidation of lipids inside cells might react with these groups, and block the oxidation process due to these free radicals.

Total flavonoids of chrysanthemum could also reduce serum levels of triglyceride, total cholesterol, low-density lipoprotein cholesterol, non-esterified fatty acid in rats with lipid metabolism disorders (26). Meanwhile, they have been suggested to increase serum high-density lipoprotein cholesterol levels as well. Evidence combined together has shown that total flavonoids of chrysanthemum could serve as protective factors, which is capable of affecting the fat metabolism-related enzymes lipoprotein lipase and transport protein Apolipoprotein B, hence play an important role in the breakdown and transport of lipids. In histochemical studies, chrysanthemum flavonoids can reduce liver lesions caused by hyper-lipidism. It also has some effect on improving arterial vascular lesions caused by hyper-lipidemia. Wild chrysanthemum total flavonoids have been shown to inhibit the growth of fissicoccal yeast, staphylococcus, hemolytic staphylococcus, tropical candida, Staphylococcus aureus and malt-obsessed monocytobacteria , and thus possess the anti-microbial activity.

Studies have also shown that quercetin extracted from chrysanthemum could protect vascular endothelial cells (VECs) by promoting their proliferation. Certain doses of quercetin could inhibit hydrogen peroxide-induced release of the lactic acid dehydrogenase in VECs, promoting the secretion of precycline from the damaged VECs. As a result, quercetin is capable of keeping endothelial cells in the blood vessels intact and has the ability to resist oxidative damage (27). In addition, quercetin could reduce the expression of proliferating cell nuclear antigen (28), and thereby inhibit the activity of DNA polymerase by interfering with the synthesis of DNA, eventually suppressing the proliferation of tumor cells (29).

Meanwhile, luteolin extracted from chrysanthemum could significantly inhibit the extracellular regulation of protein kinase ERK2 (30), reduce ovarian cancer cell metastasis, thereby exerting an anti-cancer effect (31). Moreover, scholars have found that luteolin has a potent anti-inflammatory effect on bowel inflammation in rats (32). Meanwhile, reports have also implied that luteolin could inhibit the gene transcription of the pre-inflammatory factors in dendritic cells of the bone marrow (33). It has also been found that luteolin can effectively reduce the thickness of the airway wall and smooth muscle of asthma mice by inhibiting the secretion of pro-inflammatory cytokines such as interleukin (IL)-4 and IL-5, increasing the released level of interferon (INF)-γ, and thus improving the airway remodeling in asthma model mice (34).

Additionally, investigations with mouse ear swelling and vascular permeability experiments have shown that luteolin-7-O-β-D-glucosin, apigenin-7-O-β-D-glucosin and linarin, extracted and separated from wild chrysanthemum, have anti-inflammatory effects (35). In recent years, during the search for natural anti-viral compounds, crude extracts of chrysanthemum have been shown to inhibit human immunodeficiency virus (HIV)-1 integrative enzyme. Further analysis has revealed that a type of apigenin glycoside, apigenin-7-O-β-D-(4'-caffeoyl)-glucuronide, possesses the strongest anti-HIV activity. A recent research has also indicated that monolith-clycosin-7-O-glucosin from chrysanthemum has an anti-hypertensive effect on blood pressure in rats (36).

3. Volatile oils in chrysanthemum extracts and their potential pharmacological effects

3.1 Types of volatile oils

The volatile oil ingredients of chrysanthemum are mainly composed of monoterpenes and sesquiterpenes, their oxygen-containing derivatives, aromatic compounds and alkanes (37). Among them, the main components of monoterpenes include camphor, dragon brain, 1,8-aminoyl ether, α-thujone, β-pinene, verbenone, verbenol and p-cymene. The main components of sesquiterpenes include farnesene, farnesol, zingiberene, bisabolol, cananga oil and α-cadinol. Studies have shown that the distribution of the components of different varieties of volatile oils in chrysanthemums extracts is obviously different, and is related to the origin and processing methods. An analysis on the four major medicinal chrysanthemums, namely "Hang Ju", "Gong Ju", "Chu Ju" and "Bo Ju" has found that, the most volatile oil types are "Chu Ju", containing a total of 46 types of volatile oil composition, followed by "Bo Ju", which includes 35 varieties. "Gong Ju" and"Hang Ju" possess only 18 and 6 types of volatile oils, respectively.

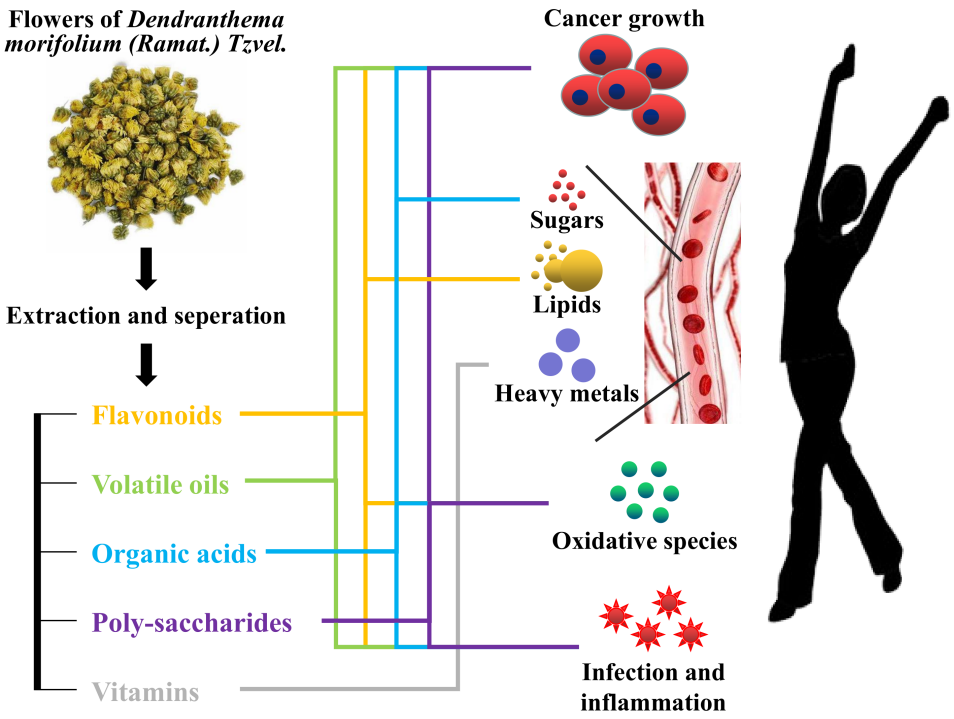


Figure 1. A summarization on the composition and possible pharmaceutical effects of Chrysanthemum extracts.

3.2 Pharmacological effects of volatile oils

In general, investigations have indicated that the volatile oil components of chrysanthemum have the ability of mutation suppression, anti-inflammation and anti-cancer effect (38), as summarized in **Table 3**.

In a study, the chrysanthemum volatile oil could inhibit sister-strand crossover induced by erythromycin and methylene sulfonate in mouse bone marrows. The authors have shown that the inhibitory effect might be exerted via reducing the split indies of sister-strand monomer exchange induced by erythromycin, suggesting that chrysanthemum volatile oil might play a role in suppressing mutation formation. A research has also indicated that the compound extracted from the chrysanthemum has a strong anti-bacterial activity . They have shown that at low concentrations, its anti-bacterial activity is gradually elevated with increasing concentrations of the extract, although the elevation is obvious at high concentrations. Further chemical analysis has revealed that the main components of the volatile oil include lemon oil, β-golden hepenene and oxidized hexapenene .

Volatile oil extracted from “Hang Ju” could significantly inhibit the swelling of the ear shell caused by xylene-induced inflammation in mice (39). Meanwhile, it could also inhibit the swelling of rats' feet caused by carrageenan-induced inflammation in a short period of time, and the level of prostaglandin E2 in the inflammatory model is significantly reduced, indicating that volatile oil may play an anti-inflammatory role by inhibiting the production of inflammatory factors. It would be quite interesting to note that an investigation has indicated that chrysanthemum volatile oil could significantly reduce the body temperature of fevered rats instead of the non-volatile oil (40). In addition, another research has revealed that low, medium and high doses of volatile oil extracted from “Hang Ju” are able to significantly reduce the elevated body temperature caused by endotoxin in rabbits in a dose-dependent manner. This study further suggests that the fever relieving mechanism may be related to changes in the content of monoamine neurotransmitters including norepinephrine, dopamine, and 5-hydroxytryptamine in the hypothalamus tissue due to the volatile oil intake (41).

Another study suggests that pyrethroids in the chrysanthemum volatile oil could be the active ingredient that possesses a significant cytotoxic effect on human nasopharyngeal cancer cell strain CNE1 by inducing in time- and dose-dependent manners.

4. Organic acids in chrysanthemum extracts and their potential pharmacological effects

4.1 Types of organic acids

Chlorogenic acid (also named 3-O-caffeoylquinic acid and caffetannic acid) and its derivatives are the main organic acid compounds discovered in chrysanthemum extracts. As a phenolic acid compound, the chlorogenic acid is made up of the coffee acid and quinine acid. Therefore, the derivatives of chlorogenic acid include caffeoylquinic acids, dicaffeoylquinic acids, and caffeoylquinic acid esters (42, 43).

Table 1. General categories of the currently reported main components from chrysanthemum and their potential pharmacological effects.

Components	Types	Pharmacological effects
Flavonoids	Mainly flavonoids (apigenin, luteolin, quercetin, etc.) and their derivatives	Antioxidant, blood lipid reduction, anti-bacterial, anti-cancer, and anti-HIV
Volatile oils	Monoterpenes, sesquiterpenes, their oxygen-containing derivatives, aromatic compounds, and alkanes	Mutation suppression, anti-inflammation, and anti-cancer
Organic acids	Caffetannic acid, quinine acid and the derivatives of chlorogenic acid	Anti-oxidant, anti-tumor, immune-modulation, and blood sugar reduction
Poly-saccharides	Polysaccharides composed of galactose, glucose, arabinose, rhamnose, mannitose, xylose, and ribose	Anti-oxidant, anti-tumor, and immune-regulation
Others	Vitamin C and metal elements	Reduce the levels of lead in both bone and blood tissues

4.2 Pharmacological effects of organic acids

Pharmacological effects of anti-oxidant, anti-tumor, immunomodulation and blood sugar reduction have been suggested to be linked with chlorogenic acid and derivatives extracted from chrysanthemum (44).

Investigations have revealed that organic acids extracted from chrysanthemum possess a wide range of anti-oxidant activities, since the chlorogenic acid and derivatives show strong inhibition and removal effect on free radicals such as 2,2-diphenyl-1-picrylhydrazyl (DPPH), lipid oxidation derivatives and hyperoxides. In addition, reducibility of these organic acids on oxidized iron even surpasses vitamin C (45). This is due to the reductive hydroxyl group in the chlorogenic acid molecule, which forms a proton with anti-oxidative ability. When combined with free radicals such as hydroxyls or super-oxygen anions, the deionized proton could stabilize these free radicals and hence protect cells and tissues from oxidation.

In a study conducted on primary cells separated from the Lewis lung cancer mouse and in human lung cancer cells A549, chlorogenic acid has been found to maintain a certain level of inhibitory effect *in vitro* and *in vivo*, with the peak inhibitory dose at 20 mg/kg (46). It is speculated that the inhibitory effects on these lung cancers is achieved both directly by inducing apoptosis in cancerous cells and indirectly by regulating immune surveillance T cells. In addition, chlorogenic acid has a significant inhibitory effect on CT26 colon cancer transplanted tumors (47), where the tumor suppression rate increases with elevated concentrations of chlorogenic acid.

The regulatory role of chlorogenic acid and derivatives from chrysanthemum on immune system is also quite obvious in some investigations. Chlorogenic acid could significantly upregulate the levels of INF-γ and tumor necrosis factor (TNF)-α in both epithelial lymphocytes and inherent lymphocytes in the intestine, and high doses of chlorogenic acid could also promote the secretion of TNF-α in peripheral monocytes in the blood. In addition, studies have shown that chlorogenic acid can promote the productions of INF-γ and TNF-α in human lymphocytes and peripheral white blood cells, and enhance the proliferation of T cells caused by influenza virus antigens.

Chlorogenic acid has also been found to effectively reduce the blood sugar levels in an alloxan-treated diabetic mouse model, and there is no significant difference in blood glucose values among the high-, medium- and low-dose groups after administration . However, it has no significant effect on blood sugar levels in normal mice, suggesting it might be directly related with the alloxan induced signaling. Another report suggests that 1,5-bis-O-caffeoylquinic acid, a derivative of chlorogenic acid, could reduce the occurrence of hepatic damage including fibrosis, and hence possesses a protective role in liver cells . The molecular mechanism remains unclear, but the investigation suggests this compound could inhibit the proliferation cycle of damaged liver cells, promote normal cell vitality, and regulate the levels of glutamicpyruvic transaminase and glutamic-oxaloacetic transaminase in the serum, thereby protecting against the pathological structural changes due to acute damage.

5. Polysaccharides in chrysanthemum extracts and their potential pharmacological effects

5.1 Types of polysaccharides

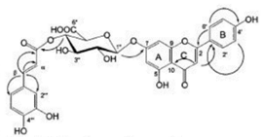
Polysaccharides are complicated and macro molecules composed of dehydrated and condensed mono-saccharides. At present, the research on the chemical structure of polysaccharides in chrysanthemum extracts has mainly focused on the composition and connection of mono-saccharides by determining their biological activities. Current understanding shows that polysaccharides from chrysanthemum are mainly composed of galactose, glucose, arabinose, rhamnose, mannitose, xylose and ribose. Among these mono-saccharides, galactose and glucose are the most abundant ones (48).

5.2 Pharmacological effects of polysaccharides

Evidence also indicates that polysaccharides from chrysanthemum extracts possess pharmacological functions including anti-oxidant, anti-tumor and immune regulation.

In vitro experiments showed that three types of

Table 2. Flavonoids in Chrysanthemum extract and their potential pharmacological effects.

Components	Chemical Name	Molecular Formula	Pharmacological effects
Total flavonoids of Chrysanthemum	Mixture	Mixture	1. Antioxidant 2. Blood lipid reduction
Wild Chrysanthemum total flavonoids	Mixture	Mixture	Anti-microbial
Quercetin	3,5,7,3',4'-Pentahydroxyflavone	$C_{15}H_{10}O_7$	1. Inhibit the proliferation of tumor cells 2. The injury of lipid peroxidation
Luteolin	3',4',5,7-Tetrahydroxyflavone	$C_{15}H_{10}O_6$	1. Inhibit the metastasis of ovarian cancer cells 2. Anti-inflammatory 3. Improve the airway remodeling in asthma model mice
Luteolin-7-O-β-D-glucosin	b-D-Glucopyranosiduronic acid,2-(3,4-dihydroxyphenyl)-5-hydroxy-4-oxo-4H-1-benzopyran-7-yl	$C_{21}H_{20}O_{11}$	Anti-inflammatory
Apigenin-7-O-β-D-glucosin	b-D-Glucopyranosiduronic acid,5-hydroxy-2-(4-hydroxyphenyl)-4-oxo-4H-1-benzopyran-7-yl	$C_{21}H_{20}O_{10}$	Anti-inflammatoryanti-hypertensive
Apigenin-7-O-β-D-(4"-caffeoyl)- glucuronide		$C_{30}H_{24}O_{14}$	Anti-HIV
Linarin	7-[[6-O-(6-deoxy-alpha-L-mannopyranosyl)-beta-D-glucopyranosyl]oxy]-5-hydroxy-4'-methoxyflavone	$C_{28}H_{32}O_{14}$	Anti-inflammatory

polysaccharides from chrysanthemum (with different molecular weights ranging from < 100 kDa, 100-300 kDa and > 300 kDa, respectively) all possess reducibility on radicals such as DPPH, hydroxyl free radicals and hyperoxide anion, indicating that these polysaccharides are able to remove free radicals and serve as anti-oxidant. Another *in vivo* study also demonstrates that these polysaccharides have potent anti-oxidative activity (49). However, after refining the polysaccharides, their free radical removal activity is reduced in the fine product, suggesting that some substances such as binding proteins might have synergistic effects on the anti-oxidative activity of polysaccharides from chrysanthemum.

Additional investigations have shown that the aforementioned three types of polysaccharides with different molecular weights have a significant inhibitory effect on human liver cancer cell line HepG-2 in a dose-dependent manner (50). The polysaccharide with medium molecular weight could also reduce the proliferation of human breast cancer cell line MCF-7 (51). Another research demonstrates that six kinds of polysaccharides isolated from chrysanthemum extracts can reduce the survival rate of the human pancreatic cancer cell line PANC-1, but do not affect or even enhance the human normal liver cell line L02, suggesting these polysaccharides can be served as a potential anti-cancer drug due to their safety.

Apart from the aforementioned organic acid components, chrysanthemum polysaccharides could significantly raise the levels of INF-γ and TNF-α in peripheral white blood cells as well. Study in cyclophosphamide-treated mice with a low immune function has shown that chrysanthemum polysaccharides in high and low-dose groups could both significantly increase the intensity of delayed allergy, increase the serum hemolytic level, and promote the production of hemolysin antibodies in these mice, indicating that chrysanthemum polysaccharides improve cellular and humoral immunities in immune-suppressive mice.

6. Others

Abundant vitamin C and metal elements have been uncovered in chrysanthemum extracts as well (52). Study has shown that the levels of lead in both bone and blood tissues is reduced in lead poisoned mice after application of chrysanthemum tea. Moreover, the amounts of hemoglobin and red blood cells are significantly higher in treated mice compared with the model group. This phenomenon could be attributed to a low solubility of ascorbic lead salt which is formed by combining vitamin C with the lead ion. In addition, another study suggests that vitamin C could accelerate the metabolism of lead thus preventing its deposition. Moreover, selenium in chrysanthemum together with some binding proteins could also be combined with lead to form stable chelates, which aids lead excretion. Meanwhile, iron, zinc and

Table 3. Pharmacological effects of essential oils from different chrysanthemum species.

Components		Pharmacological effects
Volatile Oils from the Flowers of Chamomile		Inhibit sister-strand crossover induced by erythromycin, Methylene sulfonate in mouse bone marrow
Volatile Oils from the Flowers of Chrysanthemum nankingense	Lemon oil	Anti-bacterial activity
	β-golden hepenene	
	Oxidized hexapenene	
Volatile oil extracted from "Hang Ju"		1. Inhibit the swelling of the ear shell induced by inflammation 2. Reduce the body temperature rise caused by endotoxin
Pyrethroids		Possess a significant cytotoxic effect on human nasopharyngeal cancer cell strain CNEl

calcium in chrysanthemum extracts could also antagonize the absorption of lead.

7. Conclusion and prospects

The composition and pharmacological effects of chrysanthemum extracts have been studied extensively in China, which has been briefly translated and summarized in this mini review. However, more investigations on several directions are urgently needed before a broader range of medicinal applications of chrysanthemum and its components. Firstly, many investigations have focused mainly on the pharmacological effects of chrysanthemum extract. However, not much is known about the potential toxicity in terms of dose, origin and processing method of chrysanthemum, which could be quite important for the safety issues of its application. Secondly, although many investigations have demonstrated some molecular mechanisms underlying the pharmacological effects, most of them lack the specific upstream molecular targets that are affected directly. Thus, limited information on sensitivity and specificity hinders further development of these components to potential drugs. Last but not least, due to different origins and processing methods of chrysanthemum, the compositions may vary, which also pose as obstacles on the way to the commercial utilization of chrysanthemum. Therefore, a strict criterion based on more detailed analytical and pharmacological investigations is highly warranted for chrysanthemum and its extracts.

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RESEARCH ARTICLE

Inhibition of microRNA-874 ameliorates cardiomyocyte apoptosis and improves cardiac function in the peripartum cardiomyopathy of Gαq transgenic mice

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ABSTRACT

It is widely reported that microRNAs (miRNAs, miRs) play critical roles in the occurrence and development of peripartum cardiomyopathy (PPCM). Here, we aimed to explore the biological role of miR-874 and its underlying mechanisms in PPCM. To this purpose, a mouse model of PPCM was established through cardiac-specific overexpression of Gαq. Transthoracic echocardiography and left ventricular catheterization were used to examine the cardiac functions and hemodynamics. Apoptosis of the cardiomyocytes was determined by the terminal deoxynucleotidyl transferase dUTP nick end labeling assay. The direct interaction between miR-874 and signal transducer and activator of transcription 3 (STAT3) was confirmed by luciferase assay. The expression of apoptosis-related genes and proteins was evaluated by real-time quantitative reverse transcription polymerase chain reaction and Western blot, respectively. The results demonstrated that miR-874 inhibition significantly increased the survival and cardiac functions of pregnant Gαq transgenic mice. In addition, miR-874 inhibitor ameliorated cardiomyocyte apoptosis, downregulated both mRNA and protein levels of Bax, while upregulated those of Bcl-2. Further, STAT3 was found to be a direct target of miR-874. In addition, miR-874 inhibition increased the expression of STAT3 and janus kinase 2. In summary, miR-874 inhibition could improve cardiac functions and suppress cardiomyocyte apoptosis by targeting STAT3 during PPCM in Gαq transgenic mice.

Keywords: MicroRNA-874 · PPCM · Left ventricular function · Cardiomyocyte apoptosis · STAT3

Introduction

Peripartum cardiomyopathy (PPCM) is a type of cardiac disease of unknown etiology occurred in the third trimester of pregnancy or even months after delivery, without other causes of heart failure (1). The left ventricular ejection fraction of PPCM patients is less than 45% (similar to dilated cardiomyopathy) with or without left ventricular dilatation (2). The clinical manifestations of PPCM include cardiac enlargement (mainly left ventricular enlargement), heart failure (dyspnea, fatigue and peripheral edema) and thromboembolism (such as pulmonary and/or cerebral embolism) (3). Malignant arrhythmias or sudden cardiac death was occasionally seen (3). Although the pathogenesis of PPCM is not clear, there are several potential risk factors including inflammatory response, viral infection, autoimmune response, oxidative stress and prolactin, as well as some genetic factors (4). Currently, the effective treatments of PPCM adopt standard heart failure treatment and supportive treatment. However, there is no specific treatment method for PPCM (5). Herein, it is important to explore the molecular mechanisms underlying PPCM to develop corresponding new treatment schemes.

MicroRNAs (miRNAs, miRs), as small non-coding RNAs, are believed to participate in multiple biological processes, including cell proliferation, apoptosis, differentiation and growth (6). MiRNAs are reported to

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exert important functions in various cardiovascular diseases, such as myocardial hypertrophy, myocardial infarction, heart failure, hypertension and arrhythmia (7). Most current studies on miR-874 have focused on its inhibitory role in cancer, but its role in cardiovascular diseases remains unclear. It was suggested that miR-874 inhibitors could suppress myocardial cell necrosis by targeting cysteinyl aspartate specific proteinase (caspase)-8 (8). In addition, miR-874 inhibition could protect cardiomyocytes and inhibit cardiomyocyte apoptosis caused by ischemia-reperfusion by targeting signal transducer and activator of transcription 3 (STAT3) in mice (9). Besides, STAT3 is thought to participate in cardio-protection by inhibiting reactive oxygen-induced pregnancy oxidative stress (10). Studies have shown that female mice with a cardiomyocyte-specific loss of STAT3 exhibit the PPCM phenotypes (11, 12). Reduction of STAT3 activates cathepsin D and increases oxidative stress, which resulted in the inhibition of endothelial cell migration and proliferation, as well as cell apoptosis and impairment of heart functions by cleaving prolactin into 16k Da fragments (13). Gαq, a member of the Gq/11 subfamily of G protein, is a functional unit of the Gαq protein encoded by Gnaq (14). It is documented that mice with cardiac-specific overexpression of Gαq exhibit systolic dysfunction and baseline cardiac hypertrophy (15). The mice were susceptible to heart failure when challenged with hemodynamic overload (15). We therefore hypothesized that regulation of miR-874 expression should be able to modulate the myocardial function and myocardial apoptosis during PPCM.

Materials and Methods

Animal model

The Gαq transgenic mice were established as previously described (16). The oocytes were microinjected with 1,000 copies of the fragment and were subsequently implanted into pseudo-pregnant females. Genomic Southern analysis was used to screen the presence of the transgene in three-week old mouse pups. Treatment with miR-874 inhibitor was conducted on the 12th day of pregnancy, and tissue samples were obtained 23 days later, on the 14th day postpartum. Pregnant Gαq transgenic mice (10 weeks old) were divided into four groups (n=24 each group): 1) age-matched, non-pregnant female wild-type mice, 2) Gαq transgenic mice, 3) pregnant Gαq transgenic mice, and 4) Gαq transgenic mice treated with miR-874 inhibitor. Animal studies were reviewed and approved by the Ethics Commitment of Shanghai First Maternity and Infant Hospital.

MiR-874 inhibition

MiR-874 antagomir negative control (antagomir-NC) and antagomir were provided by GenePharma (Shanghai, China). All the 3′-ends were conjugated to cholesterol, and the bases were 2′-OMe modified. The sequence of miR-874 antagomir was 5′-UCGGUCCCUCGGGCCAGGGCAG-3′. The chemically modified oligonucleotides (5′-CAGUACUUUUGUGUAGUACAA-3′) was used as the negative control (miR-874-NC). Mice were injected intravenously with miR-874 antagomir or its negative control (0.2 mL) for three consecutive days at a dose of 30 mg/kg body weight per injection.

Echocardiography and cardiac hemodynamics

Echocardiography was applied to determine the left ventricular function by using Vevo 2100 (Visual Sonics, Canada). Echocardiographic examination was performed on the surviving mice, and the four groups of mice were mixed and numbered for anesthesia and examination by an assistant using a double-blind method. The mice to be tested were anesthetized by inhalation of isoflurane, and the heart rate was kept constant at (400 ± 20) times/min during the examination. A heated plate was used to keep the body temperature of the mice constant during the examination. In order to ensure the imaging quality, mice were kept in a fixed position, with hair removed and skin prepared by applying an appropriate amount of ultrasonic couplant to the chest skin. The values obtained in the examination were averages of 5 consecutive cardiac cycles. A high-frequency probe was placed on the left thoracic side of the mouse, with the probe notch pointed toward the mouse's head, and rotated 30° to 45° counterclockwise to switch to M-mode. The sampling line was placed at the level of the mitral valve chordae, perpendicular to the ventricular septum and the posterior wall of the left ventricle. The scanning speed of the curve was 200 mm/s. The left ventricular fraction shortening (LVFS) and left ventricular end-diastolic dimension (LVEDD) were then obtained. Left ventricular catheterization was measured by a multichannel physiological recorder (Beijing B&E Teksystems, Beijing, China). Mice were anesthetized with light methoxyflurane and were placed supine on a heating pad. Then a pressure micro catheter was used to retrograde into the left ventricle of the mouse, which was connected to the Power Lab workstation to obtain the left ventricular end-diastolic pressure (LVEDP), left ventricular systolic pressure (LVSP), $-dp/dt_{max}$ and $+dp/dt_{max}$.

Cell apoptosis

The left ventricular tissues of the experimental mice were derived and fixed in paraformaldehyde (4%), dehydrated and embedded in paraffin. They were further cut into 3-4 μm slices, attached on a poly-lysine adhesion slide, and baked at 65 °C for 1 h. Cell apoptosis was further detected by One Step TUNEL Apoptosis Assay Kit (Beyotime, Shanghai, China) according to the manufactures' instructions.

Reactive oxygen species (ROS) measurement

The left ventricular tissues from indicated mice were freshly isolated and digested with trypsin for 15 min, and gently blown for 1 min using a pipette. The isolated cells

were then plated in 24-well plates (5×10^5 cells/well). Intracellular ROS levels were evaluated with DCFH-DA. The cells were incubated with DCFH-DA (10 μM) at 37 °C. Fluorescent signal intensity of DCF was examined at 488 nm (excitation wavelength) and 525 nm (emission wavelength).

qRT-PCR

The total RNA from isolated left ventricular tissues was extracted with Trizol reagent (Life Technologies, Carlsbad, CA). Then, the RNA was reverse transcribed into cDNA by a reverse transcription kit (Life Technologies). qRT-PCR was conducted using 2 × SYBR Green PCR Master Mix (Life Technologies) to detect the levels of Bax, Bcl-2, miR-874, and STAT3. β-actin or U6 (for miR-874) was used as the internal control. The following primers were used:
U6:
F: CTCGCTTCGGCAGCACA;
R: AACGCTTCAGAATTTGCGT.
STAT3:
F: GAAACAACCAGTCTGTGACCAG;
R: CACGTACTCCATTGCTGACAAG.
miRNA-874:
F: TGCGGCGGCCCCACGCACCAG;
R: CCAGTGCAGGGTCCGAGGT.
Bax:
F: CTGAGCTGACCTTGGAGC;
R: GACTCCAGCCACAAAGATG.
Bcl-2:
F: TGCACCTGAGCGCCTTCAC;
R: TAGCTGATTCGACCATTT.
Bcl-XL:
F: ACATCCCAGCTTCACATAACCC;
R: CCATCCCGAAAGAGTTCATTCAC.
JAK2:
F: GCAAAGGTAACTTCGGGAGTGT;
R: AGTCTCGGAGGTGCTCTTCAG.
β-actin:
F: GACGGCCAGGTCATCACTATTG;
R: AGGAAGGCTGGAAAAGAGCC.
The relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ relative quantitative method.

Western blot

Western bolt analysis was carried out following common protocol. The total protein of isolated left ventricular tissues was extracted. Sodium dodecyl sulfate lysate was added. 20 μL protein was prepared, then loaded in 12% polyacrylamide gel electrophoresis. After transferred to the membranes, the membranes with the protein were blocked by Tris-buffered saline Tween-20 containing 5% bovine serum albumin for 1 h at room temperature. The primary antibodies including anti-Bcl-2 (ab59348, 1:1000), anti-Bax (ab32503, 1:1000), anti-STAT3 (ab68153, 1:1000), anti-janus kinase 2 (JAK2) (ab108596, 1:1000) were purchased from Abcam (Cambridge, MA). GAPDH (ab8245, 1:3000) was used as a loading control. The primary antibodies were incubated at 4 °C overnight. Horseradish peroxidase-conjugated secondary antibodies (Invitrogen) were used for further incubation for 2 h.

Dual luciferase reporter gene assay

The sequences of STAT3 3′-untranslated region (UTR) including the wild-type binding site of miR-874 or the mutated binding site were cloned into the pMIR vector (Promega Corporation, WI, USA). MiR-874 mimics was purchased from GenePharma (Shanghai, China). The relative luciferase activity was measured by Luciferase Dual Assay Kit (Thermo Fisher Scientific, Waltham, MA).

Statistics

The data were shown as mean ± standard deviation (SD). Survival comparison between groups was performed by Kaplan-Meier analysis with a Log-rank (Mantel-Cox) test. To analyze data between multiple groups, one-way ANOVA followed with a Tukey's post hoc test was performed. Statistically significant difference was accepted at $p<0.05$.

Results

MiR-874 inhibitor significantly decreases the mortality of the PPCM mice

To evaluate the activity of miR-874 inhibitor on the mortality of PPCM in the Gαq mice, a Kaplan-Meier survival analysis was employed. The mice were randomly divided into four groups, with 24 mice in each group. Administration of miR-874 inhibitor on the 12th day after pregnancy was considered as the beginning of the experiment, and then the experiment was terminated after 23 consecutive days (on the 14th day after delivery). Study design of the research was shown in **Figure S1**. It was shown that the mortality in the pregnant Gαq transgenic mice was 10 out of 24, whereas miR-874 antagonist decreased the mortality to 3 out of 24 in the pregnant Gαq transgenic mice (**Figure 1**). Of note, we found that control antagomir had little effect on the survival rate of the pregnant Gαq transgenic mice (**Figure S2**). Thus, treatment with miR-874 inhibitor significantly decreased the mortality of PPCM in the Gαq transgenic mice.

MiR-874 inhibitor modulates the left ventricular function in PPCM

To further investigate the role of miR-874 in PPCM, echocardiography was applied to determine the left ventricular function in the mice. At the 14th day after delivery, LVEDD and LVFS of the age-matched, female non-pregnant wild-type (n=24) and Gαq transgenic mice (n=24), pregnant Gαq transgenic mice (n=14) and Gαq transgenic mice treated with miR-874 inhibitor (n=21) were compared. Consistent with the previous literature, the LVEDD was higher in Gαq transgenic mice than in wild-type mice before pregnancy. In addition, the pregnant Gαq transgenic mice showed a further increased LVEDD, which could be reversed by treatment with miR-

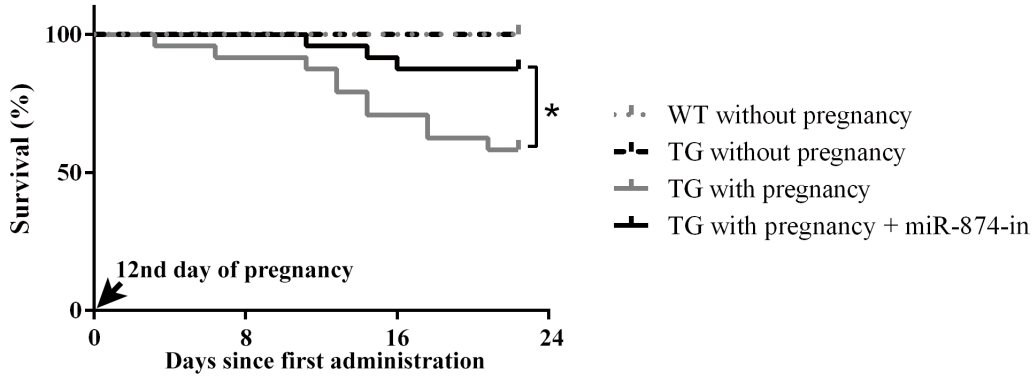

Figure 1. Kaplan-Meier survival analysis. All-cause mortality over course of experiment starting at day 12 after conception and 23 days continuous before euthanasia. All animals that entered study were included in this analysis. *p < 0.05 between the indicated groups, Log-rank (Mantel-Cox) test.

874 inhibitor (**Figure 2A**). Next, we found that the LVFS was lower in Gαq transgenic mice than in wild-type mice before pregnancy. Besides, the pregnant Gαq transgenic mice showed a further decreased LVFS, whereas treatment with miR-874 inhibitor reversed this trend (**Figure 2B**). In addition, the control antagomir had no significant effect on the left ventricular functions in the pregnant Gαq transgenic mice (**Figure S3**). Therefore, our results demonstrated that treatment with miR-874 inhibitor could partially rescue the left ventricular dilation and contractile dysfunction.

MiR-874 inhibitor modulates the left ventricular hemodynamics in PPCM

The LVSP was decreased in Gαq transgenic mice compared with the wild-type mice before pregnancy, and the pregnant Gαq transgenic mice showed a further decreased LVSP. The treatment using miR-874 inhibitor significantly increased the LVSP to a level similar to the non-pregnant Gαq transgenic mice (**Figure 3A**). Besides, the LVEDP was obviously higher in Gαq transgenic mice than in wild-type mice before pregnancy, and further increased in the pregnant Gαq transgenic mice, which was remarkably decreased by miR-874 inhibitor (**Figure 3B**). In addition, we found that +dP/dt and -dP/dt was decreased in Gαq transgenic mice compared with the wild-type mice before pregnancy, and was even further decreased in the pregnant Gαq transgenic mice. The treatment using miR-874 inhibitor rescued the reduced +dP/dt and -dP/dt during PPCM in the Gαq transgenic mice (**Figure 3C and D**).

MiR-874 inhibitor significantly suppresses the myocardial apoptosis

Since cardiomyocyte apoptosis is involved in perinatal cardiomyopathy, we examined the effect of miR-874 antagonist on cardiomyocyte apoptosis. By TUNEL staining assay, we found that the cell apoptosis was dramatically increased in Gαq transgenic mice compared with the wild-type mice before pregnancy, and the pregnant Gαq transgenic mice showed a further increase in the percentage of apoptotic cells, which was consistent with the previous literature. Notably, miR-874 inhibitor significantly decreased the percentage of apoptotic cells in the left ventricular tissues in the Gαq transgenic mice (**Figure 4A and B**). Therefore, we demonstrated that inhibition of miR-874 effectively suppressed the myocardial apoptosis.

MiR-874 inhibitor regulates the expression of Bax and Bcl-2 in PPCM of the Gαq transgenic mice

The expression of two apoptosis-related genes, Bax and Bcl-2, were further determined in the left ventricular myocardial tissues to explore the potential of miR-874 on cell apoptosis. Both the mRNA and the protein levels of Bax were increased in Gαq transgenic mice compared with the wild-type mice before pregnancy, and the pregnant Gαq transgenic mice showed a further increased the Bax expression (**Figure 5A, C, and E**). The treatment using miR-874 inhibitor significantly decreased the expression of Bax to a level similar as the non-pregnant Gαq transgenic mice (**Figure 5A, C, and E**). In addition, the mRNA and the protein levels of Bcl-2 were decreased in Gαq transgenic mice compared with the wild-type mice before pregnancy, and the pregnant Gαq transgenic mice showed a further decreased Bcl-2 expression (**Figure 5B, D, and E**). Importantly, miR-874 inhibitor significantly upregulated the expression of Bcl-2 (Figure 5B, D, and E). Various studies have shown that reactive oxygen species (ROS), such as oxygen free radicals and their derivatives, are closely related to cell apoptosis. Therefore, we examined the ROS production and Bcl-XL expression in each group. These results indicated that the ROS production was increased in the Gαq transgenic mice compared with the wild-type mice before pregnancy, and the pregnant Gαq transgenic mice showed further increased ROS production, which was significantly reversed by miR-874 inhibitor (**Figure 6A**). In addition, the mRNA level of Bcl-XL was decreased in the Gαq transgenic mice compared with the wild-type mice before pregnancy, and the pregnant Gαq transgenic mice showed further decreased Bcl-XL expression (**Figure 6B**). Importantly, miR-874 inhibitor significantly upregulated

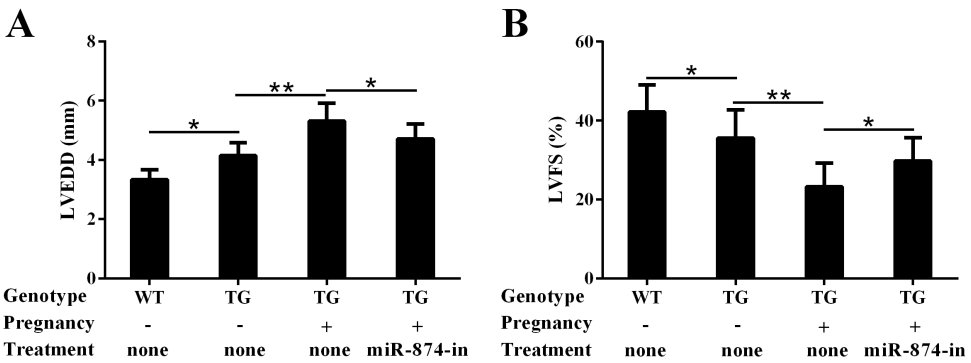

Figure 2. Echocardiographic analysis of left ventricular function. LVEDD (A) and LVFS (B) in survived mice from each group. Age-matched, female non-pregnant wild-type (WT, n=24) and Gαq transgenic mice (GT, n=24), pregnant Gαq transgenic mice (n=14) and Gαq transgenic mice treated with miR-874 antagonist (miR-874-in, n=21) were compared. Data are presented as mean ± SD. *p < 0.05, **p < 0.01 between the indicated groups. LVEDD: left ventricular end-diastolic dimension, LVFS: left ventricular fraction shortening.

the expression of Bcl-XL (**Figure 6B**).

MiR-874 directly targets and suppresses the expression of STAT3

Since miR-874 was critical in regulating cardiomyocyte apoptosis in PPCM of the Gαq transgenic mice, we examined miR-874 expression in all four groups. The expression of miR-874 was increased in Gαq transgenic mice compared with the wild-type mice before pregnancy, and the pregnant Gαq transgenic mice showed a further increase. MiR-874 inhibitor successfully downregulated the expression of miR-874 (**Figure 7A**). The mRNA levels of STAT3 and JAK2 were decreased in Gαq transgenic mice compared with wild-type mice before pregnancy, and the pregnant Gαq transgenic mice showed a further decrease in STAT3 and JAK2 expression (**Figure 7B and C**). Notably, miR-874 inhibitor significantly upregulated the expression of STAT3 (**Figure 7B and C**), suggesting a correlation between miR-874 and STAT3. Western blot analysis further confirmed that the patterns of STAT3 and JAK protein expression were consistent with their mRNA levels, respectively (**Figure 7D-F**). Next, to verify the direct interaction between miR-874 and STAT3, the predicted binding sequences of miR-874 with wild-type or mutant 3′-UTR region of STAT3 mRNA were subcloned

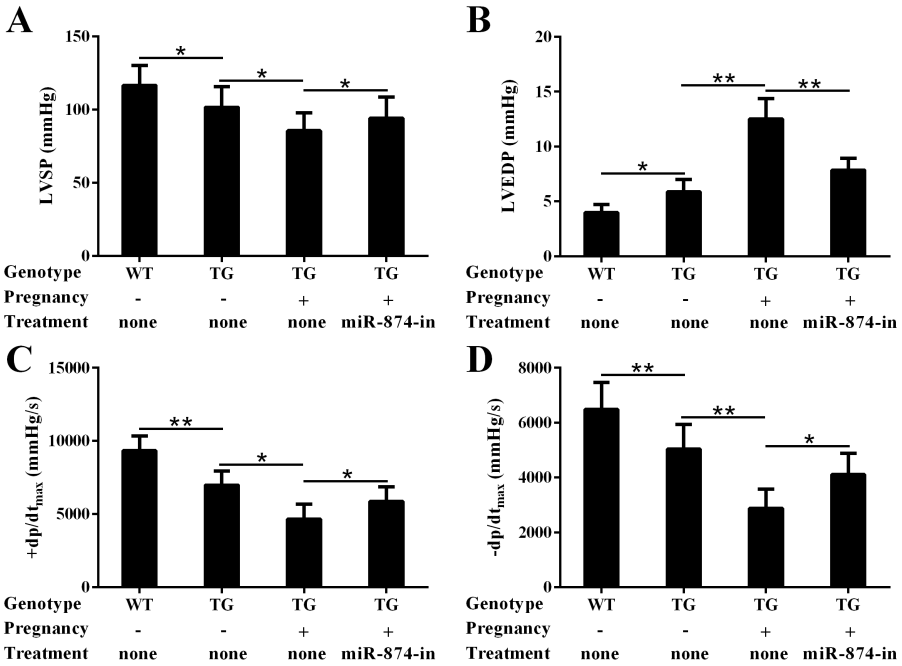

Figure 3. Analysis of left ventricular hemodynamics. LVSP (A), LVEDP (B), +dp/dtmax (C) and -dp/dtmax (D) in survived mice from each group. Age-matched, female non-pregnant wild-type (WT, n=24) and Gαq transgenic mice (GT, n=24), pregnant Gαq transgenic mice (n=14) and Gαq transgenic mice treated with miR-874 antagonist (miR-874-in, n=21) were compared. Data are presented as mean ± SD. *p < 0.05, **p < 0.01 between the indicated groups. LVSP: left ventricular systolic pressure, LVEDP: left ventricular end-diastolic pressure.

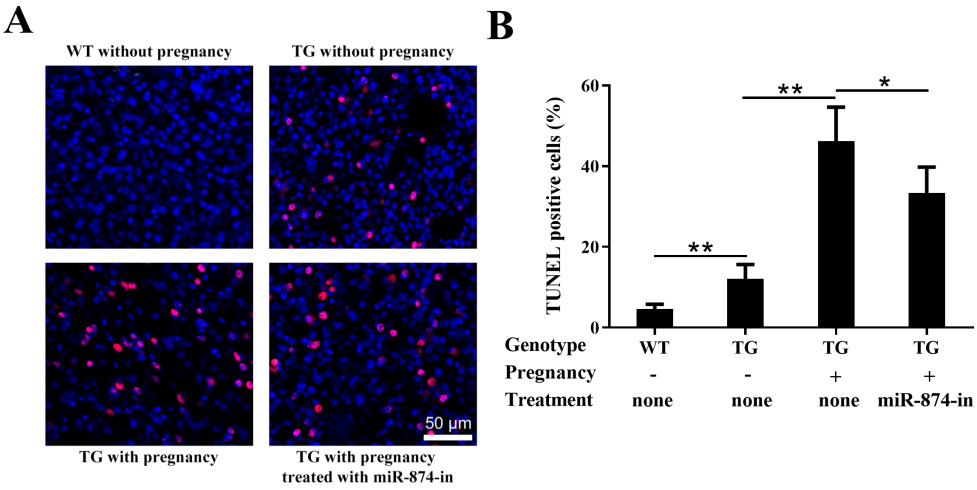

Figure 4. Inhibition of miR-874 ameliorated left ventricular cell apoptosis in PPCM of Gαq transgenic mice. (A) Representative TUNEL staining for detecting left ventricular cell apoptosis among different groups. Cells marked with red indicated apoptosis cells. (B) The percentage of apoptosis ratio among different groups from the TUNEL staining. Age-matched, female non-pregnant wild-type (WT, n=6) and Gαq transgenic mice (GT, n=6), pregnant Gαq transgenic mice (n=6) and Gαq transgenic mice treated with miR-874 antagonist (miR-874-in, n=6) were compared. Data are presented as mean ± SD. *p < 0.05, **p < 0.01 between the indicated groups.

to a luciferase reporter vector (**Figure 7G**). It was demonstrated that miR-874 mimics significantly decreased the relative luciferase activity of wild-type 3'UTR of STAT3, whereas no significant difference was observed for the mutated sequence (**Figure 7H**). Therefore, our data demonstrated that inhibition of miR-874 suppressed the myocardial apoptosis in PPCM by targeting the STAT3/JAK signaling pathway in Gαq transgenic mice.

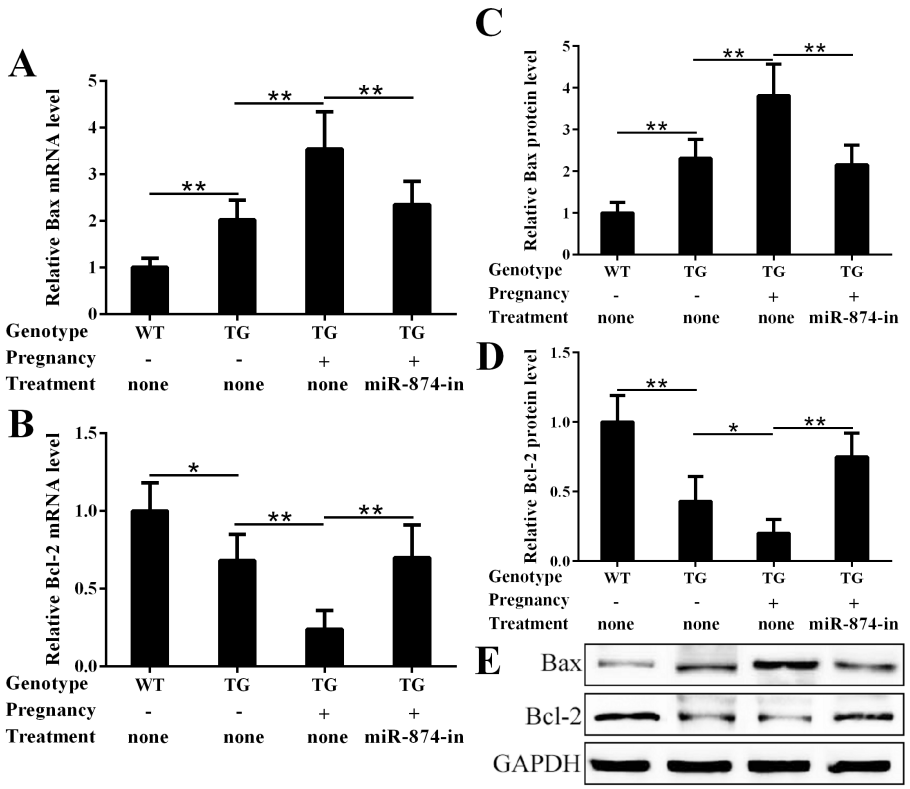

Figure 5. Inhibition of miR-874 ameliorated cardiomyocyte apoptosis in PPCM of Gαq transgenic mice. QRT-PCR was used to measure the mRNA levels of Bax (A) and Bcl-2 (B) from left ventricular myocardial tissues among different groups. Western blotting was used to measure the protein levels of Bax and Bcl-2 from left ventricular myocardial tissues among different groups (C-E). Age-matched, female non-pregnant wild-type (WT, n=18) and Gαq transgenic mice (GT, n=18), pregnant Gαq transgenic mice (n=8) and Gαq transgenic mice treated with miR-874 antagonist (miR-874-in, n=15) were compared. Data are presented as mean ± SD. *p < 0.05, **p < 0.01 between the indicated groups.

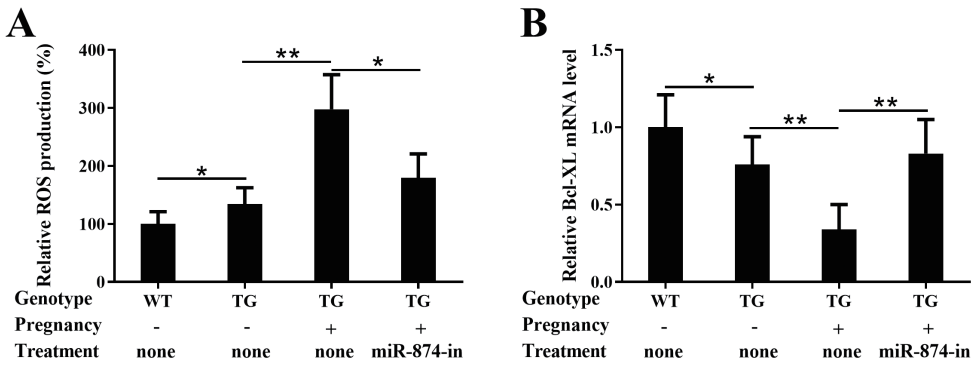

Figure 6. Measurement of ROS production in each group (A) and the relative Bcl-XL mRNA expressions in each group. Data are presented as mean ± SD. *p < 0.05, **p < 0.01 between the indicated groups.

Discussion

The generation and elimination of oxygen free radicals and active oxygen in the body are in a dynamic equilibrium state under normal circumstances, but in certain pathological situations, the generation of active oxygen beyond the body's ability to clear them will cause oxidative stress damage to tissues and cells (17). Many studies have shown that during the pathological process of heart diseases such as myocardial ischemia, cardiac remodeling and myocardial ischemia-reperfusion injury, more ROS and oxygen free radicals often accumulate. These accumulated ROS further cause cardiomyocyte necrosis and apoptosis, eventually aggravating the severity of the disease and forming a vicious cycle (18). Therefore, reducing the oxidative stress injury to cardiomyocytes and reducing apoptosis are of great clinical significance to improve the treatment effect of cardiovascular diseases and to improve cardiac functions.

MiRNAs participate in a variety of pathological and physiological processes such as apoptosis, proliferation and differentiation through the regulation of their target genes. Therefore, their roles in cardiovascular diseases have been valued by more and more researchers. MiRNAs are reported to participate in the pathogenesis of atherosclerotic plaques (21), vascular regeneration after myocardial infarction (22) and myocardial remodeling (23). Circulating miRNAs could also be used to diagnose cardiovascular diseases such as myocardial infarction (24, 25). MiR-874 has been widely studied in tumors and various targets have been identified. Wang et al. reported that miR-874 could regulate myocardial necrosis via targeting caspase-8 (8). In addition, miR-874 inhibited epithelial-mesenchymal transition and metastasis in hepatocellular carcinoma by regulating sex-determining region Y-box 12, which regulated cell fate determination and embryonic development (26). Furthermore, miR-874 alleviated inflammatory response and renal injury in diabetic nephropathy through targeting toll-like receptor-4 (27). Leong et al. reported that miR-874 expression was decreased in hepatocellular carcinoma and negatively regulated PIN1 expression, which modulated cell cycle progression, cell proliferation and apoptosis (28). These data suggest that miR-874 might regulate embryonic development and cell apoptosis in PPCM. Here, we reported that inhibition of miR-874 significantly improved cardiac functions and ameliorated cardiomyocyte apoptosis during PPCM in the Gαq transgenic mice. Inhibition of miR-874 significantly increased the survival and rescued the left ventricular dilation and contractile dysfunction in the pregnant Gαq transgenic mice. In addition, miR-874 inhibitor ameliorated cardiomyocyte apoptosis and regulated the expression of Bcl-2 and Bax.

STAT3 is an important signal transduction protein in cells, which is involved in a series of physiological activities such as apoptosis and proliferation of cells. When cells are stimulated, the SH2 domain of STAT3 binds to phosphorylated tyrosine residues on the receptor, and at the same time autophosphorylation occurs, enabling its entry into the nucleus and turning on its transcription activity (29). STAT3 is an important anti-apoptotic factor and STAT3 function in PPCM has been evaluated in various studies. The myocardial infarct area of STAT3 overexpressing myocardial tissue was significantly reduced after hypoxia-reperfusion injury (30). Overexpression of STAT3 in mouse cardiomyocytes could reduce apoptosis induced by hypoxia-reoxygenation injury (31). STAT3-deficient mouse cardiomyocytes were more sensitive to oxidative stress injury and had a higher apoptosis rate (32). Angiotensin II AT1 receptor, which is Gq protein-coupled, is a major stimulus of JAK-STAT activation (33). Furthermore, GRK2 has been implicated in STAT3 regulation (34), as well as in cardiac anti-apoptosis/inflammation mediated by the β2-adrenergic receptor (35). In the present study, we found that miR-874 inhibitor ameliorated cardiomyocyte apoptosis and regulated the expression of Bax and Bcl-2. Further, STAT3 was a direct target of miR-874, whose inhibition increased the expression and the activation of STAT3 in mouse cardiomyocytes. Our study hereby extends the knowledge on miR-874 in the context of PPCM by targeting STAT3. Other potential targets of miR-874 involved in the STAT3 signaling pathway, such as angiotensin II receptors and GRK2, need to be further investigated in future studies.

Overexpressed STAT3 could eliminate active oxygen in the body by upregulating the activity of the manganese superoxide enzyme, thereby reducing tissue damage

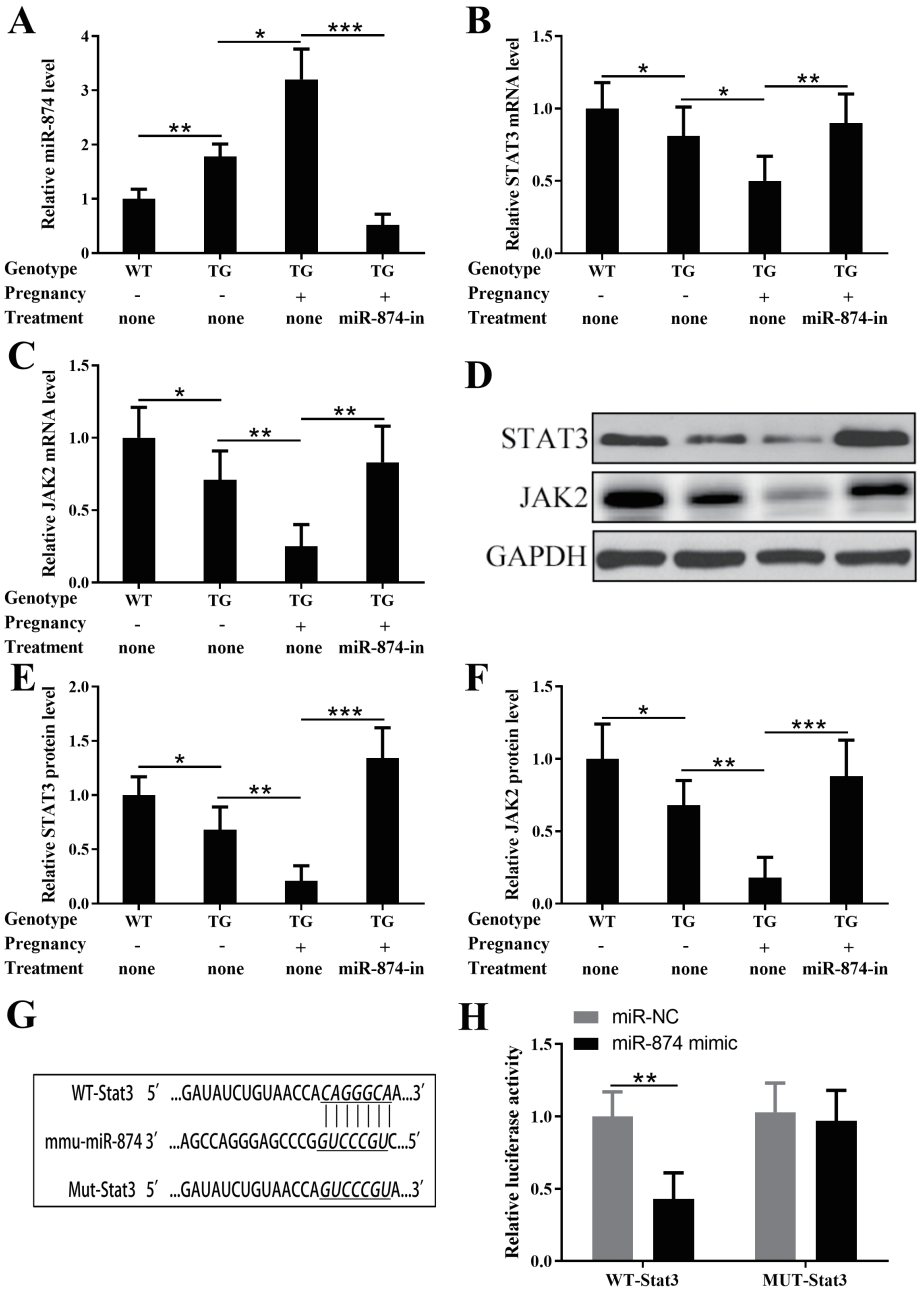

Figure 7. Inhibition of miR-874 targeted STAT3. QRT-PCR was used to measure the level of miR-874 (A), STAT3 (B) and JAK2 (C) among different groups. (D) Western blotting was used to measure the protein level of STAT3 and JAK2 among different groups and the relative expressions (E and F). (G) The suspected binding of miR-874 with the wild-type or mutant 3′-UTR region of STAT3 mRNA is shown. (H) Luciferase activity after the transfection of the wild-type or mutant 3′-UTR region of STAT3 along with miR-874 mimics. Age-matched, female non-pregnant wild-type (WT, n=18) and Gαq transgenic mice (GT, n=18), pregnant Gαq transgenic mice (n=8) and Gαq transgenic mice treated with miR-874 antagonist (miR-874-in, n=15) were compared. Data are presented as mean ± SD. *p < 0.05, **p < 0.01 and ***p < 0.001 between the indicated groups.

caused by oxidative stress (31). Prolactin is one of the important hormones to maintain normal physiological activities among pregnant women. Oxidative stress could cleavage full length prolactin into 16k Da prolactin fragments by stimulating the protein expression of cathepsin D in cardiomyocytes, while 16k Da prolactin fragments in the body could promote the apoptosis of vascular endothelial cells and prevent angiogenesis by upregulating miR-146a (36, 37). The treatment using kiosk (which could inhibit the release of prolactin) returned the plasma miR-146a levels of PPCM patients to normal levels (13, 37). In addition, animal experiments have confirmed that mice with reduced anti-oxidative stress capacity after special treatment show symptoms similar to dilated cardiomyopathy after delivery. In this study, we found that the ROS production was increased in Gαq transgenic mice compared with wild-type mice before pregnancy, and the pregnant Gαq transgenic mice showed further increased ROS production, which was consistent with previous studies. Notably, miR-874

inhibitor treatment significantly decreased the ROS production to a level similar to the non-pregnant Gαq transgenic mice. Our research suggests that miR-874 inhibitor could be utilized for the clinical treatment of PPCM, bringing new hope for the clinical management of PPCM.

Conclusion

In conclusion, our results have demonstrated that miR-874 inhibition significantly increases the survival and cardiac functions of pregnant Gαq transgenic mice. In addition, miR-874 inhibitor ameliorates cardiomyocyte apoptosis and regulates the expression of Bax and Bcl-2. Further, STAT3 is a direct target of miR-874 and miR-874 antagonist increases the expression and the activation of STAT3. Our findings reveal the potential biomarkers for cardiovascular disease and novel therapeutic targets for the treatment of PPCM.

Conflict of interest

The authors declare that they have no conflicts of interest to disclose.

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Supplementary Information

The supplemental material can be downloaded online at: https://stemedicine.org/index.php/stem/article/view/75

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