ISOOR International Society of Organoid Research

www.isoor.org info@isoor.org

Journal of Organoid & Bioscience

Volume 2 | Number 1 | June 2024

ISSN: 3029-2271

PUBLISHED BY SCIPOLIS PTE. LTD.

TABLE OF CONTENTS

Scipolis
May the Science be with you
OPEN ACCESS

JOBs
Journal of Organoid & Bioscience

Editorial

Perspective on Tumor Organoids in Drug Screening and Cancer Precision Medicine

Lingzhi Wang[1,2]

[1]National University of Singapore

[2]Editor-in-Chief, *Journal of Organoid and Bioscience*

https://doi.org/10.62714/ZLSH6629

The landscape of cancer research is swiftly evolving, presenting fresh optimism through innovative tactics that customize treatment for each patient. In 2016, Clevers unveiled a groundbreaking study in Cell, introducing organoids that emulate essential structural and functional traits of organs like the kidney, lung, gut, brain, and retina. This technology enables the replication of human organ development and various pathologies within a controlled laboratory environment. Mounting evidence underscores the potential of patient-derived organoids (PDOs) to reshape cancer treatment and revolutionize drug discovery in oncology. This commentary explores these pioneering research avenues and their profound implications for clinical oncology and pharmaceutical development.

The integration of tumor organoids into drug screening and cancer precision medicine marks a pivotal advancement in oncology research. Driehuis *et al*. have meticulously delineated protocols for establishing patient-derived cancer organoids from diverse epithelial tissues and cancers, streamlining in vitro testing of therapy sensitivity. These protocols, especially tailored for generating head and neck squamous cell carcinoma organoids, enable semi-automated therapy screens. Typically, organoid establishment and subsequent screenings can be completed within three months, with variations depending on factors like the starting material and the number of therapies examined. These protocols serve as invaluable references for developing organoids from other cancer types, enhancing the drug screening process.

Grossman *et al*. showcased the transformative capacity of PDOs in personalized oncology through the HOPE trial and subsequent investigations. By generating PDOs from patients with pancreatic ductal

adenocarcinoma (PDAC) and evaluating their drug sensitivity, researchers were able to anticipate clinical responses. This methodology tackles the intrinsic heterogeneity of human cancers, offering a functional precision medicine approach beyond static genomic features. PDOs provide a dynamic platform for evaluating diverse therapeutic agents and identifying effective combinations tailored to individual patients. The capability to categorize PDOs as sensitive or resistant to specific chemotherapy regimens and correlate these findings with patient outcomes underscores their utility as predictive models. This approach has been successfully extrapolated to other cancer types, such as head and neck squamous cell carcinoma and rectal cancer, illustrating its broad applicability in predicting treatment responses and guiding clinical decisions.

In a significant advancement, Dekkers *et al*. explored the interplay between engineered T cells and cancer organoids to enhance treatment outcomes. The BEHAV3D system enables the study of dynamic interactions between immune cells and patient-derived cancer organoids. Through live-tracking over 150,000 engineered T cells cultured with solid-tumor organoids, BEHAV3D identified a "super engager" cluster of T cells with potent serial killing capacity. This innovative system also unveiled behavior-specific gene signatures, including previously uncharacterized genes expressed by super engager T cells, offering fresh insights into effective T cell responses. Notably, type I interferon was found to prime resistant organoids for T cell-mediated killing, suggesting potential combination strategies to boost the efficacy of cellular immunotherapies. BEHAV3D stands out as a promising tool for characterizing the behavioral and phenotypic heterogeneity of cellular immunotherapies, paving the way for optimized, personalized treatments for solid tumors.

Letai *et al*. furthered the field by probing tumor vulnerabilities through functional precision oncology, directly examining live tumor cells with various drugs to uncover weaknesses. This strategy supplements genomic profiling by furnishing real-time, actionable insights into the most effective treatments. Emerging technologies enable the assessment of drug responses in a context mirroring the tumor microenvironment, overcoming the limitations of conventional static approaches. The utilization of pre-clinical models like patient-derived xenografts (PDXs) and PDOs, representing tumors from affected individuals, hones drug discovery and enhances the success of new therapies in clinical settings. The enhanced feasibility of developing models derived from affected individuals on a large scale for research has democratized these models for personalized therapy. For example, the realization that engraftment of breast and other tumors as PDXs predicted metastatic relapse sparked the notion that these models could customize therapy upon recurrence, resulting in the Functional Precision Oncology for Metastatic

Breast Cancer study. Through the integration of functional assays with genomic and transcriptomic data, researchers can construct a comprehensive understanding of tumor biology and resistance mechanisms. This holistic approach holds the promise of unearthing novel therapeutic targets and refining treatment regimens, ultimately augmenting patient outcomes. The dynamic nature of PDOs permits real-time testing and adjustments, furnishing a robust platform for precision medicine.

In conclusion, the incorporation of tumor organoids into drug screening and precision medicine represents a significant advancement in oncology. The ability to model patient-specific responses, test a variety of therapeutic agents, and identify effective treatment combinations offers a promising path toward more personalized and effective cancer treatments. As research continues to evolve, these innovative approaches will likely become integral to clinical decision-making, leading to better patient outcomes and advancing the field of oncology.

Lingzhi Wang
Editor-in-Chief, *Journal of Organoid and Bioscience*

References

1. Clevers H.Modeling Development and Disease with Organoids. Cell. 2016 Jun 16;165(7):1586-1597. doi: 10.1016/j.cell.2016.05.082.
2. Dekkers JF, Alieva M, Cleven A, *et al*. Uncovering the mode of action of engineered T cells in patient cancer organoids. Nat Biotechnol. 2023 Jan;41(1):60-69. doi: 10.1038/s41587-022-01397-w.
3. Driehuis, E, Kretzschmar, K, Clevers, H. Affiliations expand Establishment of patient-derived cancer organoids for drug-screening applications. Nat Protoc. 2020 Oct;15(10):3380-3409. doi: 10.1038/s41596-020-0379-4.
4. Grossman JE, Muthuswamy L, Ling Huang L, *et al*. Organoid Sensitivity Correlates with Therapeutic Response in Patients with Pancreatic Cancer. Clin Cancer Res. 2022.
5. Letai A, Bhola P, Welm AL. Functional precision oncology: Testing tumors with drugs to identify vulnerabilities and novel combinations. Cancer Cell. 2022 Jan 10;40(1):26-35. doi: 10.1016/j.ccell.2021.12.004.

Scipolis
May the Science be with you
OPEN ACCESS

JOBs
Journal of Organoid & Bioscience

News & Views

Industry News（Jan 2024 to May 2024）

Flora Wang[1]

[1]ISoOR Insight Team

https://doi.org/10.62714/NELL1134

Organoid Data Facilitates IND Clearance for PD-1 Knockout T Cell Therapy

January 23, 2024

MedGenCell, a biotech specialized in cell therapy has received approval for its Investigational New Drug (IND) application for a PD-1 gene-edited T cell therapy (IND number: CXSL2300758). The product is an autologous T cell preparation with PD-1 gene knockout, intended for patients aged 18 to 75 with advanced non-small cell lung cancer (NSCLC). Key data in IND package include organoid based drug efficacy data provided by AimingMed,one of the leading organoid companies in China.

MIMETAS Participates in the GRIPonMASH Program for Organ-on-a-Chip Disease Modeling

February 5, 2024

MIMETAS, a global leader in the field of organ-on-a-chip disease modeling, has participated in the GRIPonMASH initiative. The GRIPonMASH program is dedicated to transforming the detection and treatment of metabolic dysfunction-associated liver disease (MAFLD). MIMETAS is responsible for developing and providing organ-on-a-chip-based disease models to improve the prevention and care of metabolic dysfunction-associated fatty liver disease (MASH).

Autologous EpCAM Targeting CAR-T Enter Clinical Trial with Organoid Data

February 23, 2024

Immunofoco announced that its self-developed autologous CAR-T cell product targeting EpCAM (IMC001) has received approval from the China Center for Drug Evaluation (CDE) for clinical trials and has subsequently obtained a clinical research permit from the Food and Drug Administration (FDA). In the development process of this product, the organoid company D1Med provided organoid model for evaluation.

World Organoid Research Day+ 2024: A Gathering of Top Experts

February 27, 2024

The "World Organoid Research Day+ 2024" conference was held at the Hinkston Hall Conference Centre in the UK. The event brought together leading experts in the fields of organoids and organs-on-chips to share their latest research findings and advancements. The conference covered a range of topics including organoid development, disease modeling, and regenerative medicine.

DarkJade Sciences Completed Pre-A Financing Round with Notable Investors

February 27, 2024

DarkJade Sciences, a leading international developer of organoid intelligent equipment and chips, has completed its Pre-A round of financing. This round of funding is mainly used for equipment production, registration filing as well as market expansion, initiating the construction of production bases, and accelerating the promotion of multiple innovative product lines.

Luohua Biotech Secures A-Round Funding for Organ-on-a-Chip Technology Innovation

March 1, 2024

Luohua Biotech (Suzhou) has successfully completed a Series A financing round worth tens of millions of yuan. The funds will be used for the intelligent and standardized development of organoid chip technology, providing one-stop solutions for customers. Additionally, Luohua Biotech aims to develop more biomimetic drug testing models in fields such as oncology, nephrology, hepatology, dermatology, genetic diseases, and rare diseases.

NextGen Basel 1: Exploring 3D Chip Technology for Safety and Efficacy Assessment

March 1, 2024

On March 1st, the "NextGen Basel 1" themed conference was held at the Basel Technology Park in Switzerland. The conference covered topics such as "Safety and Efficacy Assessment of Multi-Organ Chips" and "3D Vascular Chips for Assessing Vascular Toxicity".

Beijing Nuoshan Tech Completes Angel Financing with SEE FUND Investment

March 4, 2024

Beijing Nuoshan Tech has completed a 10 million RMB angel round of financing. This round of financing

was exclusively invested by Infinite Fund SEE FUND, and the funds will be used for preclinical research of the company's fully functional microfluidic organoid chip.

Regenerative Bio and Signet Therapeutics Sign Strategic Collaboration Agreement

March 6, 2024

Regenerative Bio and Signet Therapeutics have officially signed a strategic cooperation agreement. Signet Therapeutics will use its organoid technology platform, particularly focusing on heart and ovarian organoids, to provide research cooperation services for Regenerative Bio's RevOrgan anti-aging intervention ingredient discovery and efficacy evaluation.

STEMCELL Technologies Organized "ORGANOID DAY 2024" at NTU: Exploring Organoid Advances

March 7, 2024

STEMCELL Technologies hosted the "ORGANOID DAY 2024" conference at Nanyang Technological University of Singapore, which brought together top experts, researchers, and industry professionals in the field of organoid research to explore the latest advancements and applications of organoid models.

2024 Organoids Conference & ISoOR 2024 Summit: A Successful Multidisciplinary Event in Shanghai

March 7-8, 2024

The ISoOR 2024 International Organoids Summit were successfully held in Shanghai. The conference was co-hosted by the International Society for Organoids Research (ISoOR), BioonGroup, the Greater Bay Area Institute of Precision Medicine (Guangzhou), and Shanghai Jiao Tong University School of Medicine Songjiang Research Institute. It covered themes such as "Organoids and Materials Science", "Organoids and Drug Development", and "Interdisciplinary Applications of Organoids". It provided a specialized academic platform for organoids researchers and individuals from all walks of society interested in the field of organoids.

Qijia Biotech Opening Ceremony and Strategic Partnerships

March 26, 2024

Qijia Biotech officially opened for business. At the opening ceremony, Qijia Biotech signed strategic cooperation agreements with partners such as EverPro Medical and other partner organizations. Qijia

Biotech is the world's first one-stop supplier of iPS-derived micro-organ products and technical services. With its unique iPS lineage differentiation technology, it has fully expanded the traditional organoid model in terms of lineage, structure, and function, and has revolutionarily brought the next-generation micro-organ (iORGAN) to the market. The company is committed to providing systematic solutions for scientific research, drug development, and precision medicine.

Successful Conclusion of 2024 American Association for Cancer Research (AACR) Annual Meeting

April 5-10, 2024

The 115th AACR was held in San Diego, USA. It attracted over 22,500 experts, including researchers, clinicians, and representatives from biopharmaceutical companies. Participants shared their latest advances in cancer research. Notably, 161 posters related to organoid technology were presented, highlighting the growing recognition and application of organoids in academia, industry, and medical institutions.

Tumor Organoid Drug Sensitivity Assay included in consensus on Refractory Lung Cancer

April 12, 2024

The Chinese expert consensus on refractory lung cancer has been published in the Chinese Journal of Tuberculosis and Respiratory Medicine, the official journal of the Chinese Medical Association (CJTARM). This consensus provides a robust framework for the diagnosis and treatment of refractory lung cancer. Notably, for the first time, it includes patient-derived organoid (PDO) drug sensitivity testing to enhance clinical diagnosis and treatment strategies.

OSC 2024: The 3rd Organoid Conference

April 19-21, 2024

The 3rd OSC 2024 Conference on Organoid Standardization and the Guangdong-Hong Kong-Macao Conference on Medical-Industrial Integration of Organoid and Organ Chip, hosted by the Guangdong Association of Precision Medicine Application, took place at the Tsinghua University Research Institute in Shenzhen, China. The conference focused heavily on the development of organoid and organ chip technology.

Seminar on the Application of 3D Cell Organoids and High-Content Imaging Technology

April 23, 2024

Molecular Devices hosted a seminar on the application of 3D cell organoids and high-content imaging technology. The seminar explored the progress in 3D cell and organoid research and the application of high-content imaging technology in these fields.

The ODC Committee Hosted an Organoid Industry Conference

April 24, 2024

The ODC Committee hosted an organoid industry conference themed "The Next Normal: Living With Organoids". The event was held simultaneously in Vietnam and South Korea and aimed to expand the scope and significance of organoid research.

Scipolis
May the Science be with you
OPEN ACCESS

JOBs
Journal of Organoid & Bioscience

News & Views

Innovating the Future: ISoOR 2024 Annual Meeting Achieves Remarkable Success

Martin Maldonado[1]

[1]ISoOR Secretary

https://doi.org/10.62714/DKWI1160

From March 7 to 8, 2024, the International Society of Organoid Research (ISoOR) convened its 2024 annual meeting, after the successful conference in Singapore last year. This year, the conference was held in the vibrant city of Shanghai, China, reflecting the rapid expansion of organoid industry within the country. The pivotal event was hosted by ISoOR in collaboration with Bioon and two more institutes.

Group photo of speakers

Professor Peter E. Lobie, the chairman of ISoOR, inaugurated the conference with a warm welcome to experts, scholars, and guests around the world.

As keynote speaker invited by ISoOR, Prof. Hans Clevers, pioneer of organoid field, member of the National Academy of Sciences (USA) and head of Roche Pharma Research and Early Development, delighted the audience with a talk about Organoids to Model Human Disease, followed by more than sixty scientific experts from around the world, to exchange insights in various sessions.

Moreover, Dr. Maldonado ISoOR secretary for China region spotlighted several of ISoOR's key initiatives from the previous year, such as 2023 annual meeting in Singapore, an exciting symposium for debating the latest landmarks in organoid research, and the start-point for initiation of the ISoOR Standards for Organoid Biobanks.

Prof. Peter E. Lobie (left), Prof. Hans Clevers (middle), Dr. Maldonado (right)

The ISoOR booth at the conference provided attendees access to ISoOR official publication, *the Journal of Organoid and Bioscience* (JOBs). This open-access, peer-reviewed platform encompasses a wide array of topics, including, but not limited to the Establishment of Organoid Models, Organoids in Precision Medicine, Drug Development, Regenerative Medicine, Organoid Biobanks, Policy Analysis, Current Trends in Organoid Research and Applications, and the Organoid Industry Landscape. Additionally, the latest issue of JOBs featured a white paper offering an in-depth review of organoid and organ-on-a-chip technologies, exemplifying ISoOR's commitment to empowering the organoid

community, providing researchers, industry professionals, and investors with essential knowledge for navigating the field.

ISoOR2024

The pivotal conference, which surpassed a thousand attendants, drew to a triumphant close on March 8th with organoid technology revealing vast potential in areas like disease modeling, drug development, and precision medicine, the prospects for future market growth are bright indeed. As ISoOR welcomes more members, it is poised to undertake even greater efforts to foster the advancement of organoid research.

Scipolis
May the Science be with you
OPEN ACCESS

JOBs
Journal of Organoid & Bioscience

Perspective

Will Black Box Warning Affect Development of CAR-T Therapy

Yijun Chen[1]

[1]Macera Therapeutics

https://doi.org/10.62714/UQLM1904

Cell therapy has emerged as an approved treatment and has saved many patients. As a living therapy, it serves as a role model for advancements in biology, especially in combating cancer to some extent. Despite the progress in cell therapy, one of the biggest issues on the regulatory side is that the FDA now requires a black box warning for the six cell therapies currently on the market. This announcement was made on January 22nd 2024, mandating producers to remind users about the risk of developing lymphoma. To date, all companies have put the black box warning in their product sheet.

After the announcement, there was a rapid change in the stock market for related companies. However, based on our analysis, there is no need to overreact to such regulatory changes.

1. Many popular drugs have black box warning

Many approved drugs, including some blockbusters, have black box warnings. Examples include JAK inhibitors, GLP-1 inhibitors, and TNF-alpha antibodies. Having a black box warning does not necessarily mean the drug's sales will decline. A good example is Humira, which had a black box warning but still reached annual sales of $20 billion, remaining a top seller for many years.

Another example is the ADC drug DS8201, which gained significant attention for its effectiveness against breast cancer ,especially for Her2 low expression patient. This drug had sales over $1 billion in 2023, despite having a black box warning on interstitial lung disease and embryo-fetal toxicity.

GLP-1 drugs, known for their significant effects on weight reduction, are also very popular now. The market for GLP-1 drugs are over $20 billion, while They also carry a black box warning for thyroid cancer, based on rodent studies.

2. Black box warning as a risk notification

In U.S. law, risk disclosure is crucial. Therefore, it is the duty of the FDA and biopharma companies to list all related risks; otherwise, they may face lawsuits and substantial compensation claims. Hence, the black box warning is a way to protect both the FDA and biopharma companies. Clinicians are familiar with this practice and will not overreact.

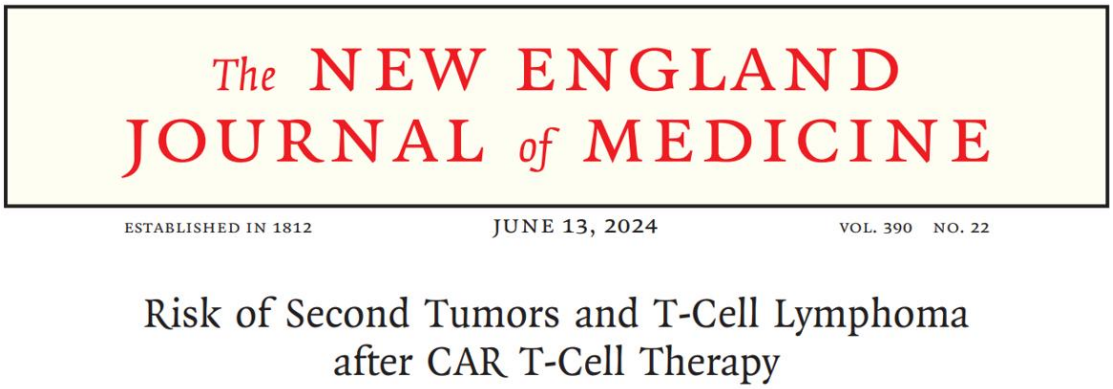
3. How black box warnings affect cell therapy

For any drug or therapy, the most important factor is the benefit/risk ratio. Currently, cell therapy has saved many terminal patients who had no other treatment options available. Cell therapy provides significant survival benefits, so the black box warning may not have a substantial impact.

On the other hand, secondary lymphoma is common among cancer patients, and there is no clear evidence to establish a direct correlation between cell therapy and lymphoma. However, as cell therapy seeks new indications, such as for autoimmune diseases, patients with non-life-threatening conditions may have more concerns about lymphoma risk.

4. Frequency of T-cell lymphoma

Researchers analyzed the outcomes for 724 people treated with CAR-T cell therapy at Stanford Health Care between 2016 and 2024. Among these individuals, the incidence of secondary blood cancers was approximately 6.5% over a median of three years of follow-up, which is roughly similar to patients who underwent stem cell transplantation rather than CAR-T cell therapy to treat their cancers [1].


The NEW ENGLAND JOURNAL of MEDICINE

ESTABLISHED IN 1812 JUNE 13, 2024 VOL. 390 NO. 22

Risk of Second Tumors and T-Cell Lymphoma after CAR T-Cell Therapy

Another research group, including Prof. Carl June, analyzed 1,500 cases and observed no instance where integrated CAR was found in T-cell lymphoma [2].

nature medicine

Brief Communication https://doi.org/10.1038/s41591-024-02826-w

T cell lymphoma and secondary primary malignancy risk after commercial CAR T cell therapy

5. Impact on CAR-T production

The concern about T-cell lymphoma arises from the use of viruses for CAR expression, which is associated with the risk of genomic integration. For instance, lentiviral vectors, despite integrating in a semi-random fashion, have an affinity for areas of the genome where active gene expression is taking place, posing a risk for insertional oncogenesis. In three cases of lymphoma, the CAR transgene has been detected in the malignant clone, indicating that the product was likely involved in the development of T-cell cancer [3].

New strategies involving targeted insertion of the CAR construct to specific loci might help reduce the risk of cancer, as well as the use of mRNA-based approaches for transient expression.

References

1. Verdun N, Marks P. Secondary Cancers after Chimeric Antigen Receptor T-Cell Therapy. N Engl J Med. 2024 Feb 15;390(7):584-586. doi: 10.1056/NEJMp2400209. Epub 2024 Jan 24. PMID: 38265704.
2. Ghilardi G, Fraietta JA, Gerson JN, Van Deerlin VM, Morrissette JJD, Caponetti GC, Paruzzo L, Harris JC, Chong EA, Susanibar Adaniya SP, Svoboda J, Nasta SD, Ugwuanyi OH, Landsburg DJ, Fardella E, Waxman AJ, Chong ER, Patel V, Pajarillo R, Kulikovskaya I, Lieberman DB, Cohen AD, Levine BL, Stadtmauer EA, Frey NV, Vogl DT, Hexner EO, Barta SK, Porter DL, Garfall AL, Schuster SJ, June CH, Ruella M. T cell lymphoma and secondary primary malignancy risk after commercial CAR T cell therapy. Nat Med. 2024 Apr;30(4):984-989. doi: 10.1038/s41591-024-02826-w. Epub 2024 Jan 24. PMID: 38266761.
3. Verdun N, Marks P. Secondary Cancers after Chimeric Antigen Receptor T-Cell Therapy. N Engl J Med. 2024 Feb 15;390(7):584-586. doi: 10.1056/NEJMp2400209. Epub 2024 Jan 24. PMID: 38265704.

Scipolis
May the Science be with you
OPEN ACCESS

JOBs
Journal of Organoid & Bioscience

Factsheet

Assessing the Efficacy and Feasibility of Conditioned Medium as a Culture Reagent for Tumor Organoid Culture: A Comprehensive Evaluation

Lingyu Hu[1], Suyao Xie[2], Mingliang You[2,3], Xiaohong Zhu[5], Lihong Bian[5], Huayang Xing[2]*, Bin Zhang[4,5]*

[1] College of Biotechnology and Bioengineering, Zhejiang University of Technology, Hangzhou, 310014, China

[2] Department of Research and Development, Hangzhou AimingMed Technologies, Hangzhou, 310000, China

[3] Hangzhou Cancer Institute, Key Laboratory of Clinical Cancer Pharmacology and Toxicology Research of Zhejiang Province, Affiliated Hangzhou Cancer Hospital, Zhejiang University School of Medicine, Hangzhou, 310002, China

[4] Department of Obstetric & Gynecology , the First Affiliated Hospital , Fujian Medical University , Fuzhou 350005, China

[5] The First Clinical College of Fujian Medical University, Fuzhou 350004, Fujian Province, China

*Correspondence: arielreal@163.com (Huayang Xing), zhangbin89@fjmu.edu.cn (Bin Zhang)

https://doi.org/10.62714/KKDL3357

Abstract

Tumor organoids are three-dimensional models of tumor cells grown in vitro which derived from primary tumors of patients. Tumor organoids highly mimic the characteristics of the primary tumors, retain tumor heterogeneity, and can be used for functional testing in vitro, including high-throughput drug screening and personalized therapeutic strategies. However, the establishment of tumor organoids facing such dilemma as low success rate and growth rate. The use of appropriate and effective culture reagent is pivotal to the success of tumor organoids culture. Nowadays, the most commonly and long-lasting used media in cell culture is conditioned medium, while its application in organoids culture has been controversial due to undefined components. In response, our study conducts a comprehensive analysis, offering compelling evidence supporting the efficacy of conditioned medium in promoting organoids

growth and expansion. This research aims to address uncertainties surrounding its use and contribute valuable insights to enhance tumor organoids culture practices.

1. Introduction

Tumor organoids are three-dimensional cell cultures derived from patient tumor tissues, which reconstitute the phenotype and molecular heterogeneity of the original tumor [1]. Previous publications have proved its clinical relevance on the cancer biology, drug responses, and personalized medicine of tumor organoids, including pancreatic, breast, lung, and gastrointestinal cancers [2-5]. The efficiency of tumor organoids establishment significantly impacts clinical applicability. Improving the success and growth rate of tumor organoids modeling is pivotal. Optimization of protocols to expedite this process is essential, ensuring timely and robust generation of tumor organoids for effective translational applications [6].

Despite the effects of clinical disassociated tumor tissues' situation, the choice of an appropriate culture medium remains crucial for the success of the tumor organoids culture. Currently, the prevailing organoids culture methods involve the use of both chemical-defined medium and conditioned medium [7]. Chemical-defined medium has precisely defined composition, providing a controlled environment for cell growth of known constituents [8, 9]. In contrast, conditioned medium is produced from factors secreted by cells during culture, introducing a mixture of complex and undefined molecules [10, 11]. Due to its regulatory compliance, definite composition and batch consistency, chemical-defined medium is preferred for drug development, clinical diagnosis, and therapy [12, 13]. However, in the process of organoids culture, some studies have indicated that conditioned medium often exhibits greater robustness and effectiveness than chemical-defined medium, with a comparatively lower cost [14,15]. For the efficient and timely translational application of tumor organoids, enhancing the robustness and productivity of the culture system becomes particularly crucial.

To evaluate the feasibility and robustness of applying conditioned medium in tumor organoids culture, we performed a comprehensive characterization with primary tumors and corresponding tumor organoids, including gastric cancer (GA), colorectal cancer (CR), cervical cancer (CC) and lung cancer (LU). Fig 1 briefly describes the experimental design of this study. We performed a comparative analysis on the organoids characterization with hematoxylin and eosin (H&E), immunohistochemical (IHC) staining and whole-exome sequencing (WES). Besides, we compared the growth situation, cell quantity and cell viability between tumor organoids cultured in conditioned medium and chemical-defined medium.

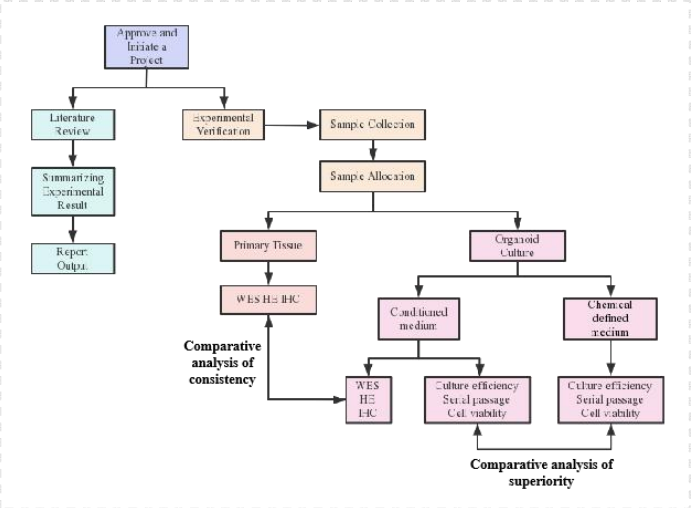


Figure 1. Experimental design of this study. We first conduct literature research to confirm the research objectives. After obtaining the cancer samples for the experiment, we separated cancer tissues for primary tissues and tumor organoids analysis, including consistency analysis and comparative analysis. WES, whole-genome sequencing; HE, hematoxylin-eosin staining; IHC, immunohistochemistry.

2. Materials and methods

2.1 Collection of patient samples

The collection of tumor tissues for the organoids generation was performed at The First Affiliated Hospital of Fujian Medical University according to the guidelines of Research Ethics Committee (Ethics project number: MRCTA, ECFAH of FMU(2021)435), following both national and local laws. Written informed consent was also obtained from all patients. The main inclusion criteria were patients with clinically locally advanced or metastatic cancer, aged 18 years or older, with fresh tissues available through either biopsy or surgical resection of the primary or metastatic lesions, and with malignant effusion samples collected using a sterile drainage bag. Samples from biopsy or surgical resection were kept in MasterAim Tissue Preservation Medium (AimingMed Hangzhou) and shipped to the laboratory at 4 degrees within 48 hours.

2.2 Preparation of organoid culture media

The chemical-defined medium used for GA, CR and CC organoids culture were configured as described in the studies, respectively [16-18]. The conditioned medium groups use the same formulation but replacing WNT-3a recombinant protein with 10% MasterAim® WNT3A Conditioned Medium, R-spondin-1 (RSPO1) recombinant protein with 25% MasterAim®R-spondin Conditioned Medium. Furthermore, an equivalent amount of FBS was incorporated into the chemically defined medium, designated as the FBS control group in the experiments. The commercial conditioned medium was

purchased from Aimingmed (Hangzhou, China).

2.3 Patient sample processing and organoid culture

Tissue samples were washed 10 times with cold 1X DPBS (Vivacell, Shanghai), then cut into 1-3 mm pieces and digested into a cell suspension using MasterAim Tissue Dissociation Medium (AimingMed, Hangzhou). The suspension was filtered through a 100-μm strainer (Corning, USA), centrifuged at 300 g for 5 minutes, and the pellet was resuspended in Matrigel (Corning, USA) for seeding in a 24-well dish (Corning, USA). After solidifying for 25 minutes at 37 ℃ in a humidified incubator, organoid culture medium was added to the wells.

For passaging, organoids are digested with TrypLE Express Enzyme (Gibco, USA) into small cell clusters (20-50 μm), and digestion is stopped with double the volume of DPBS. After centrifuged at 300 g for 5 minutes, cells are resuspended in 1 mL DPBS, mixed with 20 μL AO/PI (Alit Biotech, Shanghai), and counted using an automatic cell counter (Alit Biotech, Shanghai) for total cells and viability. Cells are then centrifuged, resuspended in Matrigel, and seeded into a 24-well dish as described above.

2.4 Hematoxylin-eosin (H&E) and Immunohistochemical (IHC) staining

Organoids were fixed overnight at 4 ℃ with 4% paraformaldehyde, embedded in paraffin, and sectioned into 4-μm slices. These sections were stained with hematoxylin for 8 minutes and eosin for 10 seconds, then dehydrated, dried, and sealed for analysis. For immunohistochemistry (IHC), sections underwent antigen retrieval by boiling in EDTA (pH 8.0) for 8 minutes, blocked with 3% hydrogen peroxide and 5% BSA, and incubated with primary antibodies including Ki67 (Abcam, USA), p53 (HUABIO, Hangzhou), p40 (CST, USA), p16 (HUABIO, Hangzhou) and CDX2 (HUABIO, Hangzhou) overnight at 4 ℃. This was followed by HRP-conjugated secondary antibodies and DAB visualization, with final hematoxylin staining for 3 minutes and sequential alcohol dehydration before sealing with neutral resin for imaging.

2.5 Whole-exome sequencing library construction and sequencing

WES was performed in Novogene Bioinformatics Technology Co., Ltd (Beijing, China). First, DNA was extracted from tumor tissue samples and tumor-derived organoids then fragmented by the Covaris S220 (Covaris, USA) to an average size of 180-280 bp. Next, the DNA fragment was end repaired and phosphorylated followed by A-tail and paired adaptor ligation at the 3' end. Selectively enriched the DNA fragments which containing adapter molecules ligated on both ends by PCR then capture the exons of these DNA. Subsequently, index tags were added to the captured libraries by PCR reaction for sequencing preparation. At last, the products were purified and qualified then sequenced on Illumina platforms according to the experimental requirements.

2.6 Sequence data quality control and analysis

To ensure the downstream bioinformatics analyses were quality and reliability, the following criteria were applied to control the quality of data:

1. Paired reads were excluded if contained adapter contamination.

2. Paired reads were excluded if over 10% of bases were uncertain in either read.

3. Paired reads were excluded if one read contained more than 50% low-quality bases (Phred quality < 5).

For sequence data analysis, BWA-MEM was used to map the clean sequence reads to the human genome (hg38). Picard and GATK were applied to perform deduplication and base quality recalibration respectively. The variants were identified with muTect2 and annotated with Annovar. The R package "maftools" was employed for visualizing SNVs, while the mutation spectrum was depicted utilizing the R package "Mutational Patterns."

3. Results

3.1 Conditioned medium increases establishment efficiency across various tumor organoid types

To assess the impact of conditioned medium on tumor organoids establishment, a comparison experiment was conducted involving 9 tumor organoids derived from gastric (GA), colorectal (CR), and cervical (CC) cancers. This was designed to exclude the influence of tumor and individual heterogeneity in the evaluation. Additionally, to exclude the effects of FBS present in the conditioned medium, an equivalent amount of FBS was incorporated into a chemically defined medium, designated as the FBS control group in the findings.

Fig 2 illustrates that conditioned medium enhances both the formation and quantity of organoids across all 9 tumor organoids, a finding corroborated by cell count data (Table 1). Notably, while FBS control groups showed improved performance in the GA1 and CR5 organoids compared to the chemically defined group, they were still outperformed by the conditioned medium. This suggests that while FBS may offer benefits in the establishment of certain organoids, it does not universally apply to all organoid types. Cell counting and viability data, as shown in Table 1, reveal that conditioned media predominantly enhance cell growth (8 out of 9 cases) rather than cell viability (3 out of 9 cases). To minimize experimental systematic errors, we considered only values exceeding 120% of the comparison group as significantly better. Overall, the findings indicate that conditioned media facilitate organoids formation and boost growth efficiency, potentially reducing the duration required for organoids drug sensitivity assays.

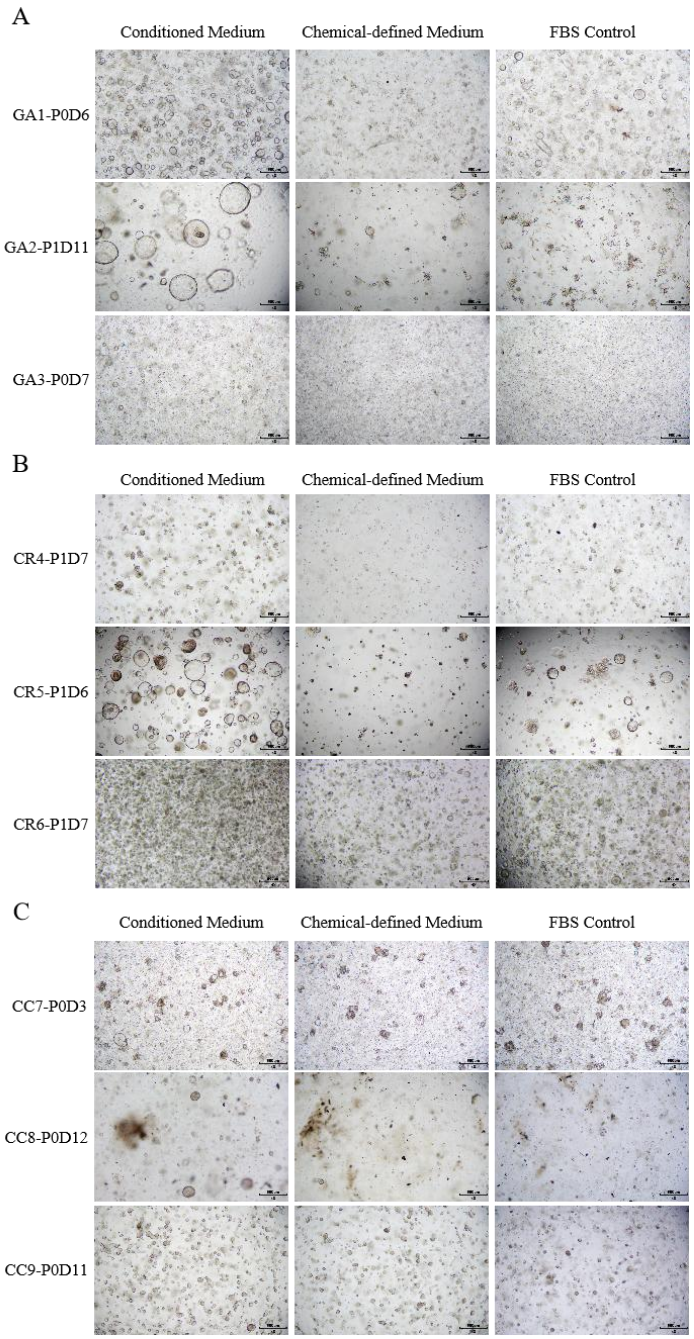


Figure 2. Growth comparison of various organoid types cultured in medium supplemented with conditioned medium and chemical-defined medium. Tumor organoids derived from (A) gastric cancer (GA) tissue of patient No.1, 2, 3, B) colorectal cancer (CR) tissue of patient No.4, 5, 6 and (C) cervical cancer (CC) tissue of patient No.7, 8, 9. Passage number and culture duration of the corresponding organoids are indicated on the graphs. Scale bar = 500 μm.

Cancer type	Patient ID	Passage number	Cell count (10^{-4})			Cell viability (%)		
			Conditioned Medium	Chemical-Defined Medium	FBS Control	Conditioned Medium	Chemical-Defined Medium	FBS Control
GA	1	P0-D6	16.4	7.0	12.0	82	90	88
	2	P1-D11	9.2	3.4	3.4	69	70	70
CR	3	P0-D7	2.7	0.3	1.0	62	4	47
	4	P1-D7	7.3	2.6	4.8	85	72	94
	5	P1-D6	10.3	2.0	1.7	39	45	42
	6	P1-D7	55.8	3.0	19.2	67	30	43
CC	7	P0-D3	7.7	5.9	5.7	73	62	81
	8	P0-D12	20.4	7.1	11.9	78	53	65
	9	P0-D11	4.4	4	3.4	63.3	58.9	68.5
Rate			88.9% (8/9)	0% (0/9)	0% (0/9)	33.3% (3/9)	11.1% (1/9)	22.2% (2/9)

Table 1. Comparison of organoids growth index with conditioned medium and chemical-defined medium. Cell quantity and viability during the passaging process were compared between the two groups. Values exceeding 120% of the comparison group are considered superior and are denoted in green.

3.2 Pathological analysis of tumor organoids cultured with conditioned medium

Histopathological comparison of organoids with their source tissues has been used as a benchmark for organoid characterization. H&E staining and IHC were conducted to compare their histopathology to that of the original tissue. IHC markers specific to the diagnosed cancer subtypes were chosen for this analysis. Both H&E staining and IHC confirmed that the tumor organoids preserved the histopathological features of the original tumors (Fig 3). There are atypical cells and deep nuclear staining present in colorectal cancer tissue, consistent with the characteristics of carcinogenesis, and these features are also present in corresponding organoids (Fig 3B). Additionally, there are obvious acinar structures in cervical cancer tissue, consistent with the carcinogenic features of cervical cancer. Similarly, acinus is observed in the corresponding CC organoids (Fig 3C).

Additionally, the diagnostic tumor markers were expressed at a high concordance rate in original tumors and corresponding organoids. In detail, GA organoids were marked by the expression of typical markers such as Ki67 and p53 (Fig 3A), while CR organoids exhibited strong Ki67 and CDX2 expression, consistently with the original tissue (Fig 3B). Similarly, cervical cancer markers p40 and p16 were identified in both the primary tissues and corresponding CC organoids (Fig 3C). Collectively, these findings indicate that organoids cultured in conditioned medium successfully preserve the pathological profiles of the original tumors.

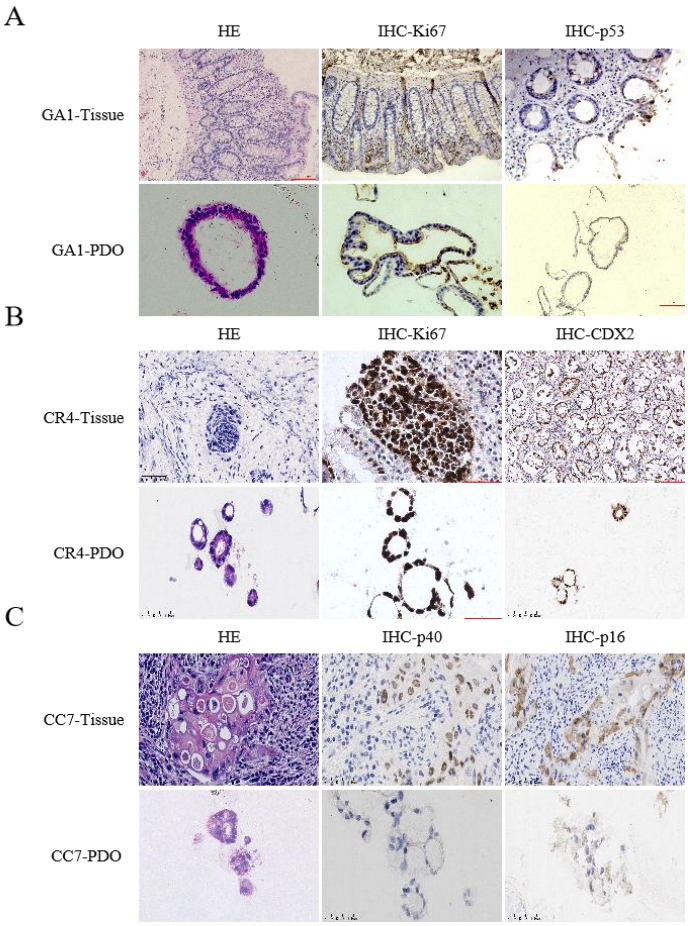


Figure 3. H&E and IHC staining of organoids cultured with conditioned medium from different patient tumor tissues. Shown are representative examples of (A) gastric cancer, (B) colorectal cancer and (C) cervical cancer. Tissues generally present tumor epithelium, while organoids are exclusively epithelial with tumor cell organization being remarkably well conserved (HE). Ki67, p53, CDX2, p40 and p16 status of original tissues are similarly well retained in the derived organoids lines. Scale bar equals 50 μm or 100 μm, and the specific bar is marked in the picture.

3.3 Genomic Characterization of primary tissues and the corresponding organoids were consistent in conditioned medium

Lastly, to further explore the genomic characteristics and understanding the genomic landscape and different genomic segments, such as single-nucleotide variants (SNVs) and mutation types of the primary tissues and the paired organoids, we performed whole-exome sequencing (WES) on several tumors and corresponding organoids. From the view of point mutation types, it is not surprisingly that the total mutational load was similar between primary tissues and corresponding organoids (Fig 4B). Furthermore, most of the SNVs in primary tissues were preserved in the paired organoids, including

mutations in TP53, KRAS, FGFR2 and KMT2C. In detail, TP53 missense mutation was detected in primary tumor tissues of gastric cancer and colorectal cancer but not in cervical cancer tissues. Similarly, TP53 missense mutation was presented in GA and CR organoids while not in CC organoids. Additionally, various gene mutation type of cancer tissue were preserved in paired organoids, including nonsense mutation of APC and splice site of WDR1 (Fig 4A).

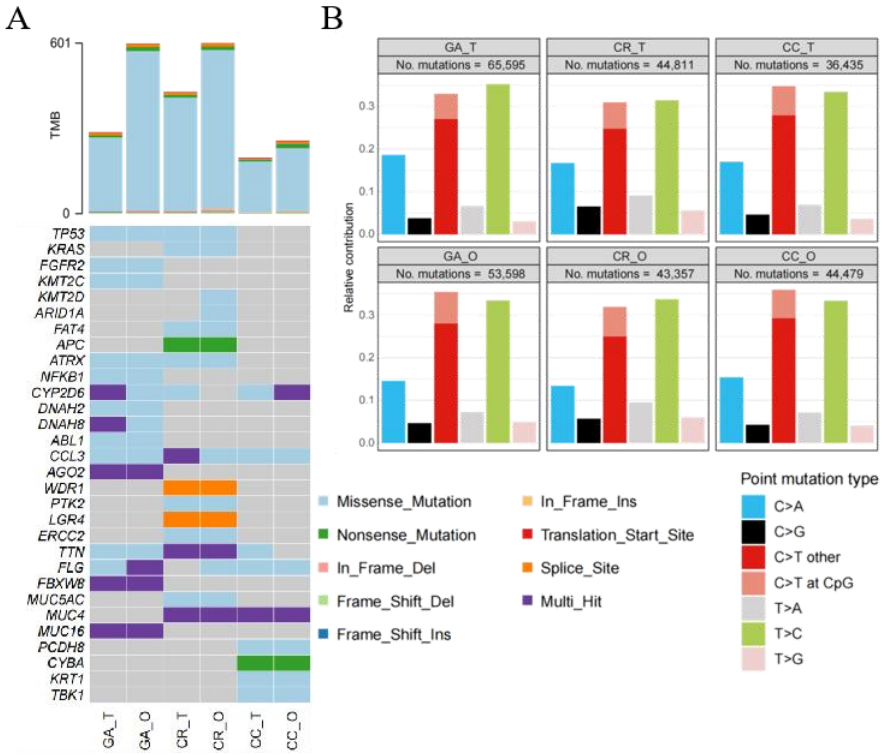


Figure 4. Oncoplot and point mutation spectrum of PDOs and tumor biopsies. A) Mutation landscape of single-nucleotide variants (SNVs) in PDOs and paired primary tumors. Top, total mutation number of each sample. Bottom, heatmap of commonly mutated genes in gastric cancer, colorectal cancer, and cervical cancer. B) The contributions of point mutation types in PDOs and paired primary tumors. The total number of point mutations per sample is indicated.

4. Discussion

Chemical-defined medium has known composition with no batch effects, which has advantages in terms of experimental reproducibility. While the content of CM various from cell type to culture condition and batch, which is too difficult to guarantee equivalent effects in each clinical study. Therefore, for new drug clinical studying and passing regulatory review, the use of chemical-defined medium is indispensable [19]. In addition, conditioned medium was generated from various cell types, most culture systems applied for cell culture contain animal products, which has raised concerns about potential viral and

mycoplasma infections, while chemical-defined medium minimize the risk of contamination from animal pathogens [20, 21]. However, conditioned medium also have many unique advantages. Firstly, chemical-defined medium has to supplement with various expensive growth factors, while the cost of conditioned medium is lower [22]. Secondly, some studies have shown that conditioned medium existed specific paracrine factors that could better stimulate endogenous repair, promote cell differentiation, and reduce cell apoptosis [23], and chemical-defined medium may not contain this kind of factors. Lastly, as described in previous studies, in chemical-defined medium, the activity of added factors could be inactivation within a few hours. Tuysuz *et al* found that Wnt3a conditioned medium was more stable than purified Wnt3a [15]. The purfied Wnt3a protein had poor performance in serum-free media due to its lipidated and hydrophobic characteristics, and the presence of CHAPS in purified Wnt3a inhibits the self-renewal of stem cells [24, 25]. Thus, in this study, we first tested the activity of WNT3A protein in MasterAim®WNT3A-Conditioned medium and in chemical-defined medium, the results confirmed that WNT3A protein in conditioned medium has better stability and higher protein activity than recombinant WNT3A protein, which is more suitable for long-term culture of organoids (Supplementary Fig 1).

In detail, WNTs and R-spondins are growth factors participate in wnt/β-catenin signaling pathway which giving shape to growing tissues while stimulating the growth and proliferation of cells, especially crucial for the activity of WNT-responsive epithelial stem cells in multiple tissues [26, 27]. In this study, we observed superior growth situation of GA, CR and CC organoids in medium supplemented with conditioned medium than chemical-defined medium (Fig 2). Over 80% of the organoids cultured with conditioned medium exhibited higher cell counts compared to those cultured with chemical-defined medium, and this promotion capability was not limited in a specific type of tumor organoids but in several including GA, CR and CC tumor organoids (Table.1). On account of tumor organoids has the ability to perform drug sensitivity testing for patients within a short time, the importance of tumor organoids modeling efficiency and success rate was obvious [28]. Thus, it is no doubt that conditioned medium has better performance and advantages to construct organoids efficiently in laboratory research then transform to clinical application timely.

To precisely transform to clinical drug sensitivity testing for patients, organoids should preserve consistency with the original tissue. Previous study has shown that in chemical-defined medium, CR tumor organoids are likely to be completely replaced by normal epithelial cells of primary tissue, resulting in a gradual loss of tumor characteristics, while conditioned medium outperformed chemical-defined medium in maintenance of genomic, epigenomic, and transcriptomic features of tumor organoids [29, 30]. In this study, cancerous features and molecular markers were also presented at a similar pattern in both tumor tissues and tumor organoids (Fig 3), and tumor organoids can maintain tumor genomic

characteristics (Fig 4). However, we observed more mutations in tumor organoids than primary cancer tissues, suggesting that original tumors could present low frequency mutations because tumor tissues contains a portion of non-tumor cells, while tumor organoids enriched tumor cells (Fig 4A). Nevertheless, the long-term deficiency of tumor environment in vitro may lead to differences between expression profiles of tumor-organoid pairs [31], whether conditioned medium can maintain genetic stability remains to be further research.

Due to the batch effects, conditioned medium has disadvantage in experimental reproducibility, for clinical diagnostic and therapeutic application, replacement of conditioned medium by chemical-defined medium would therefore be general trend. While the conditioned medium still has its own irreplaceable advantages in some aspects. Conditioned medium has the key proteins required for the culture of organoids and maintain stability of key factors, which construct organoids efficiently and transform to drug sensitivity testing application timely. However, there still are concerned about the potential variation and contamination risks to the cells due to the presence of serum in the conditioned medium [9]. To that concern, we could consider applied hPL as serum replacement in future, to minimize the potential risks of the serum. In summary, we have conducted sufficient characterization, providing a reference basis for the use of conditioned medium in organoids construction, laboratory research and other aspects.

Author contributions

Conceptualization – H.Y.X; draft preparation – L.Y.H, S.Y.X and H.Y.X; review and editing - H.Y.X, M.L.Y and B.Z.; supervision - H.Y.X; project administration - L.Y.H. and S.Y.X; funding acquisition, B.Z. All authors have read and agreed to the published version of the manuscript.

Acknowledgments

We thank all patients who participated in this study as well as their families. This study is supported by Fujian Province Finance Project (BPB-2023ZB) and Natural Science Foundation of Fujian Province (No. 2022J01702).

References

1. Clevers, H. Modeling Development and Disease with Organoids. Cell. 2016 Jun 16;165(7):1586-1597.
2. Wang H., Zhang C., Peng K., *et al*. Using patient-derived organoids to predict locally advanced or metastatic lung cancer tumor response: A real-world study. Cell Rep Med. 2023 Feb 21;4(2):100911.
3. Grünwald B., Devisme A., Andrieux G., *et al*. Spatially confined sub-tumor microenvironments in pancreatic cancer. Cell. 2021 Oct 28;184(22):5577-5592.e18.
4. Guillen K., Fujita M., Butterfield A., *et al*. A human breast cancer-derived xenograft and organoid platform for drug discovery and precision oncology. Nat Cancer. 2022 Feb;3(2):232-250.
5. Lau H., Kranenburg O., Xiao H., *et al*. Organoid models of gastrointestinal cancers in basic and translational research. Nat Rev Gastroenterol Hepatol. 2020 Apr;17(4):203-222.
6. Veninga V., Voest E. Tumor organoids: Opportunities and challenges to guide precision medicine. Cancer Cell. 2021 Sep 13;39(9):1190-1201.
7. Xu H., Jiao d., Liu A., *et al*. Tumor organoids: applications in cancer modeling and potentials in precision medicine. J Hematol Oncol. 2022 May 12;15(1):58
8. Xu J., Lian W., Chen J., *et al*. Chemical-defined medium supporting the expansion of human mesenchymal stem cells. Stem Cell Res Ther. 2020 Mar 19;11(1):125.
9. Liu Y., Song Z., Zhao Y., *et al*. A novel chemical-defined medium with bFGF and N2B27 supplements supports undifferentiated growth in human embryonic stem cells. Biochem Biophys Res Commun. 2006 Jul 21;346(1):131-9.
10. Pleguezuelos-Manzano C., Puschhof J., van den Brink S., *et al*. Establishment and Culture of Human Intestinal Organoids Derived from Adult Stem Cells. Curr Protoc Immunol. 2020 Sep;130(1):e106.
11. Lin H., Chen H., Zhao X., *et al*. Advances in mesenchymal stem cell conditioned medium-mediated periodontal tissue regeneration. J Transl Med. 2021 Nov 4;19(1):456.
12. Pei F., Jiang J., Bai S., *et al*. Chemical-defined and albumin-free generation of human atrial and ventricular myocytes from human pluripotent stem cells. Stem Cell Res. 2017 Mar:19:94-103.
13. Wu B., Yang Z., Yijie Liu., *et al*. A chemically defined system supports two distinct types of stem cell from a single blastocyst and their self-assembly to generate blastoid. Cell Prolif. 2023 Jun;56(6):e13396.
14. Dossena M., Piras R., Cherubin A., *et al*. Standardized GMP-compliant scalable production of

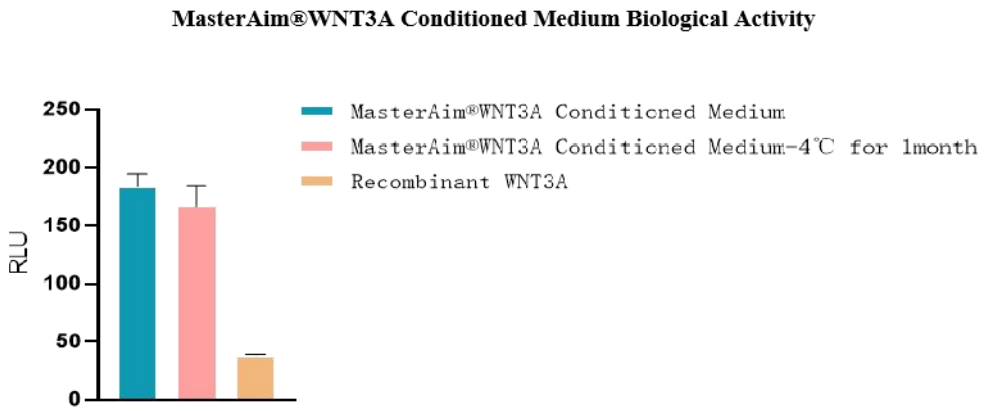
human pancreas organoids. Stem Cell Res Ther. 2020 Mar 4;11(1):94.

15. Tuysuz N., van Bloois L., van den Brink S., *et al*. Lipid-mediated Wnt protein stabilization enables serum-free culture of human organ stem cells. Nat Commun. 2017 Mar 6:8:14578.
16. Seidlitz T., Merker S., Rothe A., *et al*. Human gastric cancer modelling using organoids. Gut. 2019 Feb;68(2):207-217..
17. Mo S., Tang P., Luo W., *et al*. Patient-Derived Organoids from Colorectal Cancer with Paired Liver Metastasis Reveal Tumor Heterogeneity and Predict Response to Chemotherapy. Adv Sci (Weinh). 2022 Nov;9(31):e2204097.
18. Maru Y., Tanaka N., Ebisawa K., *et al*. Establishment and characterization of patient-derived organoids from a young patient with cervical clear cell carcinoma. Cancer Sci. 2019 Sep;110(9):2992-3005.
19. Yang R and Yu Y. Patient-derived organoids in translational oncology and drug screening. Cancer Lett. 2023 May 28:562:216180.
20. Sachs N., de Ligt J., Kopper O., *et al*. A Living Biobank of Breast Cancer Organoids Captures Disease Heterogeneity. Cell. 2018 Jan 11;172(1-2):373-386.e10.
21. Stacey G., Cobo F., Nieto A., *et al*. The development of 'feeder' cells for the preparation of clinical grade hES cell lines: challenges and solutions. J Biotechnol. 2006 Oct 1;125(4):583-8.
22. Chang Y., Wu K, Harnod T., *et al*. Comparison of the Cost and Effect of Combined Conditioned Medium and Conventional Medium for Fallopian Tube Organoid Cultures. Cell Transplant. 2023 Jan-Dec:32:9636897231160216.
23. Alidoust L., Akhoondian M., Atefi A., *et al*. Stem cell-conditioned medium is a promising treatment for Alzheimer's disease. Behav Brain Res. 2023 Aug 24:452:114543.
24. Mihara E., Hirai H., Yamamoto H., *et al*. Active and water-soluble form of lipidated Wnt protein is maintained by a serum glycoprotein afamin/α-albumin. Elife. 2016 Feb 23:5:e11621.
25. Shin W., Wu A., Min S., *et al*. Spatiotemporal Gradient and Instability of Wnt Induce Heterogeneous Growth and Differentiation of Human Intestinal Organoids. iScience. 2020 Aug 21;23(8):101372.
26. Nusse R., Clevers, H. Wnt/b-Catenin Signaling, Disease, and Emerging Therapeutic Modalities. Cell. 2017 Jun 1;169(6):985-999.
27. Xu S., Chan R., Li T., *et al*. Understanding the regulatory mechanisms of endometrial cells on activities of endometrial mesenchymal stem-like cells during menstruation. Stem Cell Res Ther. 2020 Jun 17;11(1):239.
28. Yao T and Asayama Y. Animal-cell culture media: History, characteristics, and current issues.

Reprod Med Biol. 2017 Mar 21;16(2):99-117.

29. Kopper O., Witte C., Lõhmussaar K., *et al*. An organoid platform for ovarian cancer captures intra- and interpatient heterogeneity. Nat Med. 2019 May;25(5):838-849.
30. Wang R., Mao Y., Wang W., *et al*. Systematic evaluation of colorectal cancer organoid system by single cell rna seq analysis. Genome Biol. 2022 Apr 28;23(1):106.
31. Soysal S., Tzankov A., Muenst S. Role of the Tumor Microenvironment in Breast Cancer. Pathobiology. 2015 Sep;82(3-4):142-52.

Supplementary Material



Supplementary Figure 1. WNT3A conditioned medium protein titer evaluation. WNT3A conditioned medium protein titer evaluation. The luciferase reporter activities in 293-T was detected with a TOP/FOP-Flash reporter assay. 293T cells were supplemented with the MasterAim®WNT3A-Conditioned medium, the MasterAim®WNT3A-Conditioned medium which stored at 4 ℃ for a month or recombinant WNT3A. All data are presented as mean ± SD (n=3).

Scipolis
May the Science be with you
OPEN ACCESS

JOBs
Journal of Organoid & Bioscience

Protocol

Analysis of the Killing Activity of Immune Cells with Tumor Organoids

Yi Zhou[1]*

[1]Hangzhou AimingMed Technology

*Correspondence: zhouyi@aimingmed.com

https://doi.org/10.62714/MLNP5184

The tumor microenvironment (TME) is considered a key factor in the occurrence and development of tumors. It not only exists and affects the structure, function, and metabolism of the tissue where the tumor is located, but also relates to the intrinsic environment of the tumor cells themselves. Positive responses to immunotherapy often depend on the interaction between tumor cells and the immunoregulation within the TME. Under these interactions, the TME plays a significant role in either suppressing or enhancing immune responses. However, there are currently no mature research methods for characterizing or modeling the TME.

Organoids are in vitro three-dimensional cell culture systems that can replicate the parental tissue or organ, featuring complex spatial structures of self-assembly, including cell-to-cell and cell-to-extracellular matrix interactions. Ideally, they also include interactions between cells and immune cells. However, the limitation of tumor organoids derived from patient tumor tissues is that, although a lower proportion of immune cells can be detected in the initial few generations (usually the first 1-2 generations) of organoids, these immune cells' quantity and composition hardly represent the composition of the tumor immune microenvironment *in situ*.

Co-culturing immune cells with tumor organoids further explores the interaction between tumors and the immune system. However, the co-culture system of tumor organoids and immune cells cannot fully characterize the TME. By co-culturing specific cells within the TME with tumor organoids, some characteristics of the TME are partially simulated. Co-culturing tumor organoids with different immune cells (including TILs, CAR-T, CIK, or specific components of the TME) allows for the direct detection of

the infiltration and killing effect of immune cells on the organoids, which is significant for research on tumor immunotherapy and cell therapy products.

This protocol provides the side-by-side steps for co-culturing lung cancer organoids and cytokine-induced killer cells (CIK) cells using MasterAim® co-culture related products*. Researchers can refer to this protocol to co-culture other types of tumor organoids and immune cells and carry out further detection and analysis.

*The co-culture medium has been verified for the culture of immune cells such as TILs, NK cells, and CIK cells. The genetically engineered cells, including CAR-T cells and CAR-M cells have not been verified with this medium.

Reagents and consumables used in this protocol

- MasterAim® Lung Cancer Organoids and Immune Cell Co-culture Kit (#10-100-492)
- MasterAim® Lung Cancer Organoid Kit (#10-100-060)
- MasterAim® Tissue Preservation Medium (#100-049)
- MasterAim® Tissue Dissociation Medium I (#100-047)
- MasterAim® Tissue Dissociation Medium II (#100-048)
- MasterAim® Anti-Adherence Solution (#100-291)
- MasterAim® primary enhancer (#100-008)
- Fetal Bovine Serum (FBS) (#100-236)
- Dulbecco's Modified Eagle Medium (DMEM, #100-134)
- Live cell dyes (depend on experiential requirement, dyes used in this protocol are for references only)
- Green cell dyes recommended: Calcein-AM, CMFDA, Caspase3/7
- Red cell dyes recommended: CMTPX, Mito-Red
- Dulbecco's Phosphate-Buffered Saline (DPBS, no calcium, no magnesium, no phenol red) Buffer (#100-183)
- Red Cell Lysis Buffer (#B541001-0100, Sangon Biotech)
- Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix (#356231, Corning)
- Pen-Strep Solution (#C3423-0100, Vivacell)
- TrypLE Express Enzyme (1X), Phenol Red (#12605-010, Gibco)
- 96-well Clear Ultra-Low Attachment Multiple Well Plates (#3474, Corning)
- 24-well Clear Ultra-Low Attachment Multiple Well Plates (#3473, Corning)

- Costar® 24-well Clear TC-treated Multiple Well Plates (#3524, Corning)
- Costar® 6-well Clear TC-treated Multiple Well Plates (#3516, Corning)
- X-VIVO15 Serum-free Medium with Gentamicin and Phenol Red (#04-418Q, Lonza)
- Recombinant Human IL-2 Protein (#GMP-TL777, T&L Biotechnology)
- Anti-Human CD3 mAb (#GMP-TL101, T&L Biotechnology)
- Anti-Human CD8 mAb (#GMP-TL102, T&L Biotechnology)
- Ficoll (#LTS1077, TBD Science)

Protocol

Part I Establishment of Lung Cancer Organoids

1. Reagents Preparation

1.1 Washing solution: Add 1% Pen-Strep (PS) Solution to DPBS buffer, named as DPBS-PS, pre-cool before using.

1.2 One day ahead, place MasterAim® Lung Cancer Organoid Culture Complete Medium: Place the MasterAim® Lung Cancer Organoid Basal Culture Medium and the MasterAim® Lung Cancer Organoid Culture Medium Supplement at 4°C to thaw. Transfer all the supplement into the basal medium, mix well, and it becomes the lung cancer organoid culture complete medium.

Note:

(1) The complete organoid culture medium does not contain antibiotics. Add the antibiotics (i.e. PS) before use.

(2) It is recommended to aliquot the complete medium after preparation. Keep the un-used aliquots at -20°C. After thawing, keep at 4°C, and use up within 2 weeks. Avoid repeated freeze-thaw cycles.

(3) For the establishment of primary organoids, 1X MasterAim® Primary Enhancer can be added to the culture medium to increase the culture efficacy. After passaging, organoids can be directly cultured in MasterAim® Lung Cancer Organoid Complete Culture Medium with antibiotics.

2. Tissue Processing

Note:

*For all steps processing with cells or tissues, all tubes and pipette tips **MUST** be pre-rinsed with MasterAim® Anti-Adhesion Solution (#100-291) to reduce cell loss.*

2.1 Tissue should be kept in cold MasterAim® Tissue Preservation Medium upon arrival at the lab.

2.2 Depending on the size of the tissue, place the tissue into a 15/50 mL falcon tube. Wash with 5/15 mL DPBS-PS for 3 - 5 times until the supernatant is clear. The washing solution and tissue preservation medium can be collected, centrifuged, and observed for the presence of cells. If present, the cells can be washed, centrifuged, and collected separately.

3. Tissue Dissociation

3.1 Transfer the cleaned tissue to an EP tube, add 200 µL of DPBS-PS or MasterAim® Tissue Dissociation Medium I (#100-047), and chop the tissue with scissors to 0.5-1 mm^3 fragments, ensuring the chopped tissue pieces appear uniform.

3.2 Use a pre-rinsed Pasteur pipette to transfer the chopped tissue fragments from the EP tube to a 15 mL falcon tube, add 4 mL MasterAim® Tissue Dissociation Medium I (#100-047). Place the tube on a horizontal shaker for 30 - 45 mins, 120 rpm, at 37°C. Observe under a microscope every 15 mins. Stop the digestion once significant number of cells leaking from the tissues under observation. Usually, digestion of lung cancer samples should not exceed 1 h.

Note:

After step 3.1, observe for cell leaking out under microscope. If significant number of cells leak, it indicates the tissue digests easily, and digestion time might need to be shortened; if the tissue is highly fibrotic, the cell yield may be lower, and digestion time could be extended.

3.3 After digestion with MasterAim® Tissue Dissociation Medium I, add 8 mL DPBS-PS to stop the digestion, centrifuge at 300 g for 5 mins, discard the supernatant. Add 2 mL MasterAim® Tissue Dissociation Medium II (#100-048) to the pellet, then digest on a horizonal shaker for 10 - 15 mins, 120 rpm, at 37°C. Observe under a microscope every 5 mins. Stop the digestion when most cell clumps are comprised of 5 - 20 cells.

4. Primary organoids initiation

4.1 After digestion, add 4 mL DPBS-PS to stop the digestion, centrifuge at 300 g for 5 mins, and collect the cells.

4.2 Discard the supernatant and resuspend the cells in 2 mL DPBS-PS.

4.3 (Optional, small biopsy samples may choose not to filter) Pre-rinse a 100 µM cell strainer, filter the cell suspension through the 100 µM filter into a new 50 mL pre-rinsed falcon tube. Wash the cell suspension tube with DPBS-PS several times for filtration. Centrifuge the filtrates at 300 g for 5 mins.

4.4 Discard the supernatant, avoiding contact with the cell pellet. Use a pipette tip to remove the bottom

liquid.

4.5 (Optional step) If cell pellet appears red, add 1 - 2 mL red blood cell lysis solution (volume can be adjusted based on the size of cell pellet), use 1 mL pipette tip to suspend the pellet thoroughly, incubate at room temperature for 6 min. Add 4 - 6 mL DPBS-PS to stop the lysis, then centrifuge at 300 g for 5 mins.

Note:*Lysis time should not exceed 8 min.*

4.6 Cell Counting: Resuspend the cell pellet obtained in the previous step with 1 mL DPBS-PS, and count the number of cell clusters using an automatic cell counter or a manual counting plate.

4.7 Centrifuge at 300 g for 5 mins. According to the counting results, resuspend the cell pellet obtained in the previous stage in appropriate volume of complete organoid medium, the recommended seeding density is 4*10^6 cells/mL. Add 1.5 volume of Matrigel to the suspension to mix the cell fragments, try not involving bubbles by only pressing to the first stop of the pipettor. This step **MUST** be operated on ice to prevent Matrigel solidify.

Note: *This step requires the use of ice-cold pipette tips and pre-cold medium, Matrigel* ***MUST*** *be thawed on ice in advance.*

4.8 Pipette 50 μL cell suspension to the center of a pre-warmed 24-well plate. To prevent bubbles, only press the pipettor to the first stop when using, and a solidified droplet (domes) should form in the center of each well.

Note: *The culture plate* ***MUST*** *be pre-warmed in a 37°C incubator for more than half an hour. The pipette tip* ***MUST*** *not touch the bottom of the culture plate.*

4.9 Leave the plate at 37°C for 5 mins, then invert the plate and leave it for 25 mins to allow the Matrigel to solidify.

4.10 Gently add 500 μL of pre-warmed MasterAim® Lung Cancer Organoid Culture Medium to each well along the side walls of the wells, avoiding direct addition to the domes.

4.11 Add DPBS-PS to other wells without cells to maintain humidity during culture.

Note*: Do not seed organoids into the outermost wells.*

4.12 Cover the culture plate and cultivate at 37°C and 5% CO_2.

4.13 Change the culture medium every 2 - 4 days, observe the growth, and record with photos until passaging.

4.14 Generally, organoids can be observed forming 3 - 4 days after seeding. Before passaging, ensure most organoids are larger than 100 μM. Generally, the P0 generation will undergo the first passaging after 7 - 21 days.

Part II Preparation of CIK Cells from human peripheral blood mononuclear cells (PBMCs)

1. Obtain PBMCs from freshly collected human whole blood samples using density gradient centrifugation with Ficoll. At least 10 mL of fresh whole blood is recommended for the experiment.

2. Resuspend PBMCs in X-VIVO15 medium supplemented with 10% FBS at a concentration of 1-2*10^6/mL. Add IL-2 (6000IU/mL) and mix well, then transfer the cells to a 24-well/6-well TC-treated plate based on the volume of the culture medium.

3. After three days, transfer the cell suspension to a new 24-well/6-well TC-treated plate, and add CD3 antibody (500 ng/mL) and CD28 antibody (500 ng/mL). Culture in a 37°C, 5% CO_2 incubator.

Note: *During the early activation phase of the culture process, a significant number of cells will adhere to the well. It is appropriate to collect the culture by pipetting. Discard the fully adhered cells.*

4. When the culture medium turns yellow or the cell concentration approaches saturation (i.e., greater than 2*10^6/mL), add medium to adjust the cell concentration to between 1-2*10^6/mL. Depending on the amount of medium, transfer to a larger cell culture vessel.

Note: *After 7 days of step 3, the cells can be used for co-culture experiments. For optimal results, it is recommended to use CIKs with a viability greater than 80% for co-culture. Due to immunocyte exhaustion, long-term expansion and cryopreservation are not feasible. It is suggested that CIKs cultured for more than 30 days should not be used for co-culture experiments. Below is a comparison image before and after activation.*

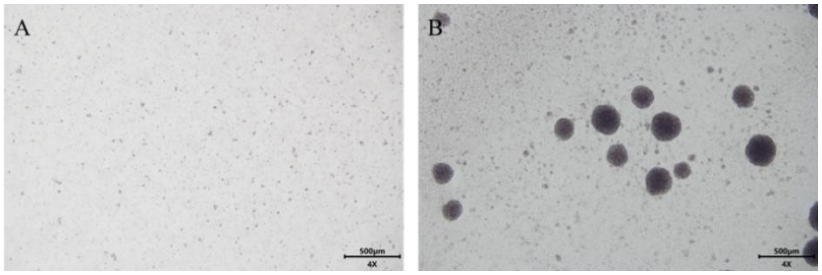


Figure 1. Bright field images of CIK cell before and after activation. A. PBMCs before activation B. After adding CD3/CD28 for 7 days, activated CIKs form colonies.

Part III. Using Lung Cancer Organoids to Evaluate the Cytotoxicity of CIK Cells

1. Reagents Preparation

1.1 One day ahead, place the MasterAim® Lung Cancer Organoid and Immune Cell Co-Culture Basal Medium (#100-493), MasterAim® Lung Cancer Organoid and Immune Cell Co-Culture Supplement 1 (#100-494), and MasterAim® Lung Cancer Organoid and Immune Cell Co-Culture Supplement 2 (#100-495) at 4°C to thaw.

1.2 Transfer all the Supplement 1 into the basal culture medium, mix well to create the partially complete co-culture medium.

1.3 Supplement 2 and MasterAim® primary enhancer is added before use, with a ratio of 120 μL of the Supplement 2 per 10 mL of the partially complete co-culture medium. It is suggested to add 0.5X MasterAim® primary enhancer throughout the entire co-culture process of organoids and immune cells.

Note:

(1) This medium does not contain antibiotics; please add the antibiotics according to experimental requirements.

(2) It is recommended to aliquot appropriately for storage at -20°C. After opening, store at 4°C and use within 2 weeks. Avoid repeated freeze-thaw cycles.

2. Cytotoxicity Analysis of CIK Cells with Lung Cancer Organoids

Note*: The following protocol uses a 96-well plate culture system as an example. Adjust the culture vessel with required cell numbers and culture volumes accordingly. This protocol uses IncuCyte's real-time imaging for the analysis of immune cell cytotoxicity, refer to the IncuCyte operation manual for specific procedures. Or choose different analysis methods according to experimental needs.*

2.1 Preparation of lung cancer organoids for co-culture:

One day ahead, disperse the cultured organoids with cold DPBS-PS. Try to be gentle to maintain organoid integrity. Typically, resuspend 3 - 4 50 μL domes of lung cancer organoids in 1 mL DPBS-PS, approximately 800 organoids, which can be used for 1 mL co-culture cell suspension. After centrifugation at 300 g for 5 mins, resuspend the organoids in 1 mL MasterAim® lung cancer organoid complete culture medium and culture in a 24-well clear Ultra-Low Attachment plate.

2.2 The next day, stain and count the suspended organoids and immune cells.

Note:*Current literature mentions an effector to target ratio between 1:5 to 1:20 for immune cells and organoids; this experiment uses a 1:10 ratio, which can be adjusted for different experimental purposes to achieve optimal results.*

2.2.1 Lung cancer organoid counting process: Considering that the organoids need to be intact during co-culture, they should not be digested into single cells. Therefore, at least 100 μL of organoid suspension needs to be used for cell counting, which will not be used in co-culture. Use TrypLE to digest the organoid suspension into single cells for counting. Based on the number of single cells in the 100 μL system, calculate the number of cells in organoids from step 2.1. The required cell number for 96-well

plate is 10^4 per well, and 2*10^5 for 24-well plate. Calculate the total required number of organoids for different experimental groups.

2.2.2 Lung cancer organoids' staining: Centrifuge at 300 g for 5 mins and collect the needed lung cancer organoids in a 1.5 mL EP tube, with the staining quantity ranging from 800 to 5000 organoids per tube. Discard the supernatant, resuspend the cells in 1 mL 37°C pre-warmed DMEM, add CMTPX to a final concentration of 2 μM, incubate in a 37°C incubator in the dark for 30 mins. After staining, centrifuge the cells at 300 g for 5 mins, wash the cells 2 - 3 times with 1 mL DPBS. After the last wash, suspend in 1 mL MasterAim® lung cancer organoid culture medium in a 24-well clear Ultra-Low Attachment plate for 4 hours, then wash the cells 2 - 3 times with 1 mL DPBS, and collect the organoids.

2.2.3 Counting of CIK cells: 4 hours after staining the organoids, calculate the required number of CIK cells according to the cell number of organoids in step 2.2.1, and transfer the required number of cells to a 1.5 mL EP tube.

2.2.4 CIK cells' staining: Centrifuge at 300 g for 5 mins and collect the CIKs in a 1.5 mL EP tube, the appropriate number of cells for staining per tube is 5 - 10*10^5 cells. Discard the supernatant, resuspend the cells in 1 mL 37°C pre-warmed DMEM, add CMFDA to a final concentration of 2 μM, incubate in a 37°C incubator in the dark for 15 mins. After staining, centrifuge the cells at 300 g for 5 mins, wash the cells 3 - 5 times with 1 mL DPBS, and collect the cells.

Note: *Steps 2.2.2 and 2.2.4 are fluorescence staining steps. The laboratory can adjust the staining scheme and the type of live cell dye according to needs. If fluorescence staining is not performed, proceed directly to step 2.3 and subsequent steps.*

2.3 After staining the lung cancer organoids and CIK cells, resuspend an appropriate amount of MasterAim® lung cancer organoids and immune cells co-culture complete culture medium according to the experimental group. In general, it is recommended to mix the volume of organoids and immune cells at 10:1. The total culture volume per well in a 96-well plate is recommended to be 100 - 150 μL per well.

2.4 First, add organoids to a 96-well clear Ultra-Low Attachment plate, wait for 30min in a 37°C incubator, then slowly drop CIK cells along the liquid interface and use the pipette tip to gently stir, mixing the entire culture system.

2.5 Place the cell culture plate in an incubator or imaging microscope for subsequent experiments. The experimental data for part IV is obtained through real-time imaging with IncuCyte S3, with image collection interval set to 2 hours, totaling 72 hours.

Part IV AimingMed Internal Data

1. MasterAim® Lung Cancer Organoid and Immune Cell Co-culture Kit (#10-100-492) Data

Using lung cancer organoids and T cells as example, AimingMed evaluated the effects of lung cancer organoid culture medium, T cell culture medium, and MasterAim® lung cancer organoid and immune cell co-culture medium (hereinafter referred to as "co-culture medium") on T cells and lung cancer organoids. As shown in Fig 2A and 2B, the co-culture medium can maintain the viability of T cells, whereas T cells cultured in the lung cancer organoid medium group showed significantly lower viability than those cultured in the immune cell medium ($p<0.01$). This result indicates that the tumor organoid medium is not suitable for the culture of T cells, while the co-culture medium can maintain the viability of T cells during the experimental window.

Next, we tested the effect of the various culture media on the viability of lung cancer organoids. As observed in Fig 2C, lung cancer organoids began to disintegrate after 8 hours of culture in the T cell culture medium, indicating that the T cell culture medium is not suitable for the growth of organoids. Meanwhile, using the Incucyte S3, we evaluated the impact of the co-culture medium on the survival of lung cancer organoids. As shown in Fig 2D/E, compared to MasterAim® lung cancer organoid culture medium, there was no significant difference in growth rate with the MasterAim® lung cancer organoid and immune cell co-culture medium. In summary, the MasterAim® organoid and immune cell co-culture kit, upon testing, can maintain the viability of both cells within the experimental window, providing more reliable data.

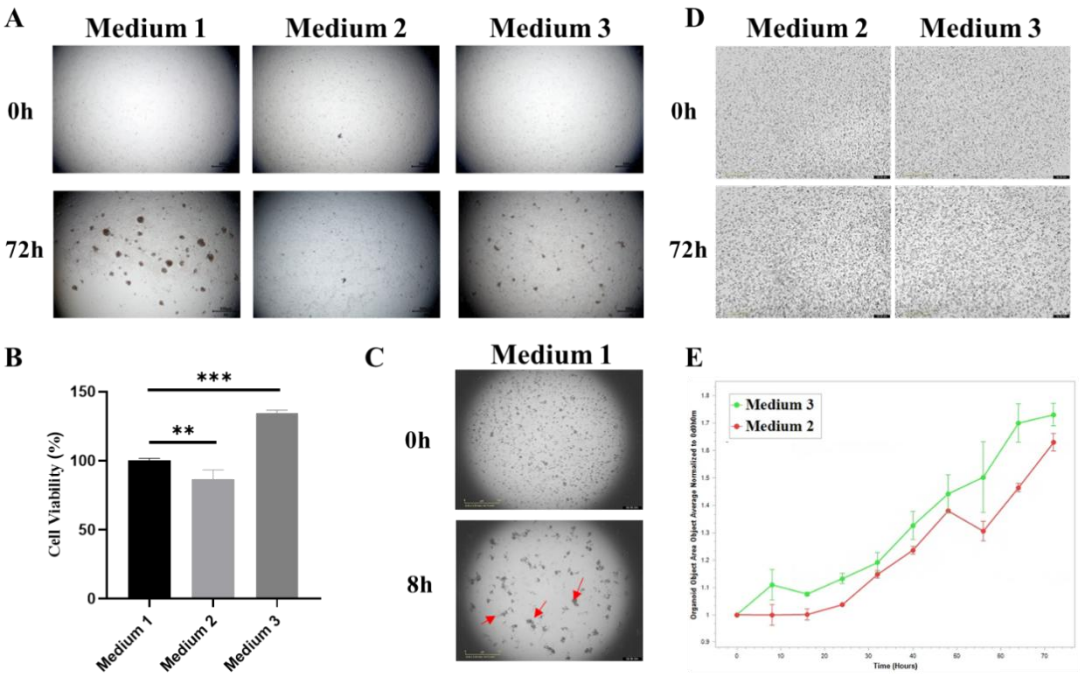


Figure 2. The impact of different culture systems on immune cells and lung cancer organoids. (A) Bright-field images of the effects of different culture media on T cells for 72h; (B) CCK-8 assay statistical differences in growth among different groups. Medium 1 - T cell culture medium, Medium 2 - MasterAim® lung cancer organoid culture medium; Medium 3 - MasterAim® lung cancer organoid and immune cell co-culture medium. t-test, **p < 0.01, ***p < 0.001; (C) Bright-field images of lung cancer organoids cultured in T cell medium for 8h, with red arrows indicating apoptotic organoids; (D) Bright-field observations of the impact of different culture media on lung cancer organoids at 0 hours and 72h; (E) Statistical curve of the growth area of organoids in different groups.

2. MasterAim® Colon Cancer Organoid and Immune Cell Co-culture Kit (#10-100-504) Data

Using this protocol, colon cancer organoids and CIK cells are co-cultured at an effector-to-target ratio of 1:10 to observe the killing effect of CIK cells on the organoids within 72 hours. AimingMed utilizes the Incucyte® S3 live-cell imaging system for real-time observation of CIK cell infiltration and killing of the organoids, facilitating the selection of the optimal experimental endpoint for evaluating their killing efficacy.

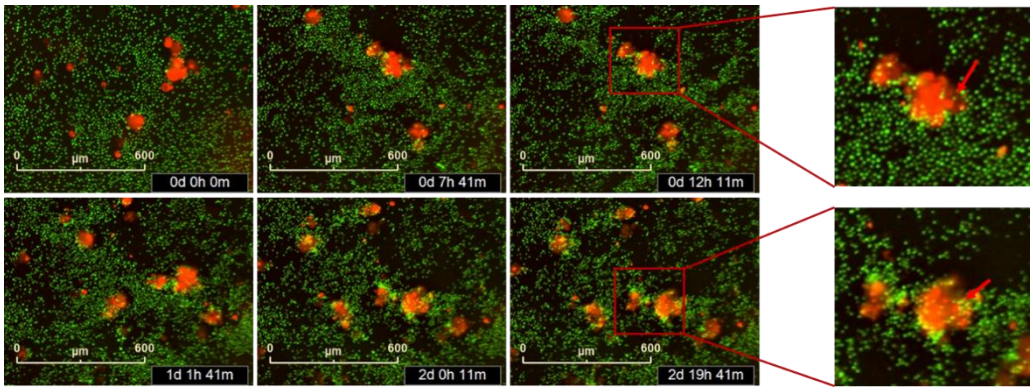


Figure 3. Detection of CIK cell-mediated killing and infiltration of colon cancer organoids using the Incucyte® S3 real-time imaging system. Fluorescence images of colorectal cancer organoids (red) and CIK cells (green) after 72 hours of co-culture. Before co-culture, organoids and CIK cells were labeled with red and green probes, respectively. 12 hours after co-culture, CIK cells can be observed aggregating around and infiltrating (red arrows) the colon cancer organoids.

Part V. Tips

1. It is recommended to culture cancer organoids derived from primary tissues using MasterAim® cancer organoid culture medium, as demonstrated in this experimental protocol.

2. The co-culture system used in this protocol is a suspension culture, which seems to allow immune

cells to attack and kill the organoids more easily compared to methods involving a matrix gel.

3. The suspension culture method does not allow for medium changes. Due to medium consumption, the optimal window for the entire culture system is 72 hours; do not exceed 96 hours, as the organoids and immune cells may undergo apoptosis due to insufficient nutrients.

4. The density of co-cultured organoids can significantly vary depending on the culture generation and certain parameters of the source organoids, so seeding density is flexible; it can be optimized based on the initial sample and growth conditions. If there is no experience with co-culture, the seeding density provided in this protocol can be used, with subsequent experiments optimized based on the results.

5. It has been verified that the MasterAim® lung cancer organoid and immune cell co-culture medium is suitable for immune cells including CIK, T cells, TILs, etc.

6. This experimental protocol only presents a co-culture protocol for immune cells and tumor organoids. If evaluating the efficacy of immune checkpoint inhibitors, an additional group of immune cells + tumor organoids + PD-1/PD-L1 can be added to compare with the immune cells + tumor organoids group.

7. Immune kill efficacy with tumor organoids can be evaluated not only by fluorescence labeling for invasion and killing effects but also any established detection methods for cell killing indicators, include FACS, qPCR, ELISA, etc.

Scipolis
May the Science be with you
OPEN ACCESS

JOBs
Journal of Organoid & Bioscience

Review

Unlocking the Potential of Organoid Models in Ferroptosis: A Breakthrough in Cancer Research

Dingci Lu[1,2], Min Shu[1], Zhaowu Ma[1]*

[1] Department of Clinical Laboratory, The Second Hospital of Jingzhou, Jingzhou, Hubei 434000, P.R. China

[2] School of Basic Medicine, Health Science Center, Yangtze University, Nanhuan Road 1, Jingzhou, Hubei 434023, China

*Correspondence: mazw@yangtzeu.edu.cn (Zhaowu Ma)

https://doi.org/10.62714/SQAN3746

Abstract

Ferroptosis, a regulated form of cell death driven by iron-dependent lipid peroxidation, has emerged as a critical mechanism in cancer biology. Understanding the molecular mechanisms and experimental models of ferroptosis is essential for developing novel therapeutic strategies. Organoid models, which closely mimic the architecture and functionality of human tissues, offer a promising platform for studying ferroptosis in various cancer types. This review explores the construction and application of organoid models, highlighting their advantages in cancer research. We discuss the link between ferroptosis and organoids in various cancers, including colorectal cancer, pancreatic cancer, liver cancer, breast cancer, gastric cancer, ovarian cancer, and other cancers. Furthermore, we examine the therapeutic implications of ferroptosis in organoid models, emphasizing the potential for these models to enhance our understanding of cancer biology and improve treatment outcomes.

Keywords: Ferroptosis, Organoid Models, Cancer research

1. Introduction

The advent of organoid models has revolutionized cancer research, providing a sophisticated platform that closely mimics the in vivo environment. Organoids, derived from stem cells or primary tissues, are three-dimensional (3D) cell culture systems derived from stem cells or primary tissues, capable of

self-organizing into structures that recapitulate the architecture and function of their tissue of origin(1, 2). This has significant implications for understanding cancer biology, drug screening, and personalized medicine(3-5). This innovative technology has opened new avenues for studying complex biological processes, including regulated cell death.

Ferroptosis, a form of regulated cell death characterized by iron-dependent lipid peroxidation, has emerged as a critical process in cancer biology(6). Unlike apoptosis or necrosis, ferroptosis is driven by the accumulation of lipid peroxides and is tightly regulated by intracellular iron metabolism and antioxidant systems, such as glutathione peroxidase 4 (GPX4) and the cystine/glutamate antiporter system Xc-(7). The interplay between ferroptosis and cancer has been extensively studied, revealing its potential to overcome drug resistance and enhance the efficacy of existing treatments. Ferroptosis has garnered significant attention in recent years due to its distinct mechanisms and implications in cancer biology.

The integration of organoid models into ferroptosis research offers unprecedented opportunities to dissect these molecular mechanisms in a more physiologically relevant context. Organoids derived from various cancer types, have demonstrated their utility in modeling tumor heterogeneity, drug responses, and disease progression(8). These models provide a robust platform for investigating the therapeutic potential of ferroptosis induction in cancer treatment.

As we delve deeper into the interplay between ferroptosis and organoid models across different cancer types, it becomes evident that these systems hold significant promise for advancing our understanding of cancer biology and developing novel therapeutic strategies. This review will explore the molecular mechanisms of ferroptosis, the construction and application of organoid models, and their implications in various cancer types, ultimately highlighting the therapeutic potential of ferroptosis in organoid-based cancer research.

2. The Link between Organoids and Ferroptosis

2.1 Construction and application of organoid models

Organoid models have revolutionized biomedical research by providing a more accurate representation of human tissues and organs in vitro(5, 9). These 3D structures are derived from stem cells or primary tissues and can mimic the architecture and functionality of their in vivo counterparts. The construction of organoid models involves several key steps, including the isolation of stem cells, embedding them in a suitable extracellular matrix, and providing the necessary growth factors and signaling molecules to promote differentiation and self-organization. For instance, pancreatic ductal adenocarcinoma (PDAC) organoids can be generated using a 3D Matrigel system, which supports the growth and differentiation of

both human and mouse PDAC cells(10).

The application of organoid models extends across various fields of biomedical research, including cancer biology, drug screening, and personalized medicine. In cancer research, organoids derived from patient tumors, known as patient-derived organoids (PDOs), have been particularly valuable. These models retain the genetic and phenotypic characteristics of the original tumors, making them excellent tools for studying tumor heterogeneity, drug responses, and resistance mechanisms. Organoid models are also instrumental in understanding the molecular mechanisms underlying cancer progression and treatment resistance using organoid model of patient-derived xenografts (PDX). Furthermore, organoid models have been used to explore the interactions between cancer cells and the tumor microenvironment(11).

Currently, the construction and application of organoid models represent a significant advancement in cancer research. These models offer a more physiologically relevant platform for studying tumor biology, testing new drugs, and developing personalized treatment strategies. By faithfully recapitulating the complexity of human tissues and tumors, organoid models hold great promise for advancing our understanding of cancer and improving patient outcomes.

2.2 The potential applications of organoid models in ferroptosis

Organoid models have emerged as a revolutionary tool in cancer research, particularly in the study of ferroptosis, a form of regulated cell death characterized by iron-dependent lipid peroxidation. The application of organoid models in ferroptosis research offers several promising avenues for understanding cancer biology and developing novel therapeutic strategies. One significant application of organoid models in ferroptosis research is their use in drug screening and development. Organoids derived from various cancer types, have been instrumental in identifying compounds that can induce ferroptosis in cancer cells(12). Accumulating findings highlight the potential of organoid models to facilitate the discovery of new ferroptosis-inducing agents and optimize existing therapies.

Another critical application of organoid models is in understanding the mechanisms of chemoresistance and developing strategies to overcome it. Chemoresistance remains a major challenge in cancer treatment, and ferroptosis has been identified as a potential pathway to sensitize resistant cancer cells to chemotherapy. Current studies underscore the utility of organoid models in elucidating the molecular underpinnings of chemoresistance and identifying novel therapeutic targets(13, 14). Organoid models also offer a valuable platform for personalized medicine, which hold great promise for tailoring treatments to achieve optimal therapeutic outcomes. Therefore, the integration of organoid technology with ferroptosis research holds great potential for advancing our understanding of cancer biology and developing innovative therapeutic strategies to combat cancer.

3. Various Cancer Organoid Models in Ferroptosis

The integration of organoid models into ferroptosis research represents a new frontier in cancer research, offering a powerful platform for elucidating complex biological processes and developing targeted therapies. In the following sections, we focus on the expanding landscape of organoid models in ferroptosis and its significant contributions in personalized medicine (Fig 1 and Tab 1).

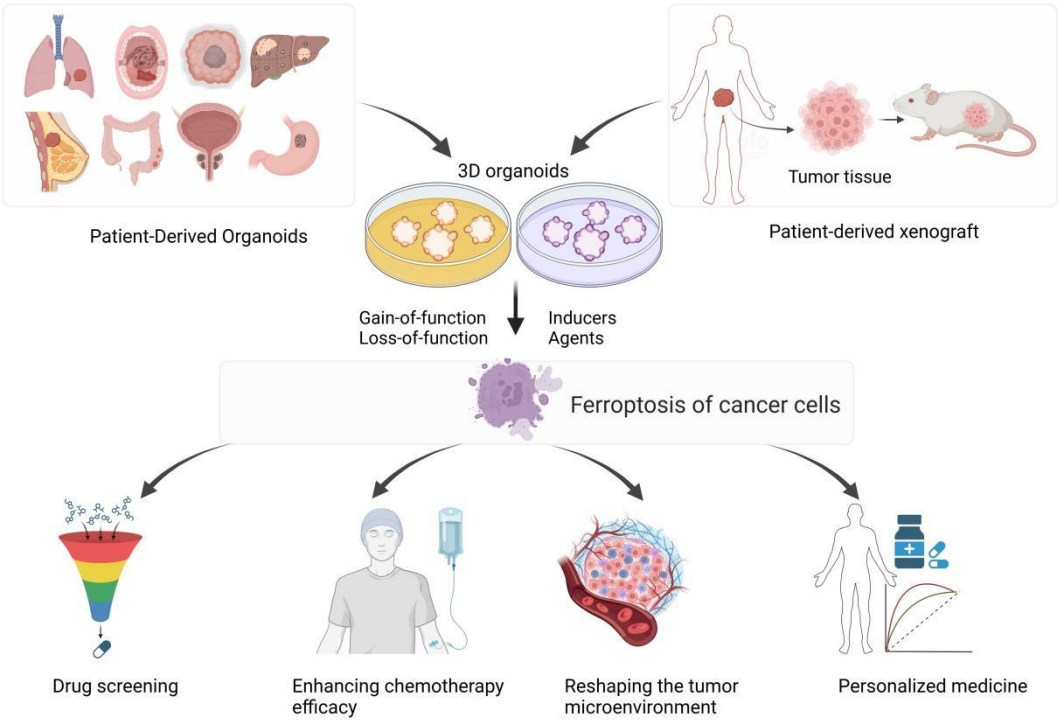


Figure 1. The roles of cancer organoid models and ferroptosis. The integration of patient-derived organoids and patient-derived xenografts into ferroptosis research, represents a new frontier in cancer research, offering a powerful platform for elucidating complex biological processes and developing therapeutic strategies.

3.1 Colorectal cancer

Colorectal cancer (CRC) has been extensively studied using organoid models to understand the mechanisms of ferroptosis and its therapeutic potential. CRC-derived organoids have shown promise in overcoming acquired drug resistance by inducing ferroptosis. For instance, studies have demonstrated that the loss of colonic epithelial Hmox1 promotes ferroptosis, suggesting a potential target for therapeutic intervention(15). Additionally, intestinal stem cell organoids have highlighted the role of IFNγ as a key cytokine capable of arresting cancer stemness and triggering GPX4-dependent ferroptosis(16). Patient-derived organoids have also been utilized to show that compounds like curcumin and

andrographis exhibit anti-tumor effects via activation of ferroptosis(12). Moreover, CRC organoids have been used to demonstrate that adipocyte-derived exosomes containing MTTP reduce ferroptosis susceptibility in CRC, promoting chemoresistance to oxaliplatin(13). These findings underscore the potential of ferroptosis as a therapeutic strategy in CRC.

Organoid models have provided valuable insights into the mechanisms of ferroptosis in CRC and its potential as a therapeutic target. Studies have shown that targeting specific pathways, such as LGR4 and β-catenin/Wnt-signaling, can overcome acquired drug resistance in CRC by inducing ferroptosis(17) . Additionally, natural compounds like andrographis have been found to sensitize CRC to chemotherapy through the activation of ferroptosis(18). The combination of Vitamin C and cetuximab has also shown promising results in inhibiting the emergence of drug-resistant cells in CRC models by triggering a synthetic lethal metabolic cell death program involving ferroptosis(19). These findings suggest that targeting ferroptosis and metabolic vulnerabilities could be effective strategies to enhance the efficacy of therapies in CRC and overcome drug resistance.

3.2 Pancreatic cancer

Pancreatic cancer, particularly PDAC, has been a challenging malignancy to treat due to its aggressive nature and resistance to conventional therapies. Organoid models derived from pancreatic cancer have provided valuable insights into the role of ferroptosis in cancer therapy. For example, elevated FSP1 has been shown to protect KRAS-mutated cells from ferroptosis during tumor initiation, highlighting a potential target for therapeutic intervention(20). Patient-derived organoids have also been used to demonstrate that targeting the MCP-GPX4/HMGB1 axis can trigger immunogenic ferroptosis, offering a novel approach to enhance anti-tumor immunity(21). Additionally, combining gemcitabine with ferroptosis inducers has been shown to enhance cytotoxic effects in SMAD4-positive organoids, suggesting a potential combination therapy for pancreatic cancer(22). These studies highlight the potential of ferroptosis as a therapeutic strategy in pancreatic cancer.

The role of ferroptosis in PDAC has been extensively studied using organoid models, which provide a physiologically relevant context to investigate chemoresistance and therapeutic responses. A study using PDAC organoids demonstrated the potential of ferroptosis inducers in overcoming chemoresistance and inhibiting tumor growth, paving the way for novel treatment strategies(10). Another study revealed the potential of small molecule chimeras, such as salinomycin derivatives and dihydroartemisinin, in inducing ferroptosis in drug-tolerant PDAC cells and organoids(23). Furthermore, the mitochondrial calcium uniporter (MCU) has been identified as a driver of metastasis and a targetable vulnerability for inducing ferroptosis in pancreatic cancer. Pharmacological inhibition of the cystine transporter SLC7A11 has been shown to effectively induce tumor regression and abrogate MCU-driven

metastasis in patient-derived organoid models(24). Therefore, organoid models provide a powerful platform for dissecting these mechanisms and identifying potential therapeutic targets. Targeting key regulators such as FSP1, MCU, and SMAD4, as well as exploiting the vulnerabilities associated with ferroptosis, holds promise for improving the treatment outcomes for pancreatic cancer patients.

3.3 Liver cancer

Liver cancer, particularly hepatocellular carcinoma (HCC), has been studied using organoid models to explore the role of ferroptosis in tumor progression and treatment. Patient-derived organoids have shown that donafenib and GSK-J4 synergistically induce ferroptosis in liver cancer by upregulating HMOX1 expression, suggesting a potential combination therapy(25). Additionally, organoid models derived from HCC patients have demonstrated that metformin can restore PPARGC1A expression and enhance ferroptosis, supporting its significance in HCC pathogenesis and therapeutic intervention(26). Furthermore, HCC organoids have been used to show that the unconventional prefoldin RPB5 interactor (URI) mediates resistance to tyrosine kinase inhibitors (TKIs)-induced ferroptosis, highlighting a potential target for overcoming drug resistance. The combination of the SCD1 inhibitor aramchol with the deuterated sorafenib derivative donafenib demonstrates potent anti-tumor effects in p53-wild type HCC organoids(27).

Additionally, combining ferroptosis induction with other therapeutic strategies, such as myeloid derived suppressor cell (MDSC) blockade, has rendered primary tumors and metastases in the liver more sensitive to immune checkpoint blockade, offering a novel approach to cancer immunotherapy. This study highlights the potential of ferroptosis-induced immune responses for the treatment of primary liver tumors and liver metastases, although it does not have the same effect on CRC organoids in subcutaneous growth but reduces their metastatic growth in the liver(28). Together, the amalgamation of ferroptosis research with organoid models opens up a promising frontier in liver cancer research. These models not only offer a more precise depiction of the tumor microenvironment but also enable the exploration of innovative therapeutic targets and strategies to address drug resistance and enhance patient outcomes.

3.4 Breast cancer

Breast cancer organoid models have provided valuable insights into the role of ferroptosis in cancer research. For instance, human TNBC organoid models have demonstrated that simultaneous inhibition of FAK and ROS1 synergistically represses tumor growth by upregulating p53 signaling and inducing ferroptosis(29). Additionally, combining anti-FGFR4 and anti-HER2 therapies has been shown to induce ferroptosis in HER2-positive breast cancer. Patient-derived xenografts and organoid experiments demonstrate the synergistic effect of combining anti-FGFR4 and anti-HER2 therapies, offering a

promising combination strategy to address resistance in HER2-positive breast cancer(30). Tamoxifen, a well-known therapeutic agent for breast cancer, has been shown to induce ferroptosis in MCF-7 breast cancer organoids. This discovery underscores the potential of ferroptosis induction in enhancing the efficacy of existing breast cancer therapies and overcoming drug resistance(31). Therefore, the integration of ferroptosis research with organoid models offers a promising frontier in breast cancer research. These models not only provide a more accurate representation of the tumor microenvironment but also facilitate the exploration of novel therapeutic targets and strategies to overcome drug resistance and improve patient outcomes.

3.5 Gastric cancer

Organoid models have also been used to explore the therapeutic implications of ferroptosis in gastric cancer. A study found that cancer-associated fibroblasts (CAFs) impair the cytotoxic function of NK cells in gastric cancer by inducing ferroptosis via iron regulation. This mechanism was elucidated using a human patient-derived organoid model, where targeting CAFs with a combination of deferoxamine and FSTL1-neutralizing antibody significantly alleviated CAF-induced NK cell ferroptosis and boosted NK cell cytotoxicity against gastric cancer(11). Inhibition of the STAT3-ferroptosis regulatory axis holds promise as a therapeutic strategy for combating chemotherapy resistance and advancing gastric cancer treatment. Targeting STAT3 with W1131 induces ferroptosis, displaying significant anti-tumor effects in various models, including organoids and patient-derived xenografts, offering a potential therapeutic approach for advanced gastric cancer(14). These findings provide new insights into the potential of ferroptosis as a therapeutic target in gastric cancer, with organoid models providing a robust platform for preclinical evaluation.

3.6 Bladder cancer

Organoid models have been employed to investigate the involvement of ferroptosis in bladder cancer (BCa) as well. For example, a recent study uncovered that Phosphoglycerate Dehydrogenase (PHGDH) upregulates the expression of SLC7A11, a component of the cystine/glutamate antiporter system Xc-, which is crucial for maintaining GSH levels and preventing ferroptosis. Further functional assays found that the PHGDH inhibitor NCT-502 induced ferroptosis in BCa cell organoid models, leading to reduced tumor growth(32). Moreover, N6-Methyladenosine (m6A) modifications influence chemoresistance by regulating RNA stability and protein levels of SLC7A11, impacting ferroptosis sensitivity in bladder cancer cells. This mechanism is swiftly induced in both cisplatin-sensitive cell lines and patient-derived organoids following short-term exposure to cisplatin, revealing a shared pathway of SLC7A11 upregulation and chemoresistance. These findings underscore the significance of epitranscriptomic plasticity as a key mechanism in rapid chemoresistance development and a promising target for

therapeutic interventions(33).

3.7 Ovarian cancer

Organoid models have provided valuable insights into the role of ferroptosis in ovarian cancer. In ovarian cancer, lipid metabolic activity and redox-driven ferroptosis are regulated by fatty acid desaturases such as SCD1 and FADS2. These enzymes balance lipid metabolism and ferroptosis, influencing cancer cell survival and proliferation. For instance, ovarian cancer organoids derived from high-grade serous ovarian cancer (HGSOC) have shown that FeNP inhibits GPX4 activity, leading to the induction of ferroptosis, suggesting a potential therapeutic approach(34). Additionally, SCD1/FADS2 fatty acid desaturases have been shown to equipoise lipid metabolic activity and redox-driven ferroptosis in ascites-derived ovarian cancer cells. Targeting lipid metabolic pathways to induce ferroptosis has shown promise in overcoming resistance to conventional therapies(35).

3.8 Other cancers

Emerging evidence from patient-derived organoid models highlights the therapeutic potential of targeting ferroptosis in other cancers. Patient-derived organoids from cholangiocarcinoma have shown that combining surufatinib with photodynamic therapy induces ferroptosis and inhibits tumor growth, suggesting a potential combination therapy(36). Glioblastoma patient-derived organoid models have demonstrated that combination treatment can enhance ferroptosis through regulating HOXM1 and GPX4 expression(37). Similarly, patient-derived organoid models from oral squamous cell carcinoma have shown that DRP1 inhibition-mediated mitochondrial elongation drives ferroptosis and abolishes cancer stemness(38). Furthermore, manoalide promotes EGFR-TKI sensitivity in lung cancer by inducing ferroptosis(39), while the AR/GPX4 axis activation and inhibition of ERO1α enhance the antitumor effects in prostate cancer and laryngeal squamous cell carcinoma, respectively(40, 41). In head and neck cancer, organoid models have been instrumental in demonstrating the synergistic effects of TrxR1 inhibition and anti-PD-1 therapy, highlighting the potential of ferroptosis induction in improving cancer treatment outcomes(42). Organoid models elucidate the role of ferroptosis in drug-tolerant persister cells and provide new avenues for cancer treatment. Collectively, integrating organoid models with ferroptosis research enhances our understanding and unveils the therapeutic potential of ferroptosis in diverse cancer types.

4. Therapeutic Implications of Ferroptosis in Organoid Models

The therapeutic implications of ferroptosis in cancer treatment have attracted considerable interest, with a particular focus on utilizing organoid models. These models serve as valuable tools in ferroptosis research, enabling drug screening, improving the effectiveness of chemotherapy, shaping the tumor

microenvironment, facilitating personalized medicine, and exploring combination therapies. This section delves into the therapeutic potential of ferroptosis as observed in organoid models, shedding light on its significance in cancer therapy.

Organoid models have emerged as a powerful tool for drug screening and development, particularly in the context of ferroptosis. These three-dimensional cultures mimic the architecture and functionality of human tissues, providing a more physiologically relevant environment compared to traditional two-dimensional cell cultures. The ability to induce ferroptosis in organoid models allows for the high-throughput screening of potential therapeutic agents that can modulate this form of cell death. For instance, studies have demonstrated the use of organoid models to identify compounds that can either induce or inhibit ferroptosis, thereby offering new avenues for cancer treatment. The use of patient-derived organoids further enhances the relevance of these models, as they can capture the genetic and phenotypic diversity of tumors, allowing for personalized medicine approaches. This capability is particularly crucial in cancers such as PDAC, where traditional treatments have limited efficacy. Recent research has shown that targeting the MCP-GPX4/HMGB1 axis in PDAC organoids can effectively trigger immunogenic ferroptosis, highlighting the potential of these models in developing novel therapeutic strategies(21).

The integration of ferroptosis in organoid models offers significant potential for enhancing the efficacy of chemotherapy. Chemoresistance remains a major hurdle in cancer treatment, and ferroptosis induction has been identified as a promising strategy to overcome this challenge. For example, in gastric cancer, the inhibition of the STAT3-ferroptosis negative regulatory axis has been shown to suppress tumor growth and alleviate chemoresistance. Organoid models derived from gastric cancer patients have been used to validate these findings, demonstrating that targeting STAT3 can induce ferroptosis and restore sensitivity to chemotherapy(14). Similarly, in colorectal cancer, the use of organoids has facilitated the study of ferroptosis pathways and their role in overcoming drug resistance, providing a robust platform for testing combination therapies that include ferroptosis inducers(17).

Organoid models are instrumental in the realm of personalized medicine, particularly for identifying predictive biomarkers of ferroptosis sensitivity. By using patient-derived organoids, researchers can assess the ferroptosis susceptibility of individual tumors, enabling the customization of treatment regimens. This approach is exemplified in studies on liver cancer, where organoids have been used to explore the role of autophagy activation and m6A modification in regulating ferroptosis. These models have revealed that high levels of m6A modification correlate with increased ferroptosis sensitivity, suggesting that m6A could serve as a predictive biomarker for ferroptosis-based therapies(43). The ability to tailor treatments based on the ferroptosis profile of a patient's tumor holds promise for

improving therapeutic outcomes and minimizing adverse effects.

The tumor microenvironment plays a critical role in cancer progression and treatment resistance. Organoid models provide a unique opportunity to study the interactions between cancer cells and the tumor microenvironment, particularly in the context of ferroptosis. For instance, in gastric cancer, CAFs have been shown to impair the cytotoxic function of NK cells by inducing ferroptosis via iron regulation. Organoid models have been used to elucidate this mechanism, demonstrating that targeting CAFs can alleviate NK cell ferroptosis and enhance anti-tumor immunity(11). These findings underscore the importance of considering the tumor microenvironment in ferroptosis-based therapies and highlight the potential of organoid models in developing strategies to modulate the tumor microenvironment for improved treatment efficacy.

The use of organoid models in ferroptosis research has opened new avenues for exploring combination therapies. Combining ferroptosis inducers with other treatment modalities, such as immunotherapy or targeted therapy, can enhance therapeutic efficacy and overcome resistance mechanisms. For example, in glioma, the inhibition of SOAT1 has been shown to increase sensitivity to ferroptosis and enhance the efficacy of radiotherapy. Organoid models have been instrumental in these studies, providing a platform to test and optimize combination therapies(44). Similarly, in breast cancer, the combination of ferroptosis inducers with traditional chemotherapeutic agents has shown promise in overcoming resistance and improving treatment outcomes(45).

5. Conclusions and Future Perspectives

The exploration of ferroptosis within the context of organoid models represents a significant advancement in cancer research, offering novel insights into the mechanisms and therapeutic potential of this form of regulated cell death. Our comprehensive review highlights the intricate molecular pathways governing ferroptosis, including the pivotal roles of intracellular iron metabolism, lipid peroxidation, GPX4, and the system Xc-. Additionally, the construction and application of organoid models have been underscored as powerful tools that bridge the gap between traditional cell lines and in vivo studies, providing a more physiologically relevant environment to study cancer biology and treatment responses.

The integration of ferroptosis research with organoid technology holds transformative potential for cancer therapeutics. Organoid models, with their ability to mimic the three-dimensional architecture and cellular heterogeneity of tumors, offer a robust platform for investigating the efficacy and mechanisms of ferroptosis-inducing agents across various cancer types. Organoid models not only enhance our understanding of ferroptosis but also pave the way for the development of more effective and targeted

cancer treatments(46). However, the heterogeneity observed in ferroptosis sensitivity across different cancer types and even within subpopulations of the same cancer underscores the need for a more nuanced understanding of the underlying mechanisms.

Balancing the diverse findings from various studies, it is evident that while the induction of ferroptosis offers a promising therapeutic avenue, the complexity of iron metabolism and redox biology necessitates a tailored approach. Future research should focus on identifying biomarkers that predict ferroptosis sensitivity and resistance, thereby enabling personalized treatment strategies. Moreover, the potential side effects of ferroptosis induction, such as unintended damage to normal tissues, warrant careful consideration and further investigation.

The convergence of ferroptosis and organoid models represents a key advancement in cancer research, deeping our comprehension of cancer therapy. Leveraging the capabilities of organoid technology enables a comprehensive exploration of ferroptosis and its therapeutic capacities, facilitating the development of more potent and personalized cancer treatments. Further investigations should focus on refining these models, investigating synergistic treatment approaches, and translating these discoveries into clinical practice, thus bridging the translational gap between laboratory and clinical settings in the battle against cancer.

Acknowledgements

This work was supported by the following research grants from the National Foreign Experts Program of China (DL2023027001L); Yangtze University Science and Technology Aid to Tibet Medical Talent Training Program Project (2023YZ18).

References

1. Corsini NS, Knoblich JA. Human organoids: New strategies and methods for analyzing human development and disease. Cell. 2022;185(15):2756-69.
2. Veninga V, Voest EE. Tumor organoids: Opportunities and challenges to guide precision medicine. Cancer cell. 2021;39(9):1190-201.
3. LeSavage BL, Suhar RA, Broguiere N, Lutolf MP, Heilshorn SC. Next-generation cancer organoids. Nature materials. 2022;21(2):143-59.
4. Beydag-Tasöz BS, Yennek S, Grapin-Botton A. Towards a better understanding of diabetes mellitus using organoid models. Nature reviews Endocrinology. 2023;19(4):232-48.

5. Eichmüller OL, Knoblich JA. Human cerebral organoids - a new tool for clinical neurology research. Nature reviews Neurology. 2022;18(11):661-80.
6. Dixon SJ, Lemberg KM, Lamprecht MR, Skouta R, Zaitsev EM, Gleason CE, *et al*. Ferroptosis: an iron-dependent form of nonapoptotic cell death. Cell. 2012;149(5):1060-72.
7. Dixon SJ, Olzmann JA. The cell biology of ferroptosis. Nature reviews Molecular cell biology. 2024;25(6):424-42.
8. Meng Y, Sun J, Zhang G, Yu T, Piao H. The three-dimension preclinical models for ferroptosis monitoring. Frontiers in bioengineering and biotechnology. 2022;10:1020971.
9. Osonoi S, Takebe T. Organoid-guided precision hepatology for metabolic liver disease. Journal of hepatology. 2024;80(5):805-21.
10. Yu C, Kang R, Tang D. Organoids Models of Pancreatic Duct Adenocarcinoma. Methods in molecular biology (Clifton, NJ). 2023;2712:45-60.
11. Yao L, Hou J, Wu X, Lu Y, Jin Z, Yu Z, *et al*. Cancer-associated fibroblasts impair the cytotoxic function of NK cells in gastric cancer by inducing ferroptosis via iron regulation. Redox biology. 2023;67:102923.
12. Miyazaki K, Xu C, Shimada M, Goel A. Curcumin and Andrographis Exhibit Anti-Tumor Effects in Colorectal Cancer via Activation of Ferroptosis and Dual Suppression of Glutathione Peroxidase-4 and Ferroptosis Suppressor Protein-1. Pharmaceuticals (Basel, Switzerland). 2023;16(3).
13. Zhang Q, Deng T, Zhang H, Zuo D, Zhu Q, Bai M, *et al*. Adipocyte-Derived Exosomal MTTP Suppresses Ferroptosis and Promotes Chemoresistance in Colorectal Cancer. Advanced science (Weinheim, Baden-Wurttemberg, Germany). 2022;9(28):e2203357.
14. Ouyang S, Li H, Lou L, Huang Q, Zhang Z, Mo J, *et al*. Inhibition of STAT3-ferroptosis negative regulatory axis suppresses tumor growth and alleviates chemoresistance in gastric cancer. Redox biology. 2022;52:102317.
15. Callahan RC, Bhagavatula G, Curry J, Staley AW, Schaefer REM, Minhajuddin F, *et al*. Epithelial heme oxygenase-1 enhances colonic tumorigenesis by inhibiting ferroptosis. bioRxiv : the preprint server for biology. 2024.
16. Lv X, He FL, Dai Y, Dai X. IFNγ synergies with cold atmospheric plasma in triggering colorectal cancer cell ferroptosis via the IFNγ/IFNR2/APC/TCF4/GPX4 axis. Aging. 2023;15(17):8692-711.
17. Zheng H, Liu J, Cheng Q, Zhang Q, Zhang Y, Jiang L, *et al*. Targeted activation of ferroptosis in colorectal cancer via LGR4 targeting overcomes acquired drug resistance. Nature cancer. 2024;5(4):572-89.

18. Sharma P, Shimura T, Banwait JK, Goel A. Andrographis-mediated chemosensitization through activation of ferroptosis and suppression of β-catenin/Wnt-signaling pathways in colorectal cancer. Carcinogenesis. 2020;41(10):1385-94.
19. Lorenzato A, Magrì A, Matafora V, Audrito V, Arcella P, Lazzari L, *et al*. Vitamin C Restricts the Emergence of Acquired Resistance to EGFR-Targeted Therapies in Colorectal Cancer. Cancers. 2020;12(3).
20. Müller F, Lim JKM, Bebber CM, Seidel E, Tishina S, Dahlhaus A, *et al*. Elevated FSP1 protects KRAS-mutated cells from ferroptosis during tumor initiation. Cell death and differentiation. 2023;30(2):442-56.
21. Li G, Liao C, Chen J, Wang Z, Zhu S, Lai J, *et al*. Targeting the MCP-GPX4/HMGB1 Axis for Effectively Triggering Immunogenic Ferroptosis in Pancreatic Ductal Adenocarcinoma. Advanced science (Weinheim, Baden-Wurttemberg, Germany). 2024;11(21):e2308208.
22. Chen HD, Ye Z, Hu HF, Fan GX, Hu YH, Li Z, *et al*. SMAD4 endows TGF-β1-induced highly invasive tumor cells with ferroptosis vulnerability in pancreatic cancer. Acta pharmacologica Sinica. 2024;45(4):844-56.
23. Antoszczak M, Müller S, Cañeque T, Colombeau L, Dusetti N, Santofimia-Castaño P, *et al*. Iron-Sensitive Prodrugs That Trigger Active Ferroptosis in Drug-Tolerant Pancreatic Cancer Cells. Journal of the American Chemical Society. 2022;144(26):11536-45.
24. Wang X, Li Y, Li Z, Lin S, Wang H, Sun J, *et al*. Mitochondrial Calcium Uniporter Drives Metastasis and Confers a Targetable Cystine Dependency in Pancreatic Cancer. Cancer research. 2022;82(12):2254-68.
25. Zheng C, Zhang B, Li Y, Liu K, Wei W, Liang S, *et al*. Donafenib and GSK-J4 Synergistically Induce Ferroptosis in Liver Cancer by Upregulating HMOX1 Expression. Advanced science (Weinheim, Baden-Wurttemberg, Germany). 2023;10(22):e2206798.
26. Zhang Q, Xiong L, Wei T, Liu Q, Yan L, Chen J, *et al*. Hypoxia-responsive PPARGC1A/BAMBI/ACSL5 axis promotes progression and resistance to lenvatinib in hepatocellular carcinoma. Oncogene. 2023;42(19):1509-23.
27. Ding Z, Pan Y, Shang T, Jiang T, Lin Y, Yang C, *et al*. URI alleviates tyrosine kinase inhibitors-induced ferroptosis by reprogramming lipid metabolism in p53 wild-type liver cancers. Nature communications. 2023;14(1):6269.
28. Conche C, Finkelmeier F, Pešić M, Nicolas AM, Böttger TW, Kennel KB, *et al*. Combining ferroptosis induction with MDSC blockade renders primary tumours and metastases in liver sensitive to

immune checkpoint blockade. Gut. 2023;72(9):1774-82.

29. Tan X, Kong D, Tao Z, Cheng F, Zhang B, Wang Z, *et al*. Simultaneous inhibition of FAK and ROS1 synergistically repressed triple-negative breast cancer by upregulating p53 signalling. Biomarker research. 2024;12(1):13.
30. Zou Y, Zheng S, Xie X, Ye F, Hu X, Tian Z, *et al*. N6-methyladenosine regulated FGFR4 attenuates ferroptotic cell death in recalcitrant HER2-positive breast cancer. Nature communications. 2022;13(1):2672.
31. Ye L, Zhong F, Sun S, Ou X, Yuan J, Zhu J, *et al*. Tamoxifen induces ferroptosis in MCF-7 organoid. Journal of cancer research and therapeutics. 2023;19(6):1627-35.
32. Shen L, Zhang J, Zheng Z, Yang F, Liu S, Wu Y, *et al*. PHGDH Inhibits Ferroptosis and Promotes Malignant Progression by Upregulating SLC7A11 in Bladder Cancer. International journal of biological sciences. 2022;18(14):5459-74.
33. Hodara E, Mades A, Swartz L, Iqbal M, Xu T, Bsteh D, *et al*. m(6)A epitranscriptome analysis reveals differentially methylated transcripts that drive early chemoresistance in bladder cancer. NAR cancer. 2023;5(4):zcad054.
34. Asif K, Adeel M, Rahman MM, Caligiuri I, Perin T, Cemazar M, *et al*. Iron nitroprusside as a chemodynamic agent and inducer of ferroptosis for ovarian cancer therapy. Journal of materials chemistry B. 2023;11(14):3124-35.
35. Xuan Y, Wang H, Yung MM, Chen F, Chan WS, Chan YS, *et al*. SCD1/FADS2 fatty acid desaturases equipoise lipid metabolic activity and redox-driven ferroptosis in ascites-derived ovarian cancer cells. Theranostics. 2022;12(7):3534-52.
36. Huang YP, Wang YX, Zhou H, Liu ZT, Zhang ZJ, Xiong L, *et al*. Surufatinib combined with photodynamic therapy induces ferroptosis to inhibit cholangiocarcinoma in vitro and in tumor models. Frontiers in pharmacology. 2024;15:1288255.
37. Qu S, Qi S, Zhang H, Li Z, Wang K, Zhu T, *et al*. Albumin-bound paclitaxel augment temozolomide treatment sensitivity of glioblastoma cells by disrupting DNA damage repair and promoting ferroptosis. Journal of experimental & clinical cancer research : CR. 2023;42(1):285.
38. Wang Z, Tang S, Cai L, Wang Q, Pan D, Dong Y, *et al*. DRP1 inhibition-mediated mitochondrial elongation abolishes cancer stemness, enhances glutaminolysis, and drives ferroptosis in oral squamous cell carcinoma. British journal of cancer. 2024;130(11):1744-57.
39. Ni Y, Liu J, Zeng L, Yang Y, Liu L, Yao M, *et al*. Natural product manoalide promotes EGFR-TKI sensitivity of lung cancer cells by KRAS-ERK pathway and mitochondrial Ca(2+) overload-induced

ferroptosis. Frontiers in pharmacology. 2022;13:1109822.

40. Zhang Z, Xie T, Zhang S, Yin H, Zhang X, Zhang S, *et al*. Second generation androgen receptor antagonist, TQB3720 abrogates prostate cancer growth via AR/GPX4 axis activated ferroptosis. Frontiers in pharmacology. 2023;14:1110146.
41. Wang Z, Zong H, Liu W, Lin W, Sun A, Ding Z, *et al*. Augmented ERO1α upon mTORC1 activation induces ferroptosis resistance and tumor progression via upregulation of SLC7A11. Journal of experimental & clinical cancer research : CR. 2024;43(1):112.
42. Hsieh MS, Ling HH, Setiawan SA, Hardianti MS, Fong IH, Yeh CT, *et al*. Therapeutic targeting of thioredoxin reductase 1 causes ferroptosis while potentiating anti-PD-1 efficacy in head and neck cancer. Chemico-biological interactions. 2024;395:111004.
43. Li Y, Guo M, Qiu Y, Li M, Wu Y, Shen M, *et al*. Autophagy activation is required for N6-methyladenosine modification to regulate ferroptosis in hepatocellular carcinoma. Redox biology. 2024;69:102971.
44. Sun S, Qi G, Chen H, He D, Ma D, Bie Y, *et al*. Ferroptosis sensitization in glioma: exploring the regulatory mechanism of SOAT1 and its therapeutic implications. Cell death & disease. 2023;14(11):754.
45. Chen PH, Wu J, Xu Y, Ding CC, Mestre AA, Lin CC, *et al*. Zinc transporter ZIP7 is a novel determinant of ferroptosis. Cell death & disease. 2021;12(2):198.
46. Srbova L, Arasalo O, Lehtonen AJ, Pokki J. Measuring mechanical cues for modeling the stromal matrix in 3D cell cultures. Soft Matter. 2024;20(16):3483-98.

Table 1. Cancer organoid models in ferroptosis

Cancer types	Organoid models	Functions	Ref.
Colorectal cancer	CRC-derived organoid	Overcome acquired drug resistance in CRC by inducing ferroptosis	(17)
Colorectal cancer	Colonic epithelial organoids	Loss of colonic epithelial Hmox1 promotes ferroptosis	(15)
Colorectal cancer	Intestinal stem cell organoids	IFNγ as a key cytokine capable of arresting cancer stemness triggers GPX4-dependent ferroptosis	(16)
Colorectal cancer	Patient-derived organoids	Curcumin and andrographis exhibit anti-tumor effects via activation of ferroptosis	(12)
Colorectal cancer	CRC Organoids	Adipocyte-derived exosomes containing MTTP reduce ferroptosis susceptibility in CRC, promoting chemoresistance to oxaliplatin	(13)
Colorectal cancer	Patient-derived organoids	Andrographis-mediated chemosensitization in CRC via activation of ferroptosis	(18)
Colorectal cancer	CRC organoids	VitC disrupted iron homeostasis and increased ROS levels, ultimately leading to ferroptosis	(19)
Pancreatic cancer	Pancreatic organoids derived from a mouse model	Elevated FSP1 protects KRAS-mutated cells from ferroptosis during tumor initiation	(20)
Pancreatic cancer	Patient-derived organoids	Triggers immunogenic ferroptosis by targeting the MCP-GPX4/HMGB1 Axis	(21)

Pancreatic cancer	patient-derived organoids	Imidazole ketone erastin induces tumor regression and abrogates MCU-driven metastasis	(24)
Pancreatic cancer	SMAD4-positive organoids	Enhances cytotoxic effects by combining gemcitabine with ferroptosis inducers	(22)
Pancreatic cancer	PDAC organoids	Ferroptosis inducers inhibit tumor growth and overcome chemoresistance	(10)
Pancreatic cancer	PDAC organoids	the synthesis of small molecule chimeras of salinomycin derivatives and dihydroartemisinin, ccumulates in lysosomes and induces ferroptosis	(23)
Liver cancer	Patient-derived organoids	Donafenib and GSK-J4 synergistically induced ferroptosis in liver cancer by upregulating HMOX1 expression	(25)
Liver cancer	Organoid models derived from HCC patients	Metformin restores PPARGC1A expression and enhances ferroptosis.	(26)
Liver cancer	HCC organoids	Unconventional prefoldin RPB5 interactor (URI) mediates resistance to tyrosine kinase inhibitors (TKIs)-induced ferroptosis	(27)
Liver cancer	CRC organoids	Combining ferroptosis induction with MDSC blockade rendered primary tumors and metastases	(28)
Breast cancer	MCF-7 organoid models	Tamoxifen can induce ferroptosis in MCF-7 organoid models	(31)

Breast cancer	Human TNBC organoid models	Simultaneous inhibition of FAK and ROS1 synergistically repress tumor growth by upregulating p53 signaling and inducing ferroptosis	(29)
Breast cancer	Breast cancer organoids	Combining anti-FGFR4 and anti-HER2 therapies induce ferroptosis in HER2-positive breast cancer	(30)
Bladder Cancer	BCa cell organoid models	PHGDH inhibitor NCT-502 induces ferroptosis in BCa cell organoid models	(32)
Bladder Cancer	Patient-derived organoids	m6A modifications affect chemoresistance by controlling SLC7A11 protein levels, influencing ferroptosis sensitivity.	(33)
Head and neck cancer	HNSCC Organoid models	Targeting thioredoxin reductase 1 (TrxR1) induces ferroptosis and potentiates the efficacy of anti-PD-1 therapy	(42)
Gastric cancer	Patient-derived organoid model	CAFs impair the cytotoxic function of NK cells in gastric cancer by inducing ferroptosis via iron regulation	(11)
Gastric cancer	Patient-derived organoids	Inhibition of STAT3-ferroptosis negative regulatory axis suppresses tumor growth and alleviates chemoresistance	(14)
Ovarian Cancer	Ovarian cancer organoids	SCD1/FADS2 fatty acid desaturases equipoise lipid metabolic activity and redox-driven ferroptosis in ascites-derived ovarian cancer cells	(35)

Ovarian Cancer	Ovarian cancer organoids derived from HGSOC	FeNP inhibits GPX4 activity, leading to induction of ferroptosis.	(34)
Cholangiocarcinoma	Patient-derived organoids	Combining surufatinib with photodynamic therapy induces ferroptosis and inhibits tumor growth	(36)
Glioblastoma	GBM patients derived organoids (PDOs) models	Combination treatment can enhance ferroptosis through regulating HOXM1 and GPX4 expression.	(37)
Oral squamous cell carcinoma	Patient-derived organoid model	DRP1 inhibition-mediated mitochondrial elongation drives ferroptosis and abolishes cancer stemness	(38)
Lung cancer	Lung cancer organoids	MA promoted ferroptosis by targeting the NRF2-SLC7A11 axis and inducing mitochondrial Ca2+ overload-induced FTH1 pathways	(39)
Prostate cancer	Organoid cultures derived from prostate cancer cells	TQB3720 promotes ferroptosis through inhibition of the AR signaling pathway	(40)
Laryngeal squamous cell carcinoma	Organoid models derived from LSCC patients	Augmented ERO1α upon mTORC1 activation induce ferroptosis resistance and tumor progression via upregulation of SLC7A11	(41)

Scipolis
May the Science be with you
OPEN ACCESS

JOBs
Journal of Organoid & Bioscience

Article

Potential Role of Herbal Remedies on Mesenchymal Stem Cells: An Overview of New Therapeutic Strategies for Osteoporosis

Yincong Liu[1a], Chuncai Li[1a], Mingxing Deng[1], Yuxiao Ma[2], Zhao Jin[1]*, Chongyang Shen[1]*

[1] School of Basic Medical Sciences, Chengdu University of Traditional Chinese Medicine, Chengdu, China

[2] Department of Biology, New York University, New York, USA

[a] These authors contributed equally to this work.

*Correspondence: dr.jinzhao@cdutcm.edu.cn，scylc@163.com

https://doi.org/10.62714/XEUW8715

Abstract

Worldwide, osteoporosis（OP）affects more than 200 million people, and as people get older, the disease is more common. It is anticipated that by 2025, aging country populations will increase the prevalence by as much as 50%. Osteoporosis is a degenerative skeletal condition that causes decreased bone mass and degeneration of the bone microstructure, which raises the risk of fracture and increases bone fragility. The irreversible nature of bone loss in the body makes osteoporosis a difficult illness for doctors to treat. Mesenchymal stem cells (MSCs), a multipotential cell with the capacity to self-renew and specialize into a number of cell types, are currently the source of preference for cell-based therapies due to their crucial role in tissue repair and synthetic biology. Bone marrow-derived mesenchymal stem cells (BMSCs) have long been employed in both preclinical and clinical research to treat osteoporosis. Even while new transplantation therapies using mesenchymal stem cells (MSCs) are promising, they are also expensive and urgent issues need to be resolved about their safety, transplantation effectiveness, and standardization of manufacturing techniques. Indeed, naturally occurring phytochemical substances (herbs) offer significant anti-inflammatory, pro-differentiation, and tissue regeneration potential, and the differentiation, migration, and immunomodulatory capabilities of MSCs treated with herbal extracts show promise in diseases like osteoporosis, neurological diseases, and other tissue degenerative disorders. In the creation of novel therapeutic approaches by MSCs for the treatment of osteoporosis illnesses, they have attracted a lot of interest. In this article, we summarize the most recent findings on herbal

extracts' effects on MSCs differentiation, migration, immunosuppression, and epigenetic regulation as well as potential mechanisms of action. Additionally, based on the yin-yang theory of Chinese traditional medicine, we did a thorough analysis of the molecular phenotypic variations between yin and yang in osteoporosis with the goal of improving guidance on the pathophysiology and clinical application of osteoporosis.

Keywords: herbal remedies; mesenchymal stromal cells; osteoporosis; immunological regulation; epigenetic regulation;

1. Introduction

Due to their enormous regeneration potential, human mesenchymal stem cells (MSCs) have gained popularity in the field of bioengineering and cell therapy[1]. MSCs were first demonstrated by Friedenstein and his associates in 1976[2]. Adult MSCs have been identified in a variety of tissues, including bone marrow, the umbilical cord, placental tissues, adipose tissues, synovial membrane and fluid, peripheral blood, dental pulp, and endometrial tissues[3]. Nevertheless, MSCs from each niche retain several features in common (like as marker expression) while differing in others (like as self-renewal and differentiation capacity). According to a recent study, perinatal niches (i.e., CPJ) present a more favorable environment for maintaining primitive MSCs than adult tissues do[4]. Additionally, Bone marrow is the putative source of various adult stem cells. Adipocytes, osteocytes, and chondrocytes are just a few of the connective tissues that mesenchymal stem cells (MSCs) obtained from the bone marrow can differentiate into[5]. MSCs are a promising cell source for regenerative medicine due to their flexible and controllable differentiation potential, ability to release a range of neurotrophins, and capacity to alter the immune system of the recipient[6].

As of 2022, a total number of 1902 clinical trials on stem cell therapy worldwide were recorded in the database of the U. S. National Library of Medicine. Of them, 499 of which are related to MSCs therapy accounting for nearly one- fourth[7]. Moreover, they provide a broad range of applications in tumors, immune disorders, neuroinflammation, and the replacement of damaged tissue due to accidents. Through their immunosuppressive or immunomodulatory characteristics in the tumor microenvironment, MSCs control immune response and accelerate tumor growth[8]. MSCs can be manipulated and transformed into effective drug carriers and transfected with anticancer genes for the therapeutic intervention of osteosarcoma due to their special properties[9]. In the context of treating liver illnesses, particularly acute liver failure, MSCs and their derivative exosome have emerged as a potential strategy[10]. Recent research shows that exosomes from MSCs improved functional recovery, encouraged neurogenesis, and decreased neuroinflammation in rats following traumatic brain injury (TBI)

[11]. A novel therapeutic approach to prevent or treat sepsis and sepsis-induced liver injury has been developed as a result of research into the potential of MSCs to increase macrophage polarization and alleviate sepsis in mice models. In order to restore damaged skeletal muscle, a novel ROS-scavenging hydrogel (Gel) containing mesenchymal stem cells (MSCs) was created (Gel@MSCs)[12-14]. MSCs have been used in investigations on diabetic nephropathy (DN) to convert 'Mφ' into an anti-inflammatory phenotype and to mitigate kidney damage in DN animals' besides mitochondrial transfer[13].

Osteoporosis (OP) is a metabolic bone disease typically characterized by low bone mass and deterioration of bone tissue microstructure, which leads to an increased risk of brittle fracture[15]. According to reports, osteoporosis and fractures brought on by the condition frequently cause morbidity and mortality in the elderly[16]. Researchers advise assessing the risk of osteoporosis in all postmenopausal women and men aged 50 and older[17]. Based on the American Association of Clinical Endocrinologists/American Society of Endocrinology Clinical Practice Guidance, osteoporosis is identified by the occurrence of fragility fractures in the absence of other metabolic bone disorders and even with a normal bone mineral density (T-score). It is also identified by T-scores of 2. 5 or less in the lumbar spine (anteroposterior), female neck, entire hip, or 1/3 radius (33% radius)[18]. The pathogenesis of osteoporosis is primarily influenced by homing disorder, decreased osteogenic differentiation ability, aging of mesenchymal stem cells, an unbalanced microenvironment, and disorders of immune control[19]. The chosen cell source for cell therapy is mesenchymal stem cells (MSCs), a kind of pluripotent cell that can transform into osteoblasts, adipocytes, or chondrocytes[20]. Recently, a large number of bone marrow mesenchymal stem cells have been applied to preclinical and clinical studies of osteoporosis. Up to now, the treatment of osteoporosis by MSCs mainly focuses on osteogenic and adipogenic differentiation, immune regulation, cell migration, and epigenetic regulation to improve the quality of life for patients. However, recombinant cytokines and growth factors, which are frequently utilized as proliferation and differentiation factors in the current stem cell therapy, have a high price tag and may have negative side effects[21]. Moreover, the efficacy of MSCs therapy is presently limited by low retention and survival ratio of transplanted cells, caused by oxidative stress, nutrient deprivation and inflammatory environment at injured sites. Because of these shortcomings, researchers have been searching for effective alternatives to the proteins and cytokines required for stem cell therapy.

Numerous herbal treatments for similar diseases have been made available by conventional medical systems since the dawn of time, herbal remedies are a promising alternative and supplementary scheme, which can significantly improve the patient's condition and significantly reduce the symptoms of the disease[7]. Although the majority of the time, the exact method by which individual and combined herbal plant extracts work is yet unknown. A deeper understanding of the therapeutic mechanism may be

gained by examining the effects of various herbal extracts on stem cell differentiation, migration, immunosuppression, and epigenetic regulation. In earlier investigations, herbal extracts were found to have significant potential for MSCs proliferation, differentiation, migration, anti-aging, and immune response management. More recently, due to its demonstrated osteogenic, anti-adipogenic, homing, anti-inflammatory, and medicinal effects, the utilization of herbal treatments and their extracts for the treatment of joint problems, involving OP, has also gained widespread acceptance in modern Western medicine[22-25]. The effectiveness of herbal components or herbal combinations and their application in the treatment of various ailments, including anti-inflammatory effects on chondrocytes and proliferation of cardiomyocytes, has really been the subject of numerous research[26,27]. The availability of herbal medications for intervention and treatment to a wide variety of people at a reasonable cost and with few adverse side-effects is an essential benefit[28]. Therefore, this literature review focuses on the potential of herbs as natural stimulants between MSCs and osteoporosis and their potential in maintaining bone homeostasis, as well as in the prevention and treatment of joint diseases, especially osteoporosis, with a macroscopic description of osteoporosis from a yin-yang perspective.

2. Effects of herbal extracts on the osteogenic differentiation and adipogenic differentiation of MSCs.

Apparently, bone loss is probably could be prevented by restoring the balance between bone-forming osteoblasts and bone-resorbing osteoclasts, as well as enhancing osteogenesis and reducing adipogenesis in MSCs. Much of the research in the following sections elaborate on such herbal remedies that showed great potential in the differentiation of MSCs.

2.1 Osteogenic effects of herbal extracts

First, Herba Epimedii has been used in traditional medicine to improve breastfeeding, encourage menstruation, ease delivery, and lessen the discomfort of dysmenorrhea, a disease involving estrogenic activity. According to estrogenic activity, BM-derived MSCs' ability to differentiate into osteoblasts was significantly enhanced by upregulating the gene expression of runt-related transcription factor 2 (RUNX2) and BMP-2[29], where in research on the total flavonoids of Herba Epimedii (HETF). Second, Curcumin, a kind of phenolic natural product isolated from the rhizome of turmeric, could promote the osteogenic differentiation of MSCs by increasing the mRNA expression of RUNX2 and osteocalcin specific to osteoblasts. In vivo studies have shown that the effect of curcumin on the osteogenic differentiation of MSCs is related to the expression of heme oxygenase (HO)-1, which is consistent with the role of ALP activity and RUNX2 mRNA on the osteoblast differentiation of MSCs[30]. Third, Berberine (BBR), known as a quaternary ammonium alkaloid isolated from Coptis coptidis. The canonical Wnt/β-catenin signaling

pathway is activated by BBR, which also strongly increases the osteogenic differentiation of MSCs by enhancing RUNX2 expression in general. In contrast, DKK-1, a specific inhibitor of Wnt signaling, can effectively inhibit the effect of BBR on osteogenesis, supporting the idea that Wnt/-catenin signaling is involved in BBR-induced osteogenesis[31]. Additionally, obtainable from Chinese medicine, cnidium lactone is a primary element having pharmacological action, which has the function of warming kidneys and strengthening Yang. Researchers suggested that cnidium lactone combined with ERα and other receptors, then activated the BMP-2/SMAD signaling pathway and ultimately stimulated BMSCs differentiation and mineralization[32]. Much more interesting, the traditional Chinese medicine compound Jiawei Yanghe soup (JWYHD) contains cooked Rehmannia, cinnamon, papaya, Jianji, caulis pathology, and other Chinese herbs, which can regulate bone homeostasis in BMSCs by activating the BMP-SMAD signaling pathway, restore the dynamic balance of osteogenic-lipogenic differentiation of MSCs, improve bone microstructure and reduce bone loss in ovariectomized PMOP rats[33].

2.2 Anti-adipogenic effects of herbal extracts

Several chemical compounds of herbal origin, including quercetin, Ligustrum lucidum, arginine, resveratrol, and Icariin were identified its anti-adipogenic activities, while restoring the balance between bone anabolism and catabolism.

Since, quercetin is a compound that exists in the form of glycosides in many plants, such as the leaves of Platycladus lateralis and Morus parasitis, and an inhibitory effect on adipocyte differentiation has been demonstrated when quercetin 3-O-β-D-galactopyranoside (Q3G) was used to induce MSCs for adipogenesis, while C/EBPα and PPARγ showed significantly lower levels, which in turn affected adipogenesis[34]. Besides, Ligustrum lucidum and psoralea (LP) are used in traditional medicine to strengthen the body and strengthen the bones, tighten the urine, and nourish the liver and kidney, which are all conditions involved in the effective promotion of bone health. The study based on the pairing of two herbs showed that the addition of LP to the osteogenic medium could significantly inhibit the proliferation and adipocyte differentiation of bone marrow derived human MSCs, and the number of adipocytes was significantly reduced[35]. Furthermore, arginine reduces triglyceride (TG) levels and exerts anti-lipid activity by inhibiting the adipose transcription factor peroxisome proliferator-activated receptor γ (PPARγ). This study provides the first experimental evidence of the bone-promoting and anti-fat effects of arginine[36], similar findings that were later confirmed in studies on resveratrol and Icariin [37] [38].

Together, these data suggest that herbs or herbal extracts have a significant modulating effect on osteogenic-lipogenic differentiation of MSCs. For instance, Icariin can, as was already mentioned, encourage MSCs development into osteoblasts[29,38], while suppress formation of adipocyte-like cells,

and the ERK signaling pathway may play a critical role in the interaction between icariin and its main metabolite, icariin II, on the osteogenesis and adipogenesis of MSCs[39]. These action mechanisms are consistent with osteoblast differentiation who have increased RUNX2 and Osterix (OSX), and reduced PPARγ and TNF-α. Numerous transcription factors and signaling pathways control the development of ubiquitous mesenchymal progenitor cells into distinct kinds of skeletal-associated cells, and several studies have shown that disturbances in the dynamic balance of bone formation and bone resorption are crucial to the pathogenesis of osteoporosis. Thus, in order to maintain bone homeostasis, which is essential for controlling the balance of MSCs during the process of osteogenic and adipogenic differentiation. Furthermore, it is known that bone marrow MSCs are effective in the treatment of osteoporosis. Whether transplantation of MSCs is clinically safe and effective for treatment remains unknown; however, in vitro data from multiple researches indicates that reactivation of the osteogenic differentiation capacity of MSCs by herbs, a natural and non-toxic in vitro activator, are a viable method.

3. Effects of herbal extracts on the immunological regulation of MSCs

It has been demonstrated that the inflammatory microenvironment interferes with MSCs function and blocks their osteogenic, lipogenic, and myogenic actions[40]. Recent studies in this area have discovered that specific herbal extracts can successfully lower inflammatory cytokine production and balance the helper T-cell population to enhance the immunomodulatory function of MSCs[41-43].

Immunomodulation of MSCs by herbs could be classified as cytokine modulation, which elevates or decreases the expression of inflammatory factors, or gene modulation, which alters gene expression to improve the performance of MSCs in the clinic. Total glucosides of paeony (TGP), for example, significantly enhanced MSCs immunomodulatory action by reducing IL-6 and TNF-α expression and upregulating TGF-β and IL-10 expression[44], while MSCs treated with phytosomal curcumin (PC) may influence the immune function of hDPSC by regulating the transcriptional levels of miR genes such as miR-23, miR-155[45]. Similarly, Ginkgo biloba extract can regulate oxidative stress-mediated bone homeostasis and inhibit the expression of inflammatory cytokines such as TNF-α, IL-6, IL-1β and IL-10 in different cells and animal models closely related to bone diseases[46-48]. In addition, it has been discovered that herbal extracts with immunomodulatory and anti-angiogenic properties, like Ganoderma lucidum polysaccharide (F3) and forsytha perforatum, can also be used in pharmaceutical formulations[49,50]. Herbs, as discussed and demonstrated above, hold great promise for MSCs-mediated immunomodulation in the treatment of diseases, particularly because of their anti-inflammatory and anti-oxidant properties[40].

Herbal-Stem Cell Combination therapies such as Astragalus and the mesenchymal stem cells, which

derived from bone marrow and umbilical cord, has been applied clinically to patients who have suffered skin and other tissue damage in order to speed up wound healing. Early vitro trials demonstrated that PG2 effectively promoted the proliferation of UCMSCs and their immunosuppressive effects, but did not clarify the precise mechanism[51]. A later vitro study, Lipopolysaccharide (LPS)-induced inflammation model in MSCs[52], confirmed that the co-treatment with astragaloside and baicalin could inhibit the expression of IL-1β, IL-8 and TNF-α more effectively, and further control - group analysis revealed that they also have the capacity to suppress apoptosis, lessen inflammatory response, and encourage differentiation and proliferation of epithelial cells, findings that were later confirmed in another study[53]. The ability of MSCs to differentiate combined with their immunosuppressive effect makes them a prime option to move towards damaged tissue for healing and is considered to be an even more appealing choice for regenerative medicine; however, the majority of in vivo investigations revealed limited MSCs engraftment and trans-differentiation within diseased or damaged tissues[5], which may have an impact on the patient's healing efficiency and subsequent maintenance. Wang *et al.*,[53]have demonstrated that Astragalus and human mese etabolism depends on the interplay of immune cells and MSCs, and abnormally high inflammatory factor levels cause excessive osteoclast activation, which causes pathological bone breakdown and bone loss. Thus, the new therapeutic options serve to improve the inflammatory microenvironment, inhibit the inflammatory response and promote the immunosuppressive role of MSCs in bone repair.

4. Effects of herbal extracts on the migration of MSCs.

Current cell-based therapeutic approaches have demonstrated efficacy and broad applicability in the treatment of degenerative and injurious diseases. Despite the significant regenerative potential of MSCs in tissue repair, they have shown a high degree of variability in therapeutic efficacy[54-56]. Recent studies suggest a strong correlation between the MSCs migration potential and their therapeutic efficacy in humans, and is proposed to use highly migratory subpopulations of stem cells in cell-based therapeutics. We enumerate several studies on the effects of herbal extracts on MSCs migration in recent years, and combined herbal-MSCs therapies have shown great potential in certain conditions[54].

This migration of MSCs is regulated by a number of factors. In essence, the process of seeking is based on specific molecular interactions rather than passive distribution. Thus, high levels of expression of appropriate adhesion molecules and chemotactic factors are required in the enhancement or inhibition of migration of MSCs by herbs. For instance, by activating CXCR4, tanshinone IIA and astragaloside IV encourage the migration and homing of mesenchymal stem cells in vitro and vivo[57]. Also, the results of the wound healing assay and gene expression analysis showed that eugenol increases BM-MSCs'

capacity for migration by over-expressing c-Met[58,59]. Besides, Lin *et al*.,[60] found that naringin activates the Ras signaling pathway to increase the production of cell chemokines and the migration of MSCs. It is worth noting that herbs may not only enhance the migratory homing ability of MSCs, but also inhibit it, which gives more possibilities for MSCs to treat diseases. Such as, Matrine inhibites Heterotopic ossification (HO) via inhibition of TGF-β-induced migration and osteogenic differentiation of MSCs in mice[61]. Agathis flavonoids also decreased the levels of STAT3 expression, which suppressed the migration and differentiation of heterogeneous glioblastoma (GBM) cells[62]. Furthermore, a lower quantity of curcumin might encourage osteogenic differentiation as well as immunomodulatory gene expression in BM-MSCs[63,64],while this has not been directly linked to cell migration. It has been shown that Higher concentrations of curcumin would induce BM-MSCs death and decrease cell proliferation and migration[65], suggesting that the migratory ability of MSCs may be related to the concentration of herbal extracts.

Salidroside could improve wound healing in diabetic patients by regulating paracrine function and proliferation of MSCs in a hyperglycemic environment and it was also found that salidroside pretreatment enhanced the migration capability of MSCs impaired under hyperglycemia, which may be an effective strategy to improve the survival and therapeutic efficacy of MSCs[66]. Additionally, Furumoto *et al*. found that Mallotus philippinensis bark extracts (EMPB) improved the wound healing status of the organism due to its effective mobilization and homing promotion ability of MSCs[67], indicating that herbal extracts with good properties can improve tissue regeneration rates and can be used for stem cell therapy and tissue engineering as an alternative treatment. Similar effects were seen in cyasterone[68] and Notoginsenoside R1[69], which cyasterone might encourage MSCs homing and osteogenic differentiation through related genes such as OPN, ALP, and BMP-2 as well as Notoginsenoside R1. Moreover, resveratrol was previously shown to increases the therapeutic efficacy of UC-MSCs by enhancing cell migration and reducing neuroinflammation mediated by MAPK signaling[70], suggesting that the migratory capabilities of MSCs manipulated with herbal extracts have potential in the treatment of disorders including diabetes, neuroinflammatory diseases, and other tissue-damaging conditions, and thus undoubtedly the combined herbal- mesenchymal stem cell therapy brings light to address this challenge despite the low rate and effectiveness of MSCs homing to damaged tissues remains a major challenge for regenerative medicine.

5. Effects of herbal extracts on the Epigenetic regulation of MSCs

Epigenetic mechanisms involved in the development of osteoporosis have long been the focus of researchers, and there is growing evidence that epigenetic modifications may represent the mechanism

linking genetic and environmental factors to increased risk of osteoporosis and fracture, primarily including DNA methylation, histone modifications and non-coding RNAs (lncRNA, microRNA and CircRNA)[71]. Gaining or losing of these epigenetic modifiers alters the epigenetic patterns of osteoblasts and the cells that are associated with them, which in turn impacts bone homeostasis, the bone immune microenvironment, and the pathological alterations implicated with osteoporosis[72]. In recent years, herbs or herbal extracts have been shown to be effective in modulating epigenetic mechanisms, especially in a range of diseases closely related to bone metabolism-related processes such as osteogenic differentiation, osteogenesis, and bone reconstruction, such as osteoporosis. In view of the significant role that epigenetic mechanisms play in controlling the metabolism of bones. We enumerate the research progress of related herbal epigenetic modulation MSCs in osteogenic lipogenic differentiation and osteoporosis pathogenesis to provide fresh directions for the treatment of diseases related to bone metabolism[73].

5.1. Resveratrol (RES)

Resveratrol (trans-3,4,5-trihydroxystilbene) is a natural polyphenol phytoestrogen found in rhubarb and other natural plants with osteogenic and osteoinductive properties[74,75] that can promote the osteogenic differentiation of adipose stem cells (ASC) and bone marrow mesenchymal stem cells (BMSCs) through epigenetic regulation. For BMSCs, resveratrol can stabilize the osteogenic/osteoclastic homeostasis of BMSCs by upregulating miR-146a expression levels, thereby inhibiting the transcription of β-catenin proteins by FOXO factors and exerting anti-osteoclastogenic effects through the Sirt1/NF-κB signaling pathway （Fig 1） [76]. Interestingly, it is the res-modulated miR-193a inhibition that appears to be responsible for the activation of Srit7/NF-κB signaling pathway in the process of promoting osteogenic differentiation of BMSCs[77]. What's more, RES could also enhance the expression of miR-92b and suppressing the expression of Nox4/NF-κB signaling pathway activity and osteoclast proliferation[78]. It's worth noting that RES could regulates miR-320c expression and thus promotes the differentiation of BMSCs to osteoblasts in a dose-dependent manner, while RUNX2 may play a direct target role in this process （Fig 1） [79]. These results suggest RES-mediated epigenetic regulation is critical for BMSCs osteogenic development.

5.2. Icariin

Icariin (ICA) is the main active ingredient of the traditional Chinese herb Epimedium, which is commonly used clinically for the treatment and prevention of many health disorders such as cardiovascular disease, osteoporosis, or sexual dysfunction[80]. It is reported that BMSCs treated with ICA in the patients with steroid-associated osteonecrosis exhibited significant osteogenesis-promoting and lipogenesis-inhibiting effects in patients with steroid-associated osteonecrosis, which may be attributed to ABCB1 promoter

demethylation in BMSCs[81]. Additionally, miR-23a appears to be important in the epigenetic regulation of osteogenic-lipogenic differentiation of bone marrow MSCs under the treatment of ICA[82], as ICA promotes the proliferation and osteogenic differentiation of BMSCs by regulating the miR-23a-mediated Wnt/β-catenin signaling pathway while inhibiting adipogenesis[83], while miR-21-5p may play a similar role[84].

5.3. Kaempferol and Zingerone

Both kaempferol and zingerone are active components isolated from Zingiber officinale[85,86]. The former can promote osteogenesis of BMSCs by mediating multiple signaling pathways such as PI3K / Aktand promote osteogenic differentiation of BMSCs and improve osteoporosis by reducing miR-10a-3p and increasing CXCL12 expression levels[87], while the latter's molecular mechanism for treating bone and bone-related disease was to target Smad7 via miR-590 resulting in Runx2 protection for osteoblast differentiation[88]. It was also found that gingerone contributes to differentiation of osteoblasts through the miR-200c-3p / smad7 regulatory axis in human bone mesenchymal stem cells (hBMSCs) [89].

5.4. Neohesperidin (NH)

NH could promote the osteogenic differentiation of human BMSCs, which in vitro studies suggest is likely to inhibit the histone modifications of LncRNA SNHG1 by regulating SNHG1 gene expression and occupancies of H3K4me3 and H3K27me3[90]. Similarly, NH was found to promote the proliferation of BMSCs, meanwhile bi-directionally regulating the occupancy of H3K27me3 and H3K4me3 in another study. Whereas LncRNA HOTAIR overexpression in particular has also been shown to inhibit osteogenic differentiation, but increase lipogenic differentiation[91].

5.5. Quercetin (QUE)

QUE could not activates the Wnt/β-catenin pathway and promotes hBMSCs osteogenic differentiation via the H19/miR-625-5p axis[92], but also partially modulates it by miR-206/Cx43 pathway, including increasing the expression of Cx43 and decreasing the expression of miR-206[93]. Significantly, QUE may regulate the osteogenic and adipocytic differentiation of ERα-deficient BMSCs to promote osteogenesis of BMSCs through circRNA–miR-326-5p–mRNA axis[94].

Majority of epigenetic pathways regulate the dynamic balance of bone formation and bone resorption, and numerous preclinical researches have shown that herbs play an important role in regulating the epigenetic mechanisms of MSCs. In recent years, extensive research has been conducted in this area (Table 1). For example, Cao *et al*[95] and Wu *et al*[96] found Astragaloside IV(AS-IV) improved tibial defects in rats through down-regulating miR-124-3p via miR-21/NGF/BMP2/Runx2 pathways. Li *et al*[97] reported that miR-671 is abundantly present in small extracellular vesicles derived from Rhizoma Drynariae-pretreated[98] bone mesenchymal stem cells and regulates osteogenesis by targeting TAK1

to mediate WNT signaling. Meanwhile, Huang *et al*[99] observed elevated cell viability and improved osteogenic differentiation of BMSCs induced by psoralen[100,101]. Furthermore, Ferulic acid (FA)[102,103], artesunate (ART)[104,105], morinda officinalis polysaccharide[106,107] and puerarin[108-110] were similarly found to enhance osteogenic differentiation and inhibit lipogenic differentiation in BMSCs and to increase bone mass in bone grafted rats via epigenetic modulation.

While the epigenetic mechanisms involved in the development of osteoporosis remain unclear, epigenetic regulation of MSCs plays an integral role in the pathogenesis of osteoporosis. Supported by these findings, herbs are proposed to play an important role in regulating the epigenetics of MSCs, particularly through the regulation of non-coding RNAs, which in turn affects the proliferative activity and osteogenic differentiation of MSCs. Furthermore, herbs have been shown to mediate the expression of signaling pathways and cytokines through epigenetic regulation via histone modifications and DNA methylation. Interestingly, bone mineral density (BMD) is considered to be a common clinical measure of bone content and the gold standard for its diagnosis and treatment[111], yet most of the genome-wide association study hits for BMD were found to be in non-coding regions[112]. In summary, herbal modulation of epigenetic mechanisms of MSCs for estrogen deficiency and age-related osteoporosis is a feasible therapeutic strategy.

6. Molecular phenotypes of Yin-Yang in osteoporosis

In recent years, much has been accomplished in human research on osteoporosis, but most of these studies have focused on the microscopic rather than the systemic level. Therefore, gathering detailed information to fully describe the organism remains a major challenge[113]. Interestingly, recent cancer-related studies have increasingly invoked the ancient Chinese theory of yin-yang, and a variety of tumor-related genes and proteins have been reported to regulate various types of cancer in a yin-yang manner[114], and these studies have made the yin-yang theory popular in medical conditions worldwide[115]. The ancient Chinese theory of yin-yang is increasingly being invoked by modern researchers in an effort to move beyond the microscopic focus of disease research and to view biological phenomena at a macroscopic level[116]. Similarly, various genes, proteins and cells have been identified as having yin-yang effects by promoting or suppressing osteoporosis, which provide a more comprehensive and systematic understanding of this complex disease. The yin-yang relationship between osteoblasts and adipocytes or osteoblasts and osteoclasts in the bone marrow plays a key role in the development of osteoporosis. The balance between yin-yang has been considered as a cellular feature[117], and several yin-yang regulatory genes and proteins associated with osteoporosis have been identified in previous studies, such as mitogen-activated protein kinase, p38, PP5, RUNX2, PPARγ,

and orexin, as well as cells such as T cells, B cells, macrophages, neutrophils, and mast cells[118].

The guided differentiation of MSCs into adipocytes or osteoblasts depends on the transcription factors PPARγ and RUNX2. It was discovered that serine phosphorylation controls the activity of PPARγ and RUNX2, but in different ways. RUNX2 activation and PPARγ inactivation are caused by the same p38MAPK that mediates serine phosphorylation, whereas PPAR activation and RUNX2 inactivation are caused by protein phosphatase 5 dephosphorylating both proteins[119]. The delicate yin-yang balance between the phosphorylation and dephosphorylation of the two proteins is thought to be the mechanism that keeps MSCs undifferentiated and sensitive to differentiation factors, which suggest a synchronized and reciprocal mechanism that regulates the quick transition of MSC differentiation to osteoblasts or adipocytes. Furthermore, Wei *et al*. also demonstrated that orexin is a dual yin-yang regulator, promoting bone production through primary OX2R and leptin-mediated neuroendocrine control and inhibiting bone formation through secondary OX1R and local regulation of osteoclast development[120]. Both p38 MAPK and PP5 signaling regulating the differentiation of MSCs and the dual regulation of skeletal homeostasis by orexins are paradigms of yin-yang regulation of osteoporosis pathogenesis. The confirmation of these mechanisms could identify pharmacological targets for the treatment of bone and related metabolic diseases[113].

Bone marrow MSCs and neighboring immune cells play an important role in the establishment of the bone marrow immune microenvironment and are the site of interaction between microenvironmental components and mesenchymal cells[117]. Various immune cells are involved in the bone homeostasis of osteogenic lipogenic differentiation and osteoclastogenesis in MSCs in a direct or indirect manner[121]. The pathophysiology of osteoporosis can actually be explained by the harmony between yin and yang. In a healthy host, the immune system's balance is maintained between Tregs and inhibitory cytokines and effector cells and pro-inflammatory cytokines; this is referred to as "yang," and when homeostasis is upset, osteoporosis is typically brought on, which is referred to as "yin." The Yin-Yang hypothesis has the potential to provide a very effective explanation for the pathology of osteoporosis[122]. Various inflammation-related cells, including T cells, B cells, macrophages, neutrophils and mast cells, have yin-yang effects on osteoporosis[123], for instance, certain T lymphocyte subtypes release tumor necrosis factor alpha[124], which raises osteoblast apoptosis and, through B cells' production of nuclear factor receptor activator B ligand (RANKL), indirectly induces osteoclastogenesis. Whereas the release of interleukin 17 (IL-17) by Th17 cells leads MSCs differentiation towards osteogenesis, it is this IL-17 that indirectly stimulates osteoclastogenesis, resulting in bone loss in osteoporosis. Macrophage polarization is modified as the disease progresses, which may be significant to the pathogenesis of osteoporosis[125]. The role of macrophages on osteoblasts may also vary depending on their

polarization phenotype and the proteins and factors they produce[126].

In conclusion, recent advances in bioscience and research on osteoporosis have centered on cellular and molecular processes such cell proliferation and differentiation, apoptosis promotion and inhibition, cell migration, anti-inflammation and inflammation, and epigenetic regulatory mechanisms. As a result, the theoretical foundation is become increasingly intricate yet less coherent. Key ideas must be integrated into a macro theory or the framework would be incomplete or unstable[127]. The yin and yang hypothesis offers a macroscopic perspective of biological events (Fig 2). Additionally, Marilena *et al*. proposed a biochemical tool based on redox parameters (such as antioxidant capacity) that can be used to categorize and describe western drugs from a yin-yang perspective by fusing the yin-yang theory with contemporary antioxidant-oxidation theory[128]. However, by using LC-MS mapping, Huang *et al*. discovered that the yin-yang qualities of herbal remedies are closely related to the physical characteristics of the constituents, such as polarity and molecular mass, and that this classification has little to do with antioxidant qualities[129]. Whether the yin-yang phenotypes of osteoporosis have something in common with the yin-yang properties exhibited by herbs, and whether this could provide theoretical support for the combination of herbs with MSCs in the treatment of osteoporosis, is certainly an interesting point that we will also continue to follow and study.

7. Conclusion

The objective of this review is to assess the effect of phytochemicals (herbs) originating from plants on MSCs' capacity to treat osteoporosis while highlighting the contributions of these substances to MSCs migration, immunological control, osteogenic adipogenic differentiation, and epigenetic regulation. In aging civilizations, osteoporosis is a common occurrence that can result in acute discomfort, spine deformity, and fragility fractures. A very fascinating and insightful area of research, plant-derived phytochemicals as naturally powerful inducers and differentiation agents of MSCs have the ability to prevent bone disorders like osteoporosis and promise to produce new therapeutic approaches. Review of the literature indicates that herbal extracts have considerable effects on human bone marrow mesenchymal stem cells as stimulators of differentiation, immunological control, migration, and epigenetic regulation. There is a ton of evidence that several plants have beneficial pharmaceutical effects, such as anti-inflammatory, anti-adipogenic, and homing activities. These characteristics of MSCs treated with herb extracts could serve as a foundation for the therapy and replacement of tissue in the future for conditions like diabetes mellitus, myocardial infarction, neuropathy, and liver and brain injury.

Despite herbal medicines have been demonstrated to have anti-fat, migratory, and immunomodulatory characteristics, most of the current research concentrated on the control of MSC's osteogenic and

proliferative capability by plant extracts. In addition, investigations of MSCs from unconventional sources, such as placenta, umbilical cord, tooth pulp, and fat, are currently being examined due to the ethical considerations involved and the challenge of acquiring samples. We can find that plant sources of some compounds can be obtained through commercial gain, while others are obtained by extracting from plants, and some traditional region herbs such as traditional Chinese medicine, Indian Ayurvedic herb. Because most herbal medicines are not completely clear, in controlled clinical trials using the composition of the available components with the patient, there may be a potential difference between. Therefore, safety, toleration, and uniformity are serious considerations. Therefore, it is crucial for pharmaceutical businesses to evaluate and standardize the herbs and extracts they utilize to assure the security, effectiveness, and caliber of their pharmaceuticals before including Chinese herbal remedies in the normal treatment of osteoporosis. It is also anticipated. Once these fundamental obstacles are removed and handled, nutritional foods (herbal remedies) can be a highly beneficial replacement for hormonal medications (such selective estrogen receptor modulators), which have a number of adverse effects and are commonly used to treat osteoporosis in many parts of the world.

In osteoporosis treatment, therefore, MSCs treated with herbal extracts have the potential to induce differentiation, anti-inflammatory, pro-migration and epigenetic modulation, may produce high cost-effective, highly available, non-toxic alternative treatment application, thereby helping to control other degenerative and metabolic diseases. Though still in its infancy, the investigation of stimulants derived from natural plants has brought regenerative medicine fresh life.

Declaration

Ethics approval and consent to participate

Not applicable

Consent for publication

Not Applicable

Availability of data and materials

No dataset was generated or analyzed during this study.

Competing interests

No competing interest.

Funding

This study was supported by the National Natural Science Foundation of China (No. 82174226), Key Research Program of Chengdu Municipal Bureau of Science and Technology (2024-RC02-00025-CG),

Chengdu University of Traditional Chinese Medicine Xinglin Scholars' Fund (No. XSGG2020002)

Liu yincong1a, Li chuncai1a, Deng mingxing1, Ma Yuxiao2, Jin zhao1*, Shen chongyang

Author contributions

Shen Chongyang: conception and design, and interpretation. Liu yincong: manuscript writing; Li chuncai: formal analysis; Deng mingxing: interpretation; Ma Yuxiao: linguistic assistance. Jin zhao: manuscript writing.

Acknowledgements

We thank Dr. Chen xiongbin for linguistic assistance and pre-submission expert review.

References

1. Berebichez-Fridman, R.; Montero-Olvera, P.R. Sources and clinical applications of mesenchymal stem cells: State-of-the-art review. Sultan Qaboos Univ Med J 2018, 18, e264-e277.
2. Brown, C.; McKee, C.; Bakshi, S.; Walker, K.; Hakman, E.; Halassy, S.; Svinarich, D.; Dodds, R.; Govind, C.K.; Chaudhry, G.R. Mesenchymal stem cells: Cell therapy and regeneration potential. J Tissue Eng Regen Med 2019, 13, 1738-1755.
3. Ding, D.C.; Shyu, W.C.; Lin, S.Z. Mesenchymal stem cells. Cell Transplant 2011, 20, 5-14.
4. Sabapathy, V.; Kumar, S. Hipsc-derived imscs: Nextgen mscs as an advanced therapeutically active cell resource for regenerative medicine. J Cell Mol Med 2016, 20, 1571-1588.
5. Hwang, N.S.; Zhang, C.; Hwang, Y.S.; Varghese, S. Mesenchymal stem cell differentiation and roles in regenerative medicine. Wiley Interdiscip Rev Syst Biol Med 2009, 1, 97-106.
6. Tatullo, M.; Codispoti, B.; Pacifici, A.; Palmieri, F.; Marrelli, M.; Pacifici, L.; Paduano, F. Potential use of human periapical cyst-mesenchymal stem cells (hpcy-mscs) as a novel stem cell source for regenerative medicine applications. Front Cell Dev Biol 2017, 5, 103.
7. Kouchakian, M.R.; Baghban, N.; Moniri, S.F.; Baghban, M.; Bakhshalizadeh, S.; Najafzadeh, V.; Safaei, Z.; Izanlou, S.; Khoradmehr, A.; Nabipour, I., *et al*. The clinical trials of mesenchymal stromal cells therapy. Stem Cells Int 2021, 2021, 1634782.
8. Ahn, S.Y. The role of mscs in the tumor microenvironment and tumor progression. Anticancer Res 2020, 40, 3039-3047.

9. Chang, X.; Ma, Z.; Zhu, G.; Lu, Y.; Yang, J. New perspective into mesenchymal stem cells: Molecular mechanisms regulating osteosarcoma. J Bone Oncol 2021, 29, 100372.
10. Shokravi, S.; Borisov, V.; Zaman, B.A.; Niazvand, F.; Hazrati, R.; Khah, M.M.; Thangavelu, L.; Marzban, S.; Sohrabi, A.; Zamani, A. Mesenchymal stromal cells (mscs) and their exosome in acute liver failure (alf): A comprehensive review. Stem Cell Res Ther 2022, 13, 192.
11. Yang, Y.; Ye, Y.; Su, X.; He, J.; Bai, W.; He, X. Mscs-derived exosomes and neuroinflammation, neurogenesis and therapy of traumatic brain injury. Frontiers in Cellular Neuroscience 2017, 11.
12. Shan, H.; Gao, X.; Zhang, M.; Huang, M.; Fang, X.; Chen, H.; Tian, B.; Wang, C.; Zhou, C.; Bai, J., *et al*. Injectable ros-scavenging hydrogel with mscs promoted the regeneration of damaged skeletal muscle. J Tissue Eng 2021, 12, 20417314211031378.
13. Yuan, Y.; Yuan, L.; Li, L.; Liu, F.; Liu, J.; Chen, Y.; Cheng, J.; Lu, Y. Mitochondrial transfer from mesenchymal stem cells to macrophages restricts inflammation and alleviates kidney injury in diabetic nephropathy mice via pgc-1alpha activation. Stem Cells 2021, 39, 913-928.
14. Xu, Y.; Liu, X.; Li, Y.; Dou, H.; Liang, H.; Hou, Y. Spion-mscs enhance therapeutic efficacy in sepsis by regulating msc-expressed traf1-dependent macrophage polarization. Stem Cell Res Ther 2021, 12, 531.
15. Lane, J.M.; Russell, L.; Khan, S.N. Osteoporosis. Clin Orthop Relat Res 2000, 139-150.
16. Anam, A.K.; Insogna, K. Update on osteoporosis screening and management. Med Clin North Am 2021, 105, 1117-1134.
17. Johnston, C.B.; Dagar, M. Osteoporosis in older adults. Med Clin North Am 2020, 104, 873-884.
18. Camacho, P.M.; Petak, S.M.; Binkley, N.; Diab, D.L.; Eldeiry, L.S.; Farooki, A.; Harris, S.T.; Hurley, D.L.; Kelly, J.; Lewiecki, E.M., *et al*. American association of clinical endocrinologists/american college of endocrinology clinical practice guidelines for the diagnosis and treatment of postmenopausal osteoporosis-2020 update. Endocr Pract 2020, 26, 1-46.
19. Jiang, Y.; Zhang, P.; Zhang, X.; Lv, L.; Zhou, Y. Advances in mesenchymal stem cell transplantation for the treatment of osteoporosis. Cell Prolif 2021, 54, e12956.
20. Maqsood, M.; Kang, M.; Wu, X.; Chen, J.; Teng, L.; Qiu, L. Adult mesenchymal stem cells and their exosomes: Sources, characteristics, and application in regenerative medicine. Life Sci 2020, 256, 118002.
21. Buhrmann, C.; Honarvar, A.; Setayeshmehr, M.; Karbasi, S.; Shakibaei, M.; Valiani, A. Herbal remedies as potential in cartilage tissue engineering: An overview of new therapeutic approaches and strategies. Molecules 2020, 25.

22. Salehi, A.; Mobarhan, M.A.; Mohammadi, J.; Shahsavarani, H.; Shokrgozar, M.A.; Alipour, A. Cabbage-derived three-dimensional cellulose scaffold-induced osteogenic differentiation of stem cells. J Cell Physiol 2021, 236, 5306-5316.
23. Karadeniz, F.; Oh, J.H.; Lee, J.I.; Kim, H.; Seo, Y.; Kong, C.S. 6-acetyl-2,2-dimethylchroman-4-one isolated from artemisia princeps suppresses adipogenic differentiation of human bone marrow-derived mesenchymal stromal cells via activation of ampk. J Med Food 2020, 23, 250-257.
24. Jamshidi-Adegani, F.; Vakilian, S.; Rehman, N.U.; Al-Broumi, M.; Al-Kindi, J.; Alam, K.; Mozafarinahavandi, P.; Hasan, A.; Al-Riyami, H.; Hussain, J., *et al*. Secondary metabolites from acridocarpus orientalis inhibits 4t1 cells and promotes mesenchymal stem cells (mscs) proliferation. Mol Biol Rep 2020, 47, 5421-5430.
25. Sanap, A.; Chandravanshi, B.; Shah, T.; Tillu, G.; Dhanushkodi, A.; Bhonde, R.; Joshi, K. Herbal pre-conditioning induces proliferation and delays senescence in wharton's jelly mesenchymal stem cells. Biomed Pharmacother 2017, 93, 772-778.
26. Kalamegam, G.; Alfakeeh, S.M.; Bahmaid, A.O.; AlHuwait, E.A.; Gari, M.A.; Abbas, M.M.; Ahmed, F.; Abu-Elmagd, M.; Pushparaj, P.N. In vitro evaluation of the anti-inflammatory effects of thymoquinone in osteoarthritis and in silico analysis of inter-related pathways in age-related degenerative diseases. Front Cell Dev Biol 2020, 8, 646.
27. Ruan, X.F.; Li, Y.J.; Ju, C.W.; Shen, Y.; Lei, W.; Chen, C.; Li, Y.; Yu, H.; Liu, Y.T.; Kim, I.M., *et al*. Exosomes from suxiao jiuxin pill-treated cardiac mesenchymal stem cells decrease h3k27 demethylase utx expression in mouse cardiomyocytes in vitro. Acta Pharmacol Sin 2018, 39, 579-586.
28. Kornicka, K.; Kocherova, I.; Marycz, K. The effects of chosen plant extracts and compounds on mesenchymal stem cells-a bridge between molecular nutrition and regenerative medicine- concise review. Phytother Res 2017, 31, 947-958.
29. Zhang, D.; Liu, L.; Jia, Z.; Yao, X.; Yang, M. Flavonoids of herba epimedii stimulate osteogenic differentiation and suppress adipogenic differentiation of primary mesenchymal stem cells via estrogen receptor pathway. Pharm Biol 2016, 54, 954-963.
30. Gu, Q.; Cai, Y.; Huang, C.; Shi, Q.; Yang, H. Curcumin increases rat mesenchymal stem cell osteoblast differentiation but inhibits adipocyte differentiation. Pharmacogn Mag 2012, 8, 202-208.
31. Tao, K.; Xiao, D.; Weng, J.; Xiong, A.; Kang, B.; Zeng, H. Berberine promotes bone marrow-derived mesenchymal stem cells osteogenic differentiation via canonical wnt/beta-catenin signaling pathway. Toxicol Lett 2016, 240, 68-80.

32. Wang, Z.; Bao, H.W. Cnidium lactone stimulates osteogenic differentiation of bone marrow mesenchymal stem cells via bmp-2/smad-signaling cascades mediated by estrogen receptor. Am J Transl Res 2019, 11, 4984-4991.
33. Luo, Y.; Xia, H.; Wang, J.; Hu, Q.; Luo, Y.; Liu, J.; Yang, Z.; Li, W.; Wang, H.; Li, F., *et al*. Jiawei yanghe decoction regulates bone-lipid balance through the bmp-smad signaling pathway to promote osteogenic differentiation of bone mesenchymal stem cells. Evid Based Complement Alternat Med 2022, 2022, 2885419.
34. Oh, J.H.; Karadeniz, F.; Seo, Y.; Kong, C.S. Effect of quercetin 3-o-beta-d-galactopyranoside on the adipogenic and osteoblastogenic differentiation of human bone marrow-derived mesenchymal stromal cells. Int J Mol Sci 2020, 21.
35. Siu, W.S.; Wong, H.L.; Lau, C.P.; Shum, W.T.; Wong, C.W.; Gao, S.; Fung, K.P.; Lau, C.B.; Hung, L.K.; Ko, C.H., *et al*. The effects of an antiosteoporosis herbal formula containing epimedii herba, ligustri lucidi fructus and psoraleae fructus on density and structure of rat long bones under tail-suspension, and its mechanisms of action. Phytother Res 2013, 27, 484-492.
36. Huh, J.E.; Choi, J.Y.; Shin, Y.O.; Park, D.S.; Kang, J.W.; Nam, D.; Choi, D.Y.; Lee, J.D. Arginine enhances osteoblastogenesis and inhibits adipogenesis through the regulation of wnt and nfatc signaling in human mesenchymal stem cells. Int J Mol Sci 2014, 15, 13010-13029.
37. Lee, S.; Choi, E.; Yang, S.M.; Ryoo, R.; Moon, E.; Kim, S.H.; Kim, K.H. Bioactive compounds from sclerotia extract of poria cocos that control adipocyte and osteoblast differentiation. Bioorg Chem 2018, 81, 27-34.
38. Sheng, H.; Rui, X.F.; Sheng, C.J.; Li, W.J.; Cheng, X.Y.; Jhummon, N.P.; Yu, Y.C.; Qu, S.; Zhang, G.; Qin, L. A novel semisynthetic molecule icaritin stimulates osteogenic differentiation and inhibits adipogenesis of mesenchymal stem cells. Int J Med Sci 2013, 10, 782-789.
39. Zhang, D.; Zhao, N.; Wan, C.; Du, J.; Lin, J.; Wang, H. Icariin and icariside ii reciprocally stimulate osteogenesis and inhibit adipogenesis of multipotential stromal cells through erk signaling. Evid Based Complement Alternat Med 2021, 2021, 8069930.
40. Yang, R.; Yang, X.; Liu, S.; Ming, L.; Zhou, Z.; Liang, Y.; Zhao, Y.; Zhou, F.; Deng, Z.; Jin, Y. Activation of the wnt/beta-catenin pathway by an inflammatory microenvironment affects the myogenic differentiation capacity of human laryngeal mucosa mesenchymal stromal cells. Stem Cells Dev 2018, 27, 771-782.
41. Elseweidy, M.M.; El-Swefy, S.E.; Shaheen, M.A.; Baraka, N.M.; Hammad, S.K. Effect of resveratrol and mesenchymal stem cell monotherapy and combined treatment in management of osteoporosis

in ovariectomized rats: Role of sirt1/foxo3a and wnt/beta-catenin pathways. Arch Biochem Biophys 2021, 703, 108856.

42. Fu, Y.; Kong, Y.; Li, J.; Wang, Y.; Li, M.; Wang, Y.; Wang, Y.; Ren, F.; Ni, J.; Li, Y., *et al*. Mesenchymal stem cells combined with traditional chinese medicine (qi-fang-bi-min-tang) alleviates rodent allergic rhinitis. J Cell Biochem 2020, 121, 1541-1551.
43. Fu, L.L.; Pang, B.Y.; Zhu, Y.; Wang, L.; Leng, A.J.; Chen, H.L. Yi guan jian decoction may enhance hepatic differentiation of bone marrowderived mesenchymal stem cells via sdf1 in vitro. Mol Med Rep 2017, 16, 2511-2521.
44. Zhao, Z.; Han, Y.; Zhang, Z.; Li, W.; Ji, X.; Liu, X.; Jin, J.; Xu, S.; Cui, H.; Cheng, Z., *et al*. Total glucosides of paeony improves the immunomodulatory capacity of mscs partially via the mir-124/stat3 pathway in oral lichen planus. Biomed Pharmacother 2018, 105, 151-158.
45. Ayadilord, M.; Nasseri, S.; Emadian Razavi, F.; Saharkhiz, M.; Rostami, Z.; Naseri, M. Immunomodulatory effects of phytosomal curcumin on related-micro rnas, cd200 expression and inflammatory pathways in dental pulp stem cells. Cell Biochem Funct 2021, 39, 886-895.
46. Lee, C.W.; Lin, H.C.; Wang, B.Y.; Wang, A.Y.; Shin, R.L.; Cheung, S.Y.L.; Lee, O.K. Ginkgolide b monotherapy reverses osteoporosis by regulating oxidative stress-mediated bone homeostasis. Free Radic Biol Med 2021, 168, 234-246.
47. Li, Z.; Li, J.; Zhao, W.; Li, Y. Potential antiosteoporotic effect of ginkgo biloba extract via regulation of sirt1-nf-kb signaling pathway. Journal of King Saud University - Science 2020, 32, 2513-2519.
48. Gu, Q.; Chen, C.; Zhang, Z.; Wu, Z.; Fan, X.; Zhang, Z.; Di, W.; Shi, L. Ginkgo biloba extract promotes osteogenic differentiation of human bone marrow mesenchymal stem cells in a pathway involving wnt/beta-catenin signaling. Pharmacol Res 2015, 97, 70-78.
49. Mendi, A.; Gokcinar Yagci, B.; Sarac, N.; Kiziloglu, M.; Ugur, A.; Uckan, D.; Yilmaz, D. The effects of hypericum perforatum l. On the proliferation, osteogenic differentiation, and inflammatory response of mesenchymal stem cells from different niches. Cells Tissues Organs 2018, 205, 208-216.
50. Chen, W.Y.; Yang, W.B.; Wong, C.H.; Shih, D.T. Effect of reishi polysaccharides on human stem/progenitor cells. Bioorg Med Chem 2010, 18, 8583-8591.
51. Chao, Y.H.; Wu, K.H.; Lin, C.W.; Yang, S.F.; Chao, W.R.; Peng, C.T.; Wu, H.P. Pg2, a botanically derived drug extracted from astragalus membranaceus, promotes proliferation and immunosuppression of umbilical cord-derived mesenchymal stem cells. J Ethnopharmacol 2017, 207, 184-191.

52. Zhu, L.; Liu, Y.J.; Shen, H.; Gu, P.Q.; Zhang, L. Astragalus and baicalein regulate inflammation of mesenchymal stem cells (mscs) by the mitogen-activated protein kinase (mapk)/erk pathway. Med Sci Monit 2017, 23, 3209-3216.
53. Wang, J.; Zhang, D.; Zhu, Y.; Mo, X.; McHugh, P.C.; Tong, Q. Astragalus and human mesenchymal stem cells promote wound healing by mediating immunomodulatory effects through paracrine signaling. Regen Med 2022, 17, 219-232.
54. Szydlak, R. Biological, chemical and mechanical factors regulating migration and homing of mesenchymal stem cells. World J Stem Cells 2021, 13, 619-631.
55. Fu, X.; Liu, G.; Halim, A.; Ju, Y.; Luo, Q.; Song, A.G. Mesenchymal stem cell migration and tissue repair. Cells 2019, 8.
56. Chamberlain, G.; Fox, J.; Ashton, B.; Middleton, J. Concise review: Mesenchymal stem cells: Their phenotype, differentiation capacity, immunological features, and potential for homing. Stem Cells 2007, 25, 2739-2749.
57. Xie, J.; Wang, H.; Song, T.; Wang, Z.; Li, F.; Ma, J.; Chen, J.; Nan, Y.; Yi, H.; Wang, W. Tanshinone iia and astragaloside iv promote the migration of mesenchymal stem cells by up-regulation of cxcr4. Protoplasma 2013, 250, 521-530.
58. Fathy, M.; Okabe, M.; E, M.O.; Saad Eldien, H.M.; Yoshida, T. Preconditioning of adipose-derived mesenchymal stem-like cells with eugenol potentiates their migration and proliferation in vitro and therapeutic abilities in rat hepatic fibrosis. Molecules 2020, 25.
59. Sisakhtnezhad, S.; Heidari, M.; Bidmeshkipour, A. Eugenol enhances proliferation and migration of mouse bone marrow-derived mesenchymal stem cells in vitro. Environ Toxicol Pharmacol 2018, 57, 166-174.
60. Lin, F.; Zhu, Y.; Hu, G. Naringin promotes cellular chemokine synthesis and potentiates mesenchymal stromal cell migration via the ras signaling pathway. Exp Ther Med 2018, 16, 3504-3510.
61. Mao, D.; Pan, X.; Rui, Y.; Li, F. Matrine attenuates heterotopic ossification by suppressing tgf-beta induced mesenchymal stromal cell migration and osteogenic differentiation. Biomed Pharmacother 2020, 127, 110152.
62. Nascimento, R.P.; Dos Santos, B.L.; da Silva, K.C.; Amaral da Silva, V.D.; de Fatima Costa, M.; David, J.M.; David, J.P.; Moura-Neto, V.; Oliveira, M.D.N.; Ulrich, H., *et al*. Reverted effect of mesenchymal stem cells in glioblastoma treated with agathisflavone and its selective antitumoral effect on cell viability, migration, and differentiation via stat3. J Cell Physiol 2021, 236, 5022-5035.

63. Huang, F.; Yao, Y.; Wu, J.; Liu, Q.; Zhang, J.; Pu, X.; Zhang, Q.; Xia, L. Curcumin inhibits gastric cancer-derived mesenchymal stem cells mediated angiogenesis by regulating nf-κb/vegf signaling. Am J Transl Res 2017, 9, 5538-5547.
64. Kim, D.W.; Choi, C.H.; Park, J.P.; Lee, S.J. Nanospheres loaded with curcumin improve the bioactivity of umbilical cord blood-mesenchymal stem cells via c-src activation during the skin wound healing process. Cells 2020, 9.
65. Yang, Q.; Leong, S.A.; Chan, K.P.; Yuan, X.L.; Ng, T.K. Complex effect of continuous curcumin exposure on human bone marrow-derived mesenchymal stem cell regenerative properties through matrix metalloproteinase regulation. Basic Clin Pharmacol Toxicol 2021, 128, 141-153.
66. Ariyanti, A.D.; Zhang, J.; Marcelina, O.; Nugrahaningrum, D.A.; Wang, G.; Kasim, V.; Wu, S. Salidroside-pretreated mesenchymal stem cells enhance diabetic wound healing by promoting paracrine function and survival of mesenchymal stem cells under hyperglycemia. Stem Cells Transl Med 2019, 8, 404-414.
67. Furumoto, T.; Ozawa, N.; Inami, Y.; Toyoshima, M.; Fujita, K.; Zaiki, K.; Sahara, S.; Akita, M.; Kitamura, K.; Nakaoji, K., *et al*. Mallotus philippinensis bark extracts promote preferential migration of mesenchymal stem cells and improve wound healing in mice. Phytomedicine 2014, 21, 247-253.
68. Zhu, J.; Liu, Y.; Chen, C.; Chen, H.; Huang, J.; Luo, Y.; Zhao, K.; Chen, D.; Xu, Z.; Li, W., *et al*. Cyasterone accelerates fracture healing by promoting mscs migration and osteogenesis. J Orthop Translat 2021, 28, 28-38.
69. Wang, H.; Yan, Y.; Lan, H.; Wei, N.; Zheng, Z.; Wu, L.; Jaspers, R.T.; Wu, G.; Pathak, J.L. Notoginsenoside r1 promotes migration, adhesin, spreading, and osteogenic differentiation of human adipose tissue-derived mesenchymal stromal cells. Molecules 2022, 27.
70. Wang, X.; Wu, J.; Ma, S.; Xie, Y.; Liu, H.; Yao, M.; Zhang, Y.; Yang, G.L.; Yang, B.; Guo, R., *et al*. Resveratrol preincubation enhances the therapeutic efficacy of huc-mscs by improving cell migration and modulating neuroinflammation mediated by mapk signaling in a mouse model of alzheimer's disease. Front Cell Neurosci 2020, 14, 62.
71. Xu, F.; Li, W.H.; Yang, X.; Na, L.X.; Chen, L.J.; Liu, G.B. The roles of epigenetics regulation in bone metabolism and osteoporosis. Frontiers in Cell and Developmental Biology 2021, 8.
72. Zhang, N.D.; Han, T.; Huang, B.K.; Rahman, K.; Jiang, Y.P.; Xu, H.T.; Qin, L.P.; Xin, H.L.; Zhang, Q.Y.; Li, Y.M. Traditional chinese medicine formulas for the treatment of osteoporosis: Implication for antiosteoporotic drug discovery. Journal of Ethnopharmacology 2016, 189, 61-80.

73. Zhu, D.Q.; Li, A.W.; Lv, Y.; Fan, Q. Traditional chinese medicine: A class of potentially reliable epigenetic drugs. Frontiers in Pharmacology 2022, 13.

74. Mobasheri, A.; Shakibaei, M. Osteogenic effects of resveratrol in vitro: Potential for the prevention and treatment of osteoporosis. Ann N Y Acad Sci 2013, 1290, 59-66.

75. Raal, A.; Pokk, P.; Arend, A.; Aunapuu, M.; Jõgi, J.; Okva, K.; Püssa, T. Trans-resveratrol alone and hydroxystilbenes of rhubarb (rheum rhaponticum l.) root reduce liver damage induced by chronic ethanol administration: A comparative study in mice. Phytother Res 2009, 23, 525-532.

76. Nan, K.; Pei, J.P.; Fan, L.H.; Zhang, Y.K.; Zhang, X.; Liu, K.; Shi, Z.B.; Dang, X.Q.; Wang, K.Z. Resveratrol prevents steroid-induced osteonecrosis of the femoral head via mir-146a modulation. Annals of the New York Academy of Sciences 2021, 1503, 23-37.

77. Song, C.Y.; Guo, Y.; Chen, F.Y.; Liu, W.G. Resveratrol promotes osteogenic differentiation of bone marrow-derived mesenchymal stem cells through mir-193a/sirt7 axis. Calcified Tissue International 2022, 110, 117-130.

78. Zhang, Y.; Liu, M.W.; He, Y.; Deng, N.; Chen, Y.; Huang, J.C.; Xie, W. Protective effect of resveratrol on estrogen deficiency-induced osteoporosis though attenuating nadph oxidase 4/nuclear factor kappa b pathway by increasing mir-92b-3p expression. International Journal of Immunopathology and Pharmacology 2020, 34.

79. Zou, J.L.; Du, J.Y.; Tu, H.L.; Chen, H.J.; Cong, K.; Bi, Z.G.; Sun, J.B. Resveratrol benefits the lineage commitment of bone marrow mesenchymal stem cells into osteoblasts via mir-320c by targeting runx2. Journal of Tissue Engineering and Regenerative Medicine 2021, 15, 347-360.

80. He, C.; Wang, Z.; Shi, J. Pharmacological effects of icariin. Adv Pharmacol 2020, 87, 179-203.

81. Sun, Z.B.; Wang, J.W.; Xiao, H.; Zhang, Q.S.; Kan, W.S.; Mo, F.B.; Hu, S.; Ye, S.N. Icariin may benefit the mesenchymal stem cells of patients with steroid-associated osteonecrosis by abcb1-promoter demethylation: A preliminary study. Osteoporos Int 2015, 26, 187-197.

82. Xu, Y.X.; Jiang, Y.P.; Jia, B.; Wang, Y.Z.; Li, T. Icariin stimulates osteogenesis and suppresses adipogenesis of human bone mesenchymal stem cells via mir-23a-mediated activation of the wnt/beta-catenin signaling pathway. Phytomedicine 2021, 85.

83. Zhang, X.Y.; Li, H.N.; Chen, F.; Chen, Y.P.; Chai, Y.; Liao, J.Z.; Gan, B.; Chen, D.P.; Li, S.; Liu, Y.Q. Icariin regulates mir-23a-3p-mediated osteogenic differentiation of bmscs via bmp-2/smad5/runx2 and wnt/beta-catenin pathways in osteonecrosis of the femoral head. Saudi Pharmaceutical Journal 2021, 29, 1405-1415.

84. Lian, F.; Zhao, C.B.; Qu, J.; Lian, Y.Y.; Cui, Y.; Shan, L.; Yan, J.L. Icariin attenuates titanium particle-induced inhibition of osteogenic differentiation and matrix mineralization via mir-21-5p. Cell Biology International 2018, 42, 931-939.

85. Rajan, I.; Narayanan, N.; Rabindran, R.; Jayasree, P.R.; Manish Kumar, P.R. Zingerone protects against stannous chloride-induced and hydrogen peroxide-induced oxidative DNA damage in vitro. Biol Trace Elem Res 2013, 155, 455-459.

86. Wu, P.; Meng, X.; Zheng, H.; Zeng, Q.; Chen, T.; Wang, W.; Zhang, X.; Su, J. Kaempferol attenuates ros-induced hemolysis and the molecular mechanism of its induction of apoptosis on bladder cancer. Molecules 2018, 23.

87. Liu, H.; Yi, X.; Tu, S.T.; Cheng, C.; Luo, J. Kaempferol promotes bmsc osteogenic differentiation and improves osteoporosis by downregulating mir-10a-3p and upregulating cxcl12. Molecular and Cellular Endocrinology 2021, 520.

88. Srinaath, N.; Balagangadharan, K.; Pooja, V.; Paarkavi, U.; Trishla, A.; Selvamurugan, N. Osteogenic potential of zingerone, a phenolic compound in mouse mesenchymal stem cells. Biofactors 2019, 45, 575-582.

89. Song, Y.X.; Mou, R.; Li, Y.; Yang, T.G. Zingerone promotes osteoblast differentiation via mir-200c-3p/smad7 regulatory axis in human bone mesenchymal stem cells. Medical Science Monitor 2020, 26.

90. Zhang, C.; Yuan, S.; Chen, Y.; Wang, B. Neohesperidin promotes the osteogenic differentiation of human bone marrow stromal cells by inhibiting the histone modifications of lncrna snhg1. Cell Cycle 2021, 20, 1953-1966.

91. Yuan, S.; Zhang, C.; Zhu, Y.; Wang, B. Neohesperidin ameliorates steroid-induced osteonecrosis of the femoral head by inhibiting the histone modification of lncrna hotair. Drug Des Devel Ther 2020, 14, 5419-5430.

92. Bian, W.; Xiao, S.; Yang, L.; Chen, J.; Deng, S. Quercetin promotes bone marrow mesenchymal stem cell proliferation and osteogenic differentiation through the h19/mir-625-5p axis to activate the wnt/β-catenin pathway. BMC Complement Med Ther 2021, 21, 243.

93. Zhang, Q.H.; Chang, B.; Zheng, G.Z.; Du, S.X.; Li, X.D. Quercetin stimulates osteogenic differentiation of bone marrow stromal cells through mirna-206/connexin 43 pathway. American Journal of Translational Research 2020, 12, 2062-2070.

94. Li, X.Y.; Chen, R.M.; Lei, X.T.; Wang, P.P.; Zhu, X.F.; Zhang, R.H.; Yang, L. Quercetin regulates er alpha mediated differentiation of bmscs through circular rna. Gene 2021, 769.

95. Cao, Y.J.; Lv, Q.X.; Li, Y. Astragaloside iv improves tibial defect in rats and promotes proliferation and osteogenic differentiation of hbmscs through mir-1 24-3p.1/stat3 axis. Journal of Natural Products 2021, 84, 287-297.
96. Wu, X.H.; Dou, B.; Sun, N.Y.; Gao, J.; Liu, X.L. Astragalus saponin iv promotes osteogenic differentiation of bone marrow mesenchymal stem cells via mir-21/ngf/bmp2/runx2 pathway. Acta Histochemica 2020, 122.
97. Li, X.; Yang, L.; Zhu, X.; Zhang, R. Mir-671 abundant in small extracellular vesicles derived from rhizoma drynariae-preconditioned bone mesenchymal stem cells regulating osteogenesis by targeting tak1 mediating wnt signaling. Osteoporosis International 2021, 32, S140-S140.
98. Lin, J.; Zhu, J.; Wang, Y.; Zhang, N.; Gober, H.J.; Qiu, X.; Li, D.; Wang, L. Chinese single herbs and active ingredients for postmenopausal osteoporosis: From preclinical evidence to action mechanism. Biosci Trends 2017, 11, 496-506.
99. Huang, Y.; Hou, Q.; Su, H.; Chen, D.; Luo, Y.; Jiang, T. Mir‐ 488 negatively regulates osteogenic differentiation of bone marrow mesenchymal stem cells induced by psoralen by targeting runx2. Mol Med Rep 2019, 20, 3746-3754.
100. Wang, Y.; Hong, C.; Zhou, C.; Xu, D.; Qu, H.B. Screening antitumor compounds psoralen and isopsoralen from psoralea corylifolia l. Seeds. Evid Based Complement Alternat Med 2011, 2011, 363052.
101. Zhang, X.; Zhao, W.; Wang, Y.; Lu, J.; Chen, X. The chemical constituents and bioactivities of psoralea corylifolia linn.: A review. Am J Chin Med 2016, 44, 35-60.
102. Wang, L.Y.; Tang, Y.P.; Liu, X.; Zhu, M.; Tao, W.W.; Li, W.X.; Duan, J.A. Effects of ferulic acid on antioxidant activity in angelicae sinensis radix, chuanxiong rhizoma, and their combination. Chin J Nat Med 2015, 13, 401-408.
103. Du, K.; Li, Z.; Fang, X.; Cao, T.; Xu, Y. Ferulic acid promotes osteogenesis of bone marrow-derived mesenchymal stem cells by inhibiting microrna-340 to induce β-catenin expression through hypoxia. Eur J Cell Biol 2017, 96, 496-503.
104. Rosenthal, P.J. Artesunate for the treatment of severe falciparum malaria. N Engl J Med 2008, 358, 1829-1836.
105. Zeng, H.B.; Dong, L.Q.; Xu, C.; Zhao, X.H.; Wu, L.G. Artesunate promotes osteoblast differentiation through mir-34a/dkk1 axis. Acta Histochem 2020, 122, 151601.

106. Song, B.; Wang, F.; Wang, W. Effect of aqueous extract from morinda officinalis f. C. How on microwave-induced hypothalamic-pituitary-testis axis impairment in male sprague-dawley rats. Evid Based Complement Alternat Med 2015, 2015, 360730.

107. Wu, P.Y.; Chen, W.; Huang, H.; Tang, W.; Liang, J. Morinda officinalis polysaccharide regulates rat bone mesenchymal stem cell osteogenic-adipogenic differentiation in osteoporosis by upregulating mir-21 and activating the pi3k/akt pathway. Kaohsiung J Med Sci 2022, 38, 675-685.

108. Chen, R.; Xue, J.; Xie, M. Puerarin prevents isoprenaline-induced myocardial fibrosis in mice by reduction of myocardial tgf-β1 expression. J Nutr Biochem 2012, 23, 1080-1085.

109. Wang, S.; Zhang, S.; Wang, S.; Gao, P.; Dai, L. A comprehensive review on pueraria: Insights on its chemistry and medicinal value. Biomed Pharmacother 2020, 131, 110734.

110. Zhou, Y.; Lian, H.; Liu, K.; Wang, D.; Xiu, X.; Sun, Z. Puerarin improves graft bone defect through microrna‐155‐3p‐mediated p53/tnf‐α/stat1 signaling pathway. Int J Mol Med 2020, 46, 239-251.

111. Jindal, M. Bone density versus bone quality as a predictor of bone strength. Oroaj 2020, 12, null.

112. Zhu, M.; Yin, P.; Hu, F.; Jiang, J.; Yin, L.; Li, Y.; Wang, S. Integrating genome-wide association and transcriptome prediction model identifies novel target genes for osteoporosis. Osteoporosis International 2021, 32, 2493-2503.

113. Friedrich, K.; Dolznig, H.; Han, X.; Moriggl, R. Steering of carcinoma progression by the yin/yang interaction of stat1/stat3. Biosci Trends 2017, 11, 1-8.

114. Zhang, Y. Persisters, persistent infections and the yin-yang model. Emerg Microbes Infect 2014, 3, e3.

115. Wei, S. Yin-yang regulating effects of cancer-associated genes, proteins, and cells: An ancient chinese concept in vogue in modern cancer research. Biosci Trends 2018, 11, 612-618.

116. K, R.B.; Tay, Y. The yin-yang regulation of reactive oxygen species and micrornas in cancer. Int J Mol Sci 2019, 20.

117. Yi, Z.; Ma, T.; Liu, J.; Tie, W.; Li, Y.; Bai, J.; Li, L.; Zhang, L. The yin-yang effects of immunity: From monoclonal gammopathy of undetermined significance to multiple myeloma. Front Immunol 2022, 13, 925266.

118. Wan, Y.Y.; Flavell, R.A. 'Yin-yang' functions of transforming growth factor-beta and t regulatory cells in immune regulation. Immunol Rev 2007, 220, 199-213.

119. Stechschulte, L.A.; Lecka-Czernik, B. Reciprocal regulation of pparγ and runx2 activities in marrow mesenchymal stem cells: Fine balance between p38 mapk and protein phosphatase 5. Curr Mol Biol Rep 2017, 3, 107-113.

120. Wei, W.; Motoike, T.; Krzeszinski, J.Y.; Jin, Z.; Xie, X.J.; Dechow, P.C.; Yanagisawa, M.; Wan, Y. Orexin regulates bone remodeling via a dominant positive central action and a subordinate negative peripheral action. Cell Metab 2014, 19, 927-940.

121. Mueller, K. Inflammation. Inflammation's yin-yang. Introduction. Science 2013, 339, 155.

122. Cappellano, G.; Vecchio, D.; Magistrelli, L.; Clemente, N.; Raineri, D.; Barbero Mazzucca, C.; Virgilio, E.; Dianzani, U.; Chiocchetti, A.; Comi, C. The yin-yang of osteopontin in nervous system diseases: Damage versus repair. Neural Regen Res 2021, 16, 1131-1137.

123. Sun, H.; Calabrese, E.J.; Lin, Z.; Lian, B.; Zhang, X. Similarities between the yin/yang doctrine and hormesis in toxicology and pharmacology. Trends Pharmacol Sci 2020, 41, 544-556.

124. Obermajer, N.; Dahlke, M.H. (compl)ex-th17-t(reg) cell inter-relationship. Oncoimmunology 2016, 5, e1040217.

125. Sica, A.; Invernizzi, P.; Mantovani, A. Macrophage plasticity and polarization in liver homeostasis and pathology. Hepatology 2014, 59, 2034-2042.

126. Godson, C.; Perretti, M. Novel pathways in the yin-yang of immunomodulation. Curr Opin Pharmacol 2013, 13, 543-546.

127. Schaller, G.E.; Bishopp, A.; Kieber, J.J. The yin-yang of hormones: Cytokinin and auxin interactions in plant development. Plant Cell 2015, 27, 44-63.

128. Gilca, M.; Gaman, L.; Lixandru, D.; Stoian, I. Estimating the yin-yang nature of western herbs: A potential tool based on antioxidation-oxidation theory. Afr J Tradit Complement Altern Med 2014, 11, 210-216.

129. Huang, Y.; Yao, P.; Leung, K.W.; Wang, H.; Kong, X.P.; Wang, L.; Dong, T.T.X.; Chen, Y.; Tsim, K.W.K. The yin-yang property of chinese medicinal herbs relates to chemical composition but not anti-oxidative activity: An illustration using spleen-meridian herbs. Front Pharmacol 2018, 9, 1304.

130. Gan, L.; Leng, Y.; Min, J.; Luo, X.M.; Wang, F.; Zhao, J. Kaempferol promotes the osteogenesis in rbmscs via mediation of sox2/ mir-124-3p/pi3k/akt/mtor axis. European Journal of Pharmacology 2022, 927.

Table 1. Effects of herbal extracts on the Epigenetic regulation of MSCs

Herbal extract	Epigenetic regulation	Study Type	Findings	References
Resveratrol	Resveratrol upregulated miR-146a while enhancing miR-92b-3p and BMP-2/Smad/Runx2, inhibiting Nox4/NF-κB.	in vitro/rat BMSCs	Stabilize osteogenic/osteogenic homeostasis in BMSCs, and stimulate proliferation and osteoblast differentiation	[76-78]
Icariin	ICA demethylated the ABCB1 promoter of BMSCs and mediate activation of Wnt/ β-catenin through miR-23a.	in vitro/human BMSCs	Promote BMSCs viability and osteogenic differentiation, weakened adipogenesis	[81-84]
Kaempferol and Zingerone	Kaempferol regulated the mediation of SOX2/ miR-124-3p/PI3K/Akt/mTOR axis, while Zingerone provided Runx2 protection by targeting Smad7 with miR-590.	in vitro/human BMSCs, rat BMSCs	Promote osteogenic differentiation of BMSCs and improve osteoporosis	[87-89,130]
Neohesperidin	Neohesperidin (NH) inhibited histone modification of LncRNA SNHG1 by regulating occupation of H3K4me3 and H3K27me3.	in vitro/human BMSCs, rat BMSCs	Improve the activity of BMSC and promote the osteogenic differentiation of human BMSCs.	[90,91]
Quercetin	Quercetin regulated the H19 / miR-625-5p axis and circulates RNA-miR-326-5p-mRNA axis via miR-206/Cx43 pathway.	in vitro/rat BMSCs, human BMSCs,	Regulate osteogenic and adipocyte differentiation of ERα-deficient BMSCS.	[92-94]
Astragaloside IV	Astragaloside IV (AS-IV) increased STAT3 expression by down-regulating miR-124-3p.	in vitro/human BMSCs, rat BMSCs	Improve tibial defects and promotes the proliferation and osteogenic differentiation of BMSCs in rats.	[96]
Ferulic acid	Ferulic acid (FA) induced β-catenin expression by inhibiting miR-340-5p.	in vitro/human BMSCs	Enhance osteogenic differentiation and promotes proliferation of human BMSCs.	[103]
Rhizoma Drynariae	Rhizoma Drynariae regulated the generation of miR-671 and mediates WnT signaling.	in vitro/ BMSCs	Dry core nodules (Gu-Sui-Bu) are often used to regulate osteogenesis.	[97]
Artesunate	Artesunate (ART) modulated miR-34a/DKK1 / Wnt pathway.	in vitro/human BMSCs	Accelerate osteoblast differentiation of hBMSCs.	[105]
Psoralen	Psoralen induced negative regulation of miR-488 by targeting Runx2.	in vitro/rat BMSCs	Increase cell viability and improve osteogenic differentiation of BMSCs.	[99]
Morinda officinalis polysaccharide	Molinda polysaccharide regulated miR-21/PTEN/PI3K/AKT axis.	in vitro/rat BMSCs	strengthening bone and improving immunological function through osteogenesis and lipogenesis inhibition	[107]
Puerarin	Puerarin regulated Mir-155-3p-mediated p53 / TNF-α / STAT1 signaling.	in vitro/rat BMSCs	Promotes BMSCs differentiation and bone formation as well as bone mass increase in bone graft rats.	[110]

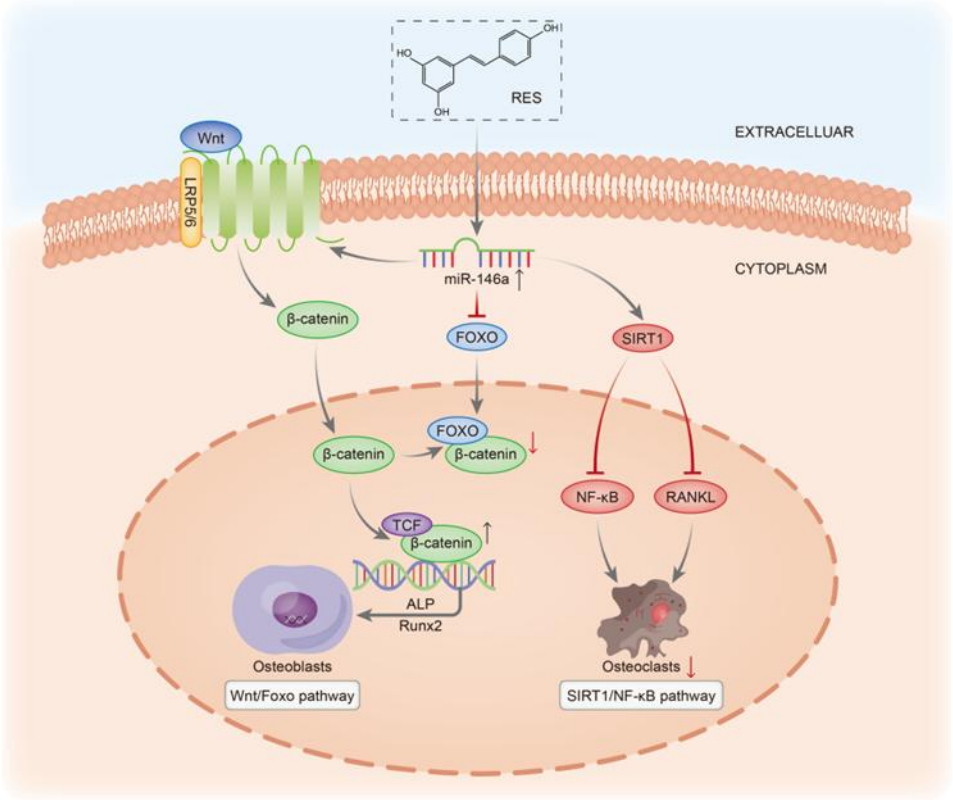


Figure 1. RES stabilizes the dynamic balance of osteogenesis/osteoclastogenesis through Wnt/Foxo and SIRT1/NF-κB pathways. RES upregulates miR-146a exp miR-146a sion levels, promotes SIRT1 expression, inhibits RANKL-induced NF-κB signaling pathway in bone marrow mesenchymal cells (BMSCs), and reduces levels of nuclear transcription factor κB (NF-κB) and the receptor activator of nuclear factor kappa - B ligand (RANKL) to suppress osteoclastogenesis. RES inhibits the expression of FOXO protein, which causes β-catenin from Wnt/TCF to FOXO-mediated transcription. RES increases the expression levels of β-catenin and Runt-related transcription factor 2 (Runx2) by attenuating the sequestration of β-catenin protein by FOXO transcription factors and promotes osteoblastogenesis by upregulating Wnt signaling.

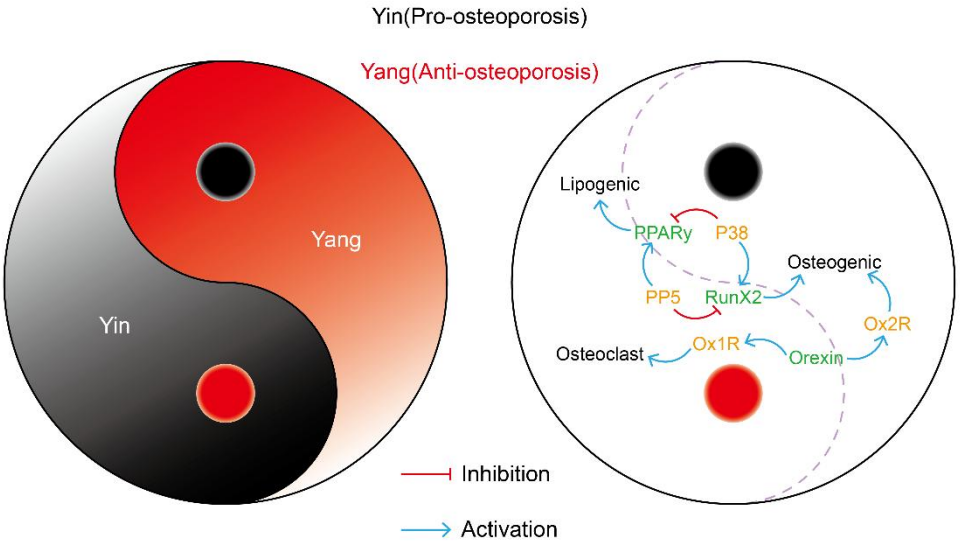


Figure 2. Yin and Yang effects of osteoporosis-related genes and proteins. Yin represents the pathological mechanism that promotes osteoporosis; Yang represents the regulatory mechanism that inhibits osteoporosis. Various genes, proteins and cells have been identified as having yin and yang effects by promoting or inhibiting the eradication of osteoporosis. Mitogen-activated protein kinase, p38, PP5, RUNX2, PPARγ, and appetitin all play an important role in osteoporosis.