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Factsheet

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

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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.

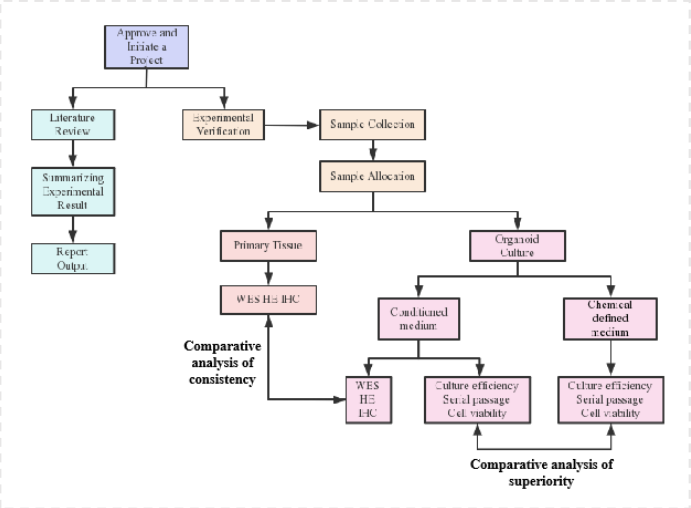

Figure 1. Experimental design of this study. We first conduct literature research to confirm the research objectives. After obtaining the cancer samples for the experiment, we separated cancer tissues for primary tissues and tumor organoids analysis, including consistency analysis and comparative analysis. 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.

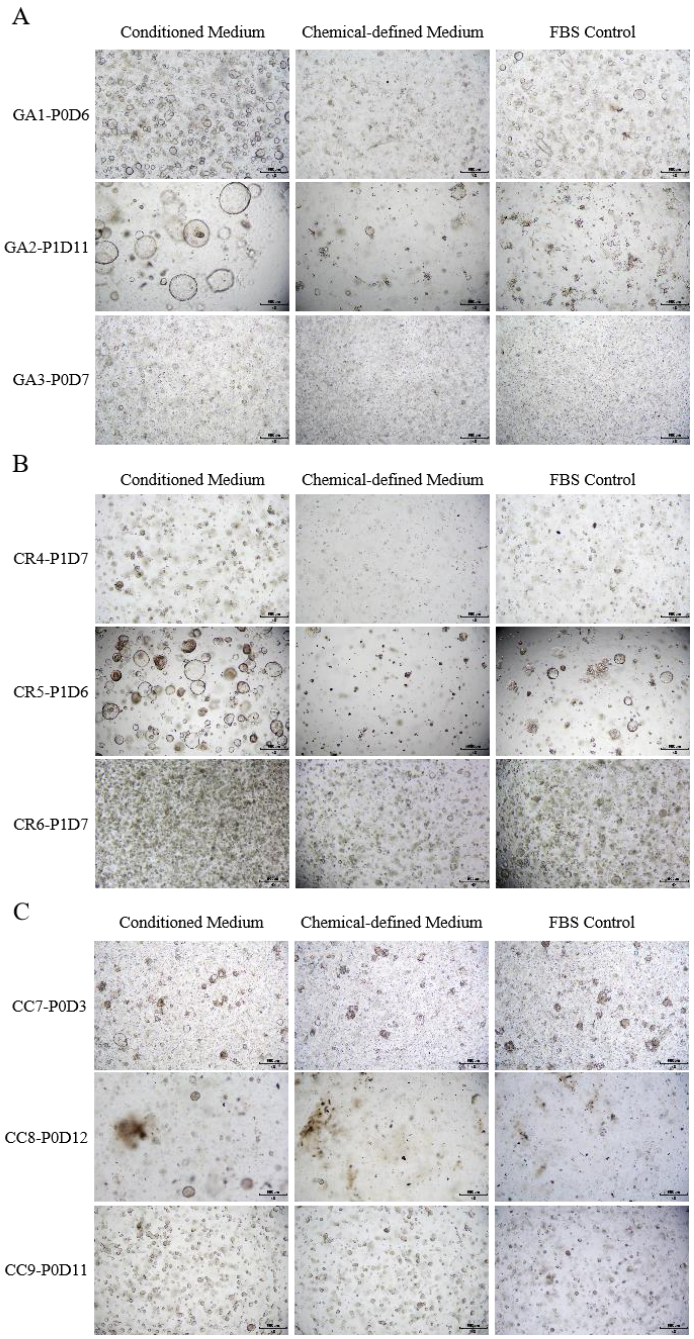

Figure 2. Growth comparison of various organoid types cultured in medium supplemented with conditioned medium and chemical-defined medium. Tumor organoids derived from (A) gastric cancer (GA) tissue of patient No.1, 2, 3, B) colorectal cancer (CR) tissue of patient No.4, 5, 6 and (C) cervical cancer (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
	3	P0-D7	2.7	0.3	1.0	62	4	47
	4	P1-D7	7.3	2.6	4.8	85	72	94
CR	5	P1-D6	10.3	2.0	1.7	39	45	42
	6	P1-D7	55.8	3.0	19.2	67	30	43
	7	P0-D3	7.7	5.9	5.7	73	62	81
CC	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.

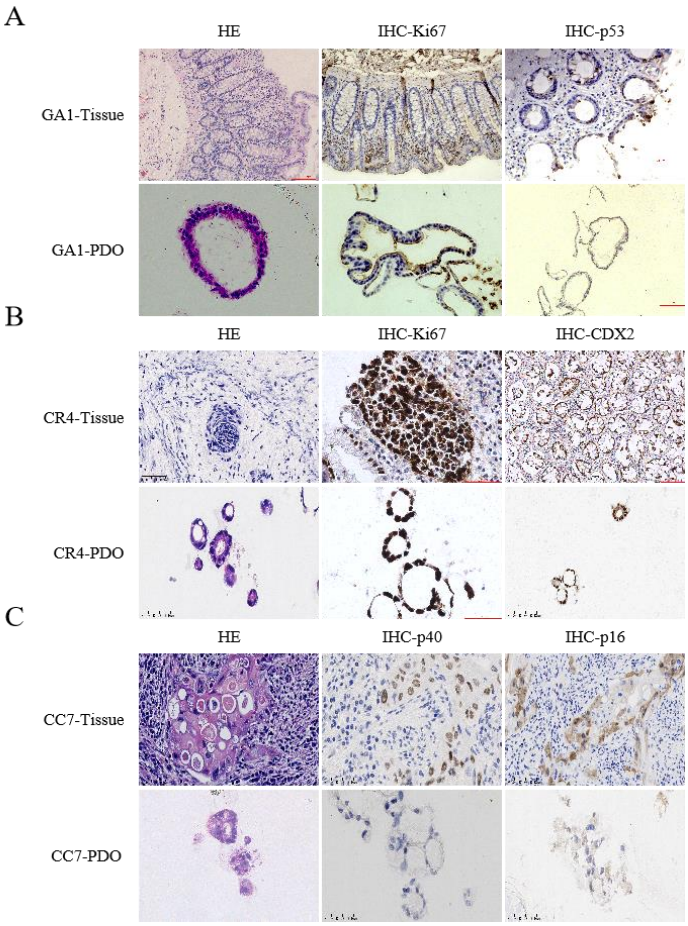

Figure 3. H&E and IHC staining of organoids cultured with conditioned medium from different patient tumor tissues. Shown are representative examples of (A) gastric cancer, (B) colorectal cancer and (C) cervical cancer. Tissues generally present tumor epithelium, while organoids are exclusively epithelial 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).

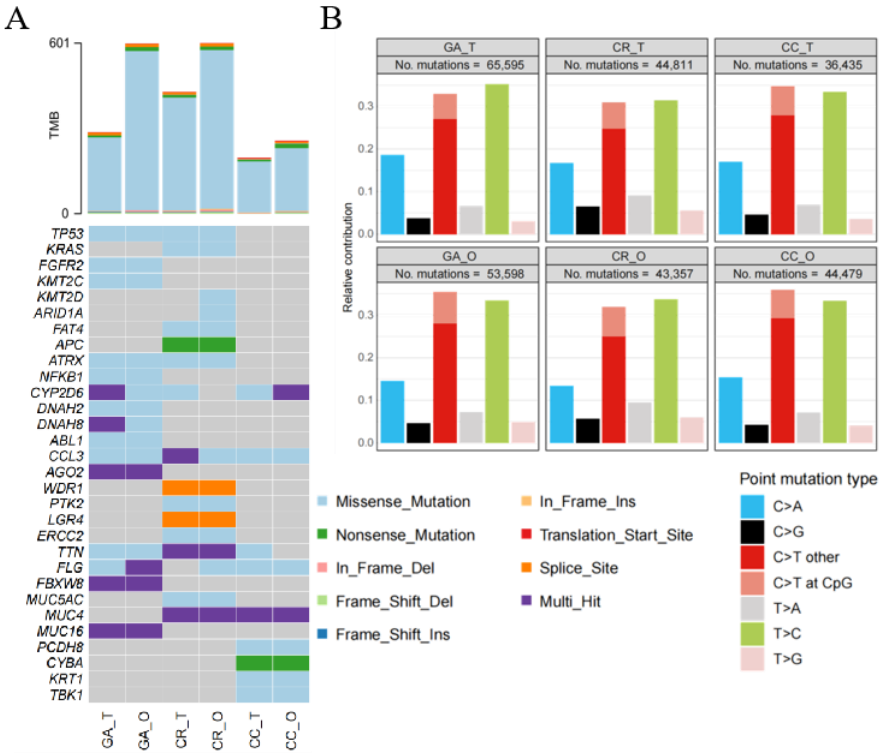

Figure 4. Oncoplot and point mutation spectrum of PDOs and tumor biopsies. A) Mutation landscape of single-nucleotide variants (SNVs) in PDOs and paired primary tumors. Top, total mutation number of each sample. Bottom, heatmap of commonly mutated genes in gastric cancer, colorectal cancer, and cervical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

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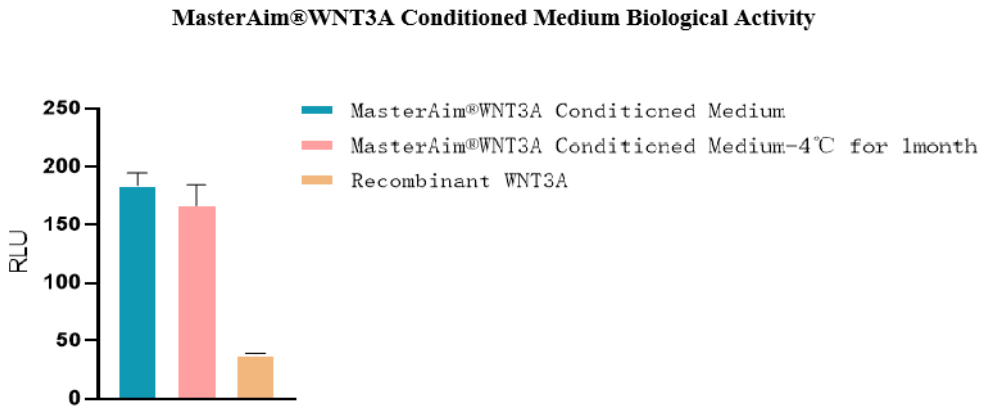
Supplementary Material



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).