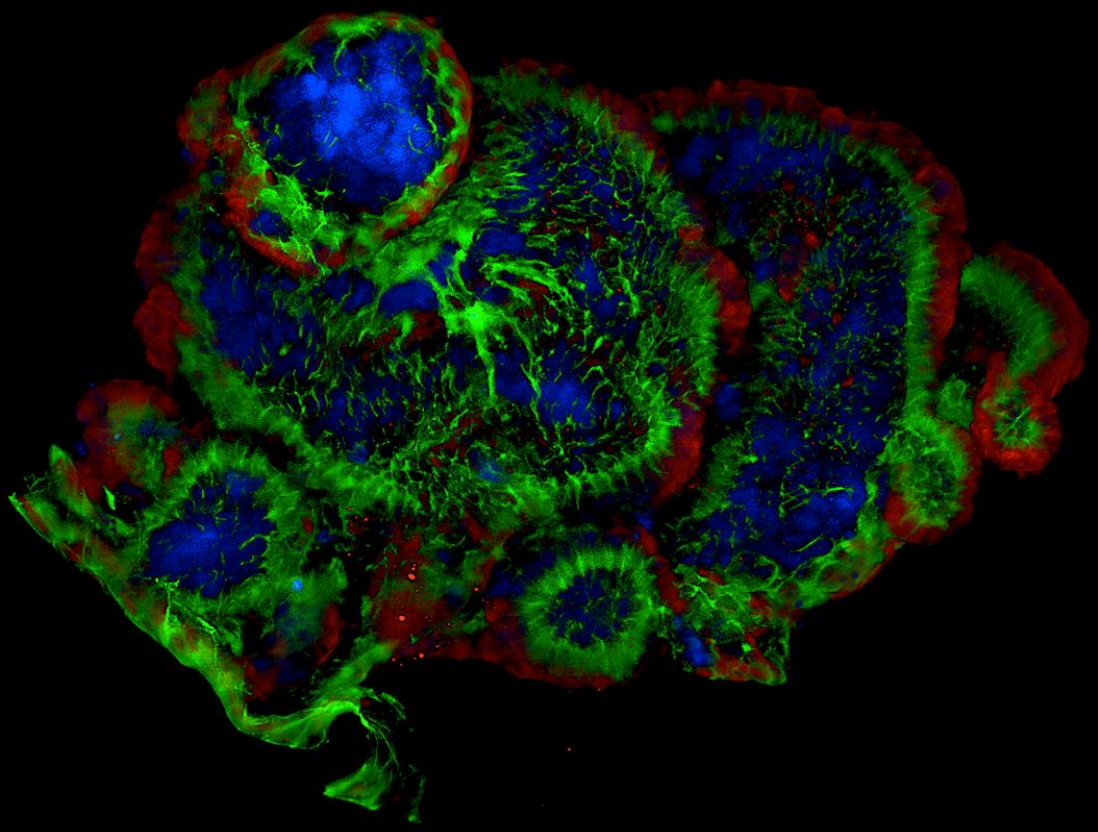
White Paper

Use of HANABI Avatar™ Organoid Growth Media in the imitation of structural, chemical, and physiological aspects of organs



Immunofluorescence image of intestinal organoids. Cells are stained for Protein kinase B, also known as AKT (red), epithelial cadherin (green) and DAPI (blue).

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Introduction

Treatment of human diseases requires tremendous amounts of preliminary research to determine biochemical causes and to develop potential therapeutics. Much of the preliminary research is conducted in 2D cell culture with immortalized cell lines as it is the most efficient method to obtain substantial initial data. However, 2D cell culture has sizable drawbacks that limit its representation of disease, which has led to the development of more representative model systems such as animal models and organoids[1]. Unfortunately, while animal models are able to recapitulate physiological complexity and human physiology in some cases, there have been a plethora of promising drugs that fail clinical trials[2]. In December of 2022, the FDA announced that animal model data would no longer be required to take drug candidates to clinical trials, opening the doors for more relevant models to be used, such as organ-on-a-chip and organoids[3]. Increased usage means that methodologies to establish and propagate organoids are going to have to improve to meet research and clinical needs. Currently, there are few commercially available media options that are affordable. Therefore, many labs use protocols found in the literature and optimize them internally, potentially resulting in a multitude of media with slightly different compositions being used across the globe. This study was conducted to compare the performance of a newly available colon organoid culture media from ADS Biotec with a media made in the lab, which was formulated after testing 14 iterations of culture media in a combined cultured strategy.

Materials & Methods

Tissue Collection

Tissues were collected from human patient samples in accordance with the Declaration of Helsinki, 2009 and did not impact any diagnostic procedures conducted for the patients. Resected tissues were staged according to WHO classification as TisN0M0 (polyposis), T2N1M0 (colorectal cancer), and T4N3M1 (peritoneal metastasis).

Establishment of Patient-derived organoids (PDOs)

Collected tissues (1cm^2) were minced mechanically using scalpels into small pieces. Resulting fragments were harvested and washed ten times with ice-cold phosphate-buffered saline (PBS; ThermoFisher Scientific) containing gentamicin (50 ng/mL, ThermoFisher Scientific) and amphotericin B (50 ng/mL, ThermoFisher Scientific) to prevent contaminations. Minced tissue samples were enzymatically digested for two hours with Liberase™ (Roche) at 37°C. A second enzymatic digestion with TrypLE™ Select Enzyme (ThermoFisher Scientific) for twenty minutes was performed on remaining fragments to separate crypt structures from basal membrane tissue. PDOs obtained were resuspended into basement membrane extract (BME) (Corning) and seeded in 48 well plates in triplicates. An average number of 200

structures/organoids per well was cultured and developed as previously described[4,5].

Organoid Culture

Culture conditions were set up in basal cell culture medium supplemented with growth factors and inhibitors as previously described[4]. Incubation was performed at 20% O_2 and 5% CO_2. After expansion, PDOs were cultured with either media optimized in the lab (Lab Made Media) or AVATAR™ Colon Organoid Media (ADS Biotech, 709038), with media replacement conducted every three days. For optimized media, ideal cell culture conditions were determined separately for each PDO sample using a combinatorial strategy in which different medias supplemented with growth and inhibitory factors were tested, while AVATAR™ Colon Organoid Media was used following manufacturer's recommendations.

Organoid Passaging

PDOs were split every 7 days as follows: PDOs were mechanically removed from BME (Corning) by pipetting, incubated in Cell Recovery Solution (Corning) for 1 hour at 4°C, washed twice with ice-cold PBS (ThermoFisher Scientific), and seeded as described above.

Growth & Viability

Organoid viability was assessed using Trypan Blue exclusion staining. Following each passaging process, organoids were removed from BME and enzymatically disrupted using TrypLE™ for 30 minutes at 37°C. Once disrupted, the single cell suspensions were washed twice with cold PBS and pelleted for 10 minutes at 300g. Pellets were resuspended in 1 mL of cold PBS. An aliquot of the cell suspension was combined with 0.4% Trypan Blue solution (Thermo Scientific), loaded on a cell counter slide, and automatically counted using the Countess II (Thermo Scientific).

Immunohistochemistry

Portions of each PDO cultures were prepared for immunohistochemistry (IHC) analysis as follows: samples were fixed in 10% formalin at room temperature for 10 minutes and then embedded in 200µl of Bio-Agar (Bio-Optica). The samples were cooled at –20°C until solidified. Resulting blocks were processed and paraffin embedded. Sections with a thickness of 3µm were then obtained for each sample and used for IHC analysis. To assess if PDOs cultured with the two different methods retained the phenotypical features of typical colorectal tissue from which they were originated, IHC characterization of Fp1, cC1 and Pc1 PDOs was performed using the following primary antibodies: anti-CK AE1/AE3 (Dako 1:100), anti-CK20 (Dako 1:500), anti-CK19 (Sigma 1:1000), anti-CDX2 (Dako 1:50), anti-Mib1 (Dako 1:400), anti-LGR5 (Origene 1:100). Briefly, slices of 3µm were cut at the microtome, dried, pre-treated with Dako PTlink (Dako) for antigen retrieval and stained using an automatic Agilent Dako Autostainer Link 48 (Dako). Images were captured using Aperio Leica ScanScope XT (Leica).

Statistical Analysis

Comparison between Lab Made Media and AVATAR™ Colon Organoid Media culture conditions were performed using R software version 4.2.2. Two-way ANOVA multiple comparisons test was used with the Bonferroni p-value adjustment. All comparison results were considered significant

when p-value was ≤0.05. IHC digital analysis was performed using QuPath software v.0.5.1 (https://qupath.github.io/). A tool was used to create a project able to identify IHC stained cells, subsequently a score was calculated by quantifying the intensity feature according to different antibody staining distributions.

Results

Extended culture of colon organoids in AVATAR™ Colon Media results in improved growth

An important indication of whether a medium has the necessary components to support tissue survival is whether organoids are growing and surviving after prolonged culture. Organoids derived from adenomas initially grew better in lab made media over three passages, then there was a dramatic shift in organoid population in passages four (28 days) and five (35 days) where organoids cultured in the AVATAR™ media were significantly higher in population than cultures in the lab made media (Fig. 1A). At 35 days post organoid establishment, there was an approximately three-fold increase in the number of organoids counted in the AVATAR™ media compared to the lab made media. When the viability of the organoid samples was examined, there was an approximately 25% decrease in the viability of organoids cultured in the AVATAR™

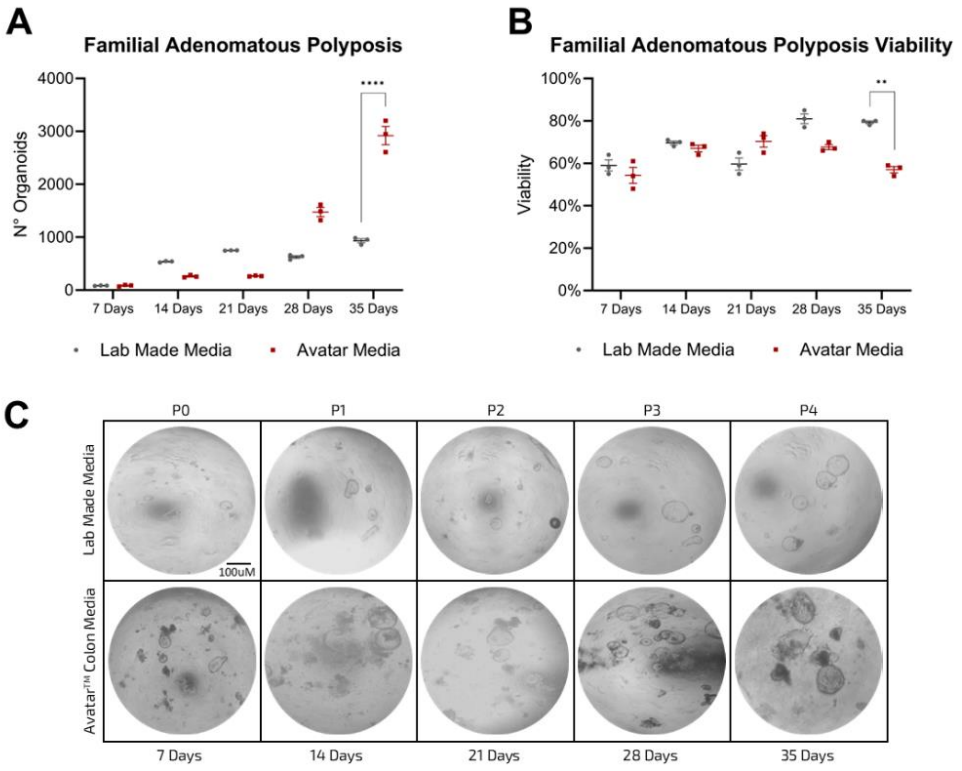

Figure 1: Avatar™ Colon Culture Media results in higher populations of adenoma organoids in extended culture. (A) Total organoid numbers cultured from adenomas over five passages. (B) Viability of adenoma organoids at each passage. (C) Representative brightfield images of adenoma organoids. ** indicates *p<0.01* and **** indicates *P<0.0001* as determined by two-tailed, two sample t-test.

media compared to the lab made media at day 35 (Fig. 1B). Representative brightfield images of organoid cultures are shown in Figure 1C. When organoids derived from colon cancer tissue were cultured, both medias appeared to support growth through the first three passages comparably. However, in passages four (28 days) and five (35 days), there was once again a dramatic increase in the growth rate of the organoids cultured in AVATAR™ media resulting in a significantly higher population of organoids cultured in the AVATAR™ media at passage five compared to the lab made media (Fig. 2A). There was an approximately two-fold increase in the number of organoids cultured in AVATAR™ media at 35 days compared to lab made media. Evaluation of viability demonstrated that organoid viability in AVATAR™ media was initially approximately half of the organoid viability in lab made media. However, the viability of organoids in the AVATAR™ media increased over the passages until it became comparable to the viability observed in lab made media (Fig. 2B). Brightfield microscopy images of the colorectal cancer organoids are depicted in Figure 2C. Metastatic disease demonstrated a noticeably different pattern of growth. As early as the first passage (7 days), there were approximately twice as many organoids cultured in AVATAR™ media as compared to organoids in the lab made media. At passage three (21 days), the population of organoids cultured in

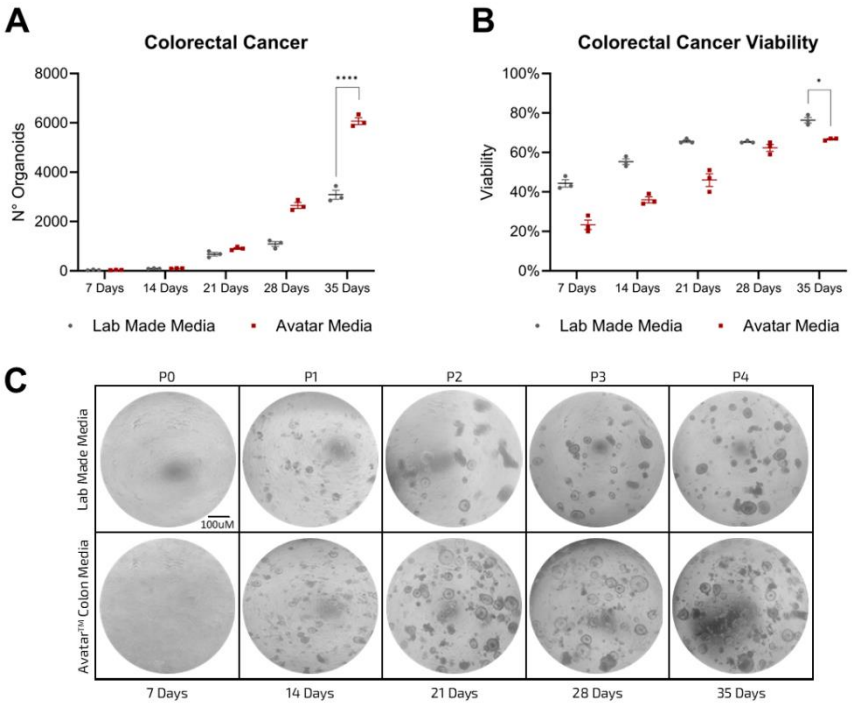

Figure 2: Avatar™ Colon Culture Media results in higher populations of colorectal cancer organoids in extended culture. (A) Total organoid numbers cultured from colorectal cancer tissue over five passages. (B) Viability of colorectal cancer organoids at each passage. (C) Representative brightfield images of colorectal cancer organoids. * indicates *P<0.05* and **** indicates *P<0.0001* as determined by two-tailed, two sample t-test.

AVATAR™ media began to increase and continued to increase through passage 5 (35 days), where there approximately 8.5 times more organoids compared to cultures in lab made media (Fig. 3A). Interestingly, the population of organoids cultured in lab made media appeared to stay relatively constant and only increased approximately two-and-a-half-fold over five passages (35 days). The viability patterns noted in the other samples was also changed in the metastatic samples as the samples cultured in AVATAR™ media were comparable to, or higher, than the samples cultured in lab made media (Fig. 3B). Comparison of brightfield microscopy suggested that the organoids cultured in the AVATAR™ media were larger than the same organoids cultured in the lab made media (Fig. 3C).

Culture with AVATAR™ media increases proliferation, but decreases stemness of some organoids

While improved organoid growth and passaging are important metrics to consider for research and biobanking, it is also important to ensure that the organoids are functionally similar to those cultured

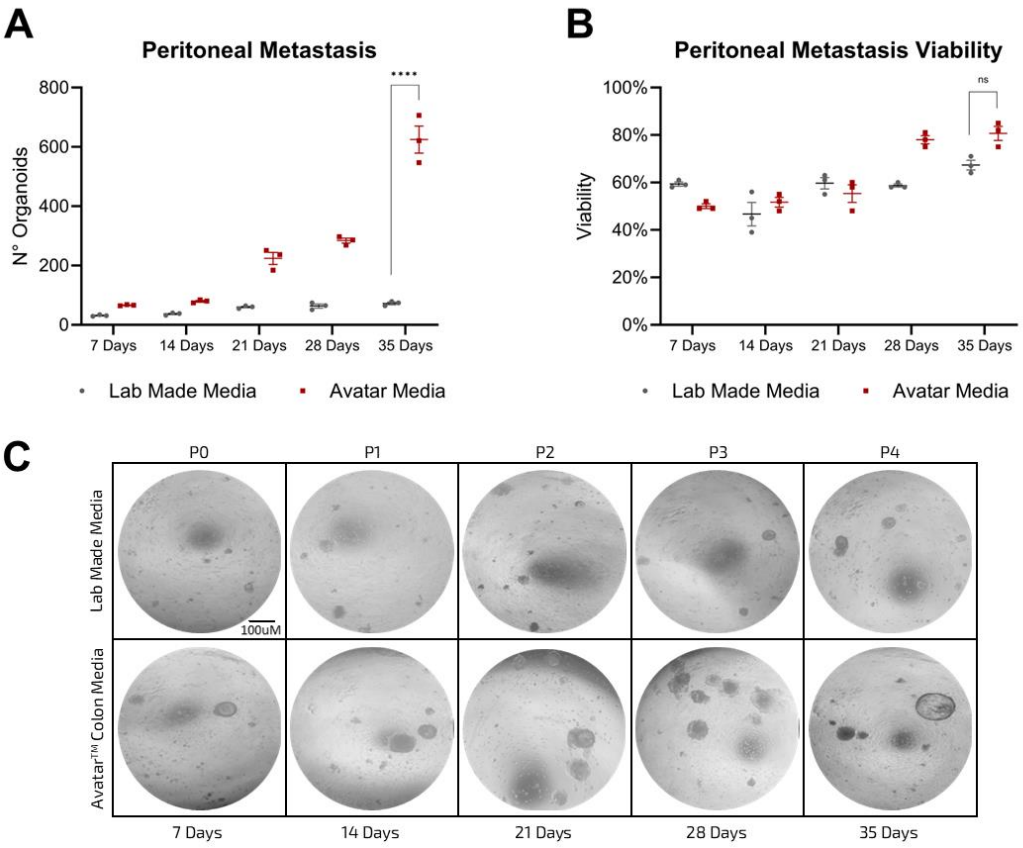

Figure 3: Avatar™ Colon Culture Media results in higher populations of peritoneal metastatic colorectal cancer organoids in extended culture. (A) Total organoid numbers cultured from peritoneal metastasis of colorectal cancer over five passages. (B) Viability of peritoneal metastasis organoids at each passage. ns indicates *not significant* and **** indicates *P<0.0001* as determined by two-tailed, two sample t-test.

in lab made media. Following the culturing of the organoid in both lab made media and AVATAR™ media, the organoids were harvested and sectioned for IHC staining to determine if there were substantial changes in several markers. Epithelial glandular markers (CKAE1/AE3, CK19, CK20), a colorectal marker (CDX2), a proliferation marker (Mib1), and an intestinal stem cell marker (LGR5) were examined. Cellular positivity, as well as expression intensity, were measured and used as the basis for determining H-Score. Positive cells were evaluated by assigning three different levels of

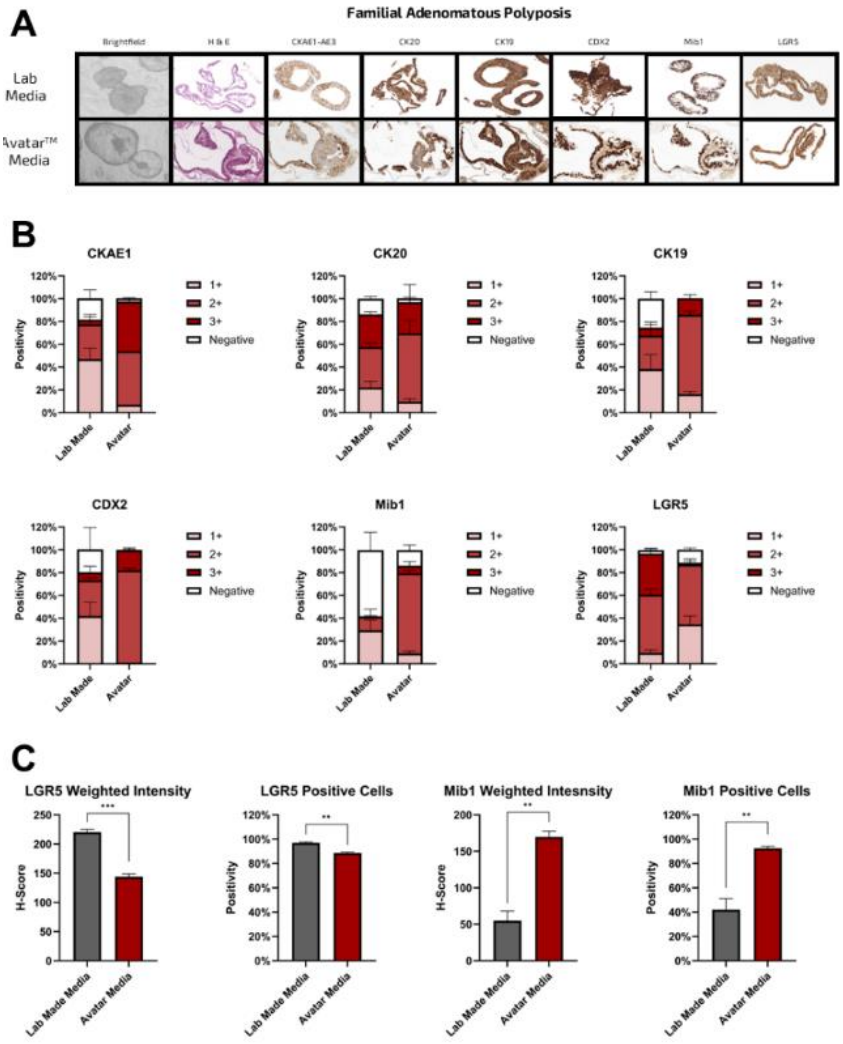

Figure 4: Adenoma organoids cultured in Avatar™ Media increased epithelial glandular, colorectal, and proliferation marker expression, but decreased stemness marker expression. (A) Representative stained sections of organiods cultured in Lab Made Media (top) or Avatar™ Media (bottom. (B) Positivity distribution of cells positive for the indicated markers in organoids cultured in Lab Made Media or Avatar™ Media. (C) H-score comparison of the stemness marker, LGR5, and proliferation marker, Mib1, for Lab Made Media and Avatar™ Media cultures. ** indicates *P<0.01* and *** indicates *p<0.001* as determined by two-tailed, two sample t-test.

positivity and one negative subcategories. Cells with 3+ had high expression, 2+ had medium expression, and 1+ had low expression. The organoids derived from the familial adenomatous polyposis sample demonstrated the most striking differences. Representative IHC images are shown in Fig. 4A. All three of the epithelial glandular markers, the colorectal marker, and the proliferation marker Mib1 demonstrated a shift to higher intensity in the AVATAR™ media, whereas the intestinal stem cell marker, LGR5, shifted lower in intensity (Fig. 4B). This was further quantified by examining both the

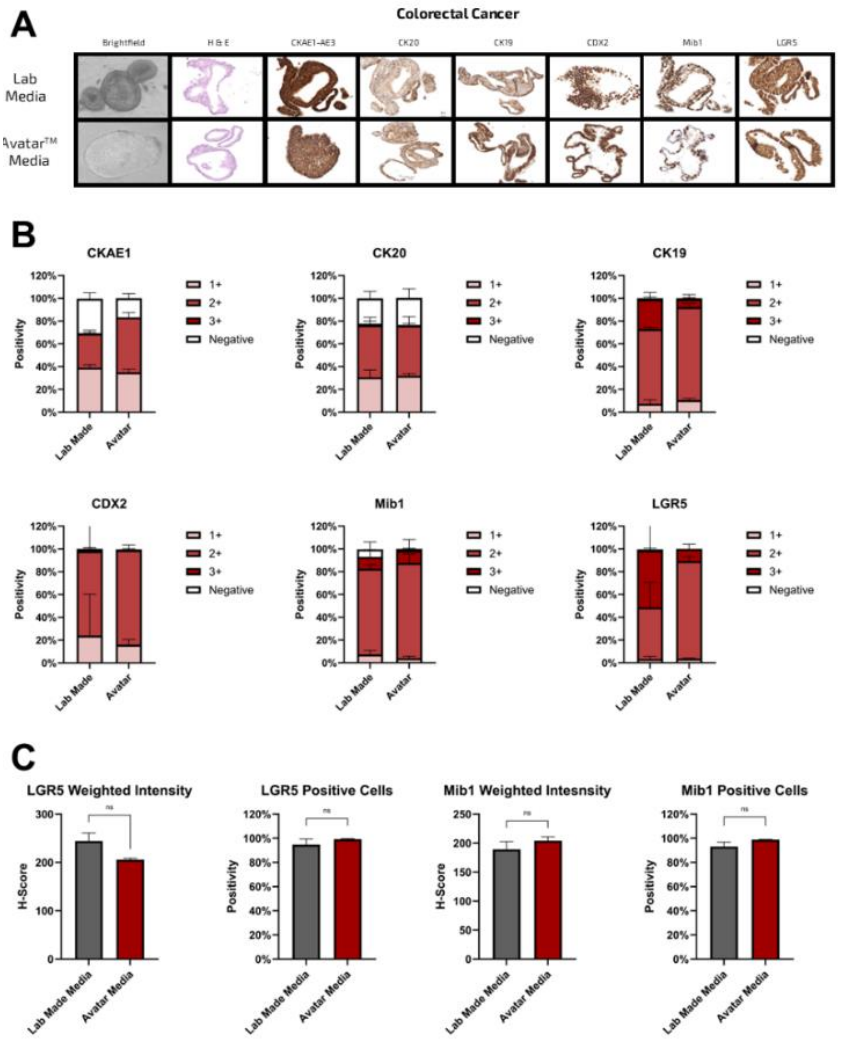

Figure 5: Colorectal cancer organoids cultured in Avatar™ Media have similar marker expression to organoids cultured in Lab Made Media. (A) Representative stained sections of organoids cultured in Lab Made Media (top) or Avatar™ Media (bottom. (B) Positivity distribution of cells positive for the indicated markers in organoids cultured in Lab Made Media or Avatar™ Media. (C) H-score comparison of the stemness marker, LGR5, and proliferation marker, Mib1, for Lab Made Media and Avatar™ Media cultures. ns indicated *not significant* as determined by two-tailed, two sample t-test.

number of positive cells and the overall positivity of the sample through use of the H-Score (Fig. 4C). While there was only a small, but significant, reduction of LGR5 positive cells, the H-Score decreased approximately 35% as more cells were negative or 1+ positive for the marker. Conversely, there was an approximately two-fold increase in the number of positive cells for Mib1 as well as an approximately three-fold increase in the H-Score, indicating organoids cultured in AVATAR™ Media were expressing Mib1 more intensely and proliferating more than organoids cultured in Lab

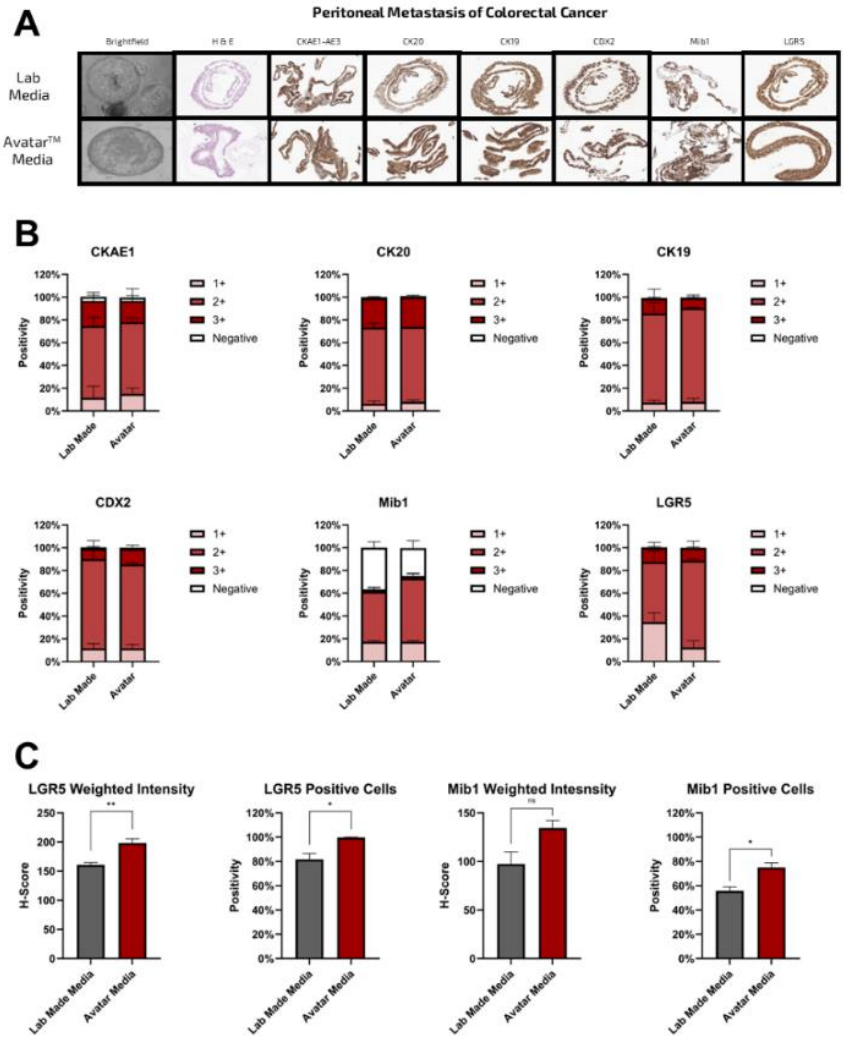

Figure 6: Metastatic colorectal cancer organoids cultured in Avatar™ have comparable glandular and colorectal marker expression, but increased proliferantion and stemness marker expression. (A) Representative stained sections of organiods cultured in Lab Made Media (top) or Avatar™ Media (bottom. (B) Positivity distribution of cells positive for the indicated markers in organoids cultured in Lab Made Media or Avatar™ Media. (C) H-score comparison of the stemness marker, LGR5, and proliferation marker, Mib1, for Lab Made Media and Avatar™ Media cultures. ns indicates *not significant*, * indicates *P<0.05* and ** indicates *p<0.01* as determined by two-tailed, two sample t-test.

Made Media.

IHC analysis was conducted on colorectal cancer organoids (Fig. 5A). The organoids were evaluated for the same markers and demonstrated a substantially lower shifts in marker expression (Fig. 5B). Like the adenoma organoids, there was an increase in proliferation marker, Mib1, expression and a decrease in stemness marker, LGR5, expression as shown by positive cells and H-Score (Fig. 5C). The increase of Mib1 expression and the decrease of LGR5 expression were both found to be not statistically significant.

Organoids derived from colorectal cancer metastasis in the peritoneum were subjected to the same processing and staining as adenoma and CRC organoids (Fig. 6A). Quantitative distribution of the positive cells within the organoids demonstrated that the expression of the epithelial glandular markers and the colorectal marker were extremely similar between the two culture conditions (Fig 6B). Interestingly, there was an increase in expression of LGR5 and Mib1, demonstrating that the organoids cultured in AVATAR™ media were made up of more proliferating cells and cells retaining stemness (Fig. 6C). Both the number of positive cells counted and the H-Score for both markers were increased in the AVATAR™ Media cultures and was found to be statistically significant.

Discussion & Summary

The Transcriptomic research lab at the National Cancer Institute Milan, with projects focused on the use of human colon organoids, evaluated their lab made media against a new colon organoid culture media from ADS Biotec. The research lab has spent considerable time and effort to develop their culture medium, a distillation of at least 14 iterations from a previously published recipe. Three different disease states of colorectal cancer (adenoma, colorectal cancer, metastasis) were utilized to examine whether the AVATAR™ Colon Organoid Media from ADS Biotec improved organoid culture. In long-term culture of each of the three conditions, the AVATAR™ media resulted in greater organoid numbers. There was a significant decrease in the viability of organoids in adenoma cultures, but that decrease improved in the cancer organoids and had disappeared in the metastatic organoids. When examining IHC markers for differences, the adenoma organoids demonstrated the greatest expression differences, with CKAE1/AE3, CK19, CK20, CDX2, and Mib1 expression increasing and LGR5 marker expression decreasing in organoids cultured in the AVATAR™ Media. Marker expression was much more similar between the two culture conditions in the cancer and metastasis organoids. The AVATAR™ Colon Organoid Media by ADS Biotec appears to be a viable alternative in colon organoid culture compared to Lab Made Media

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