

# Propagation of Human Colon, Mammary, and Lung Cancer Organoids in Growth Medium Utilizing Tissue-specific Reagent Kits and Ready-to-use Wnt-3a and R-Spondin1 Conditioned Media

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Credible leads to Incredible

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## Abstract

Three-dimensional "organoid" growth of tumors may represent a more physiologically relevant *in vitro* model system than traditional two-dimensional monolayer cultures of cancer cell lines. With the increased availability of cryopreserved human cancer organoids generated by academic laboratories, large-scale biobanking initiatives, and commercial sources, there is an unmet need for simplified, standardized, and cost-effective methods for preparation of the complex growth media required by these models. Human organoid culture media contains a variety of recombinant proteins, small molecules, and other growth factors that are costly to purchase in small-scale, time consuming to reconstitute and aliquot, and demonstrate varying stability and shelf life once prepared. Organoid culture media often also utilizes undefined conditioned media (CM) from one or more engineered cell lines that must be cultured separately, requiring additional time and resources to maintain. These lines secrete critical growth factors and the CM generated must be carefully prepared, collected, and stored. CM is subject to variability in activity levels due to batch-to-batch and protocol-to-protocol differences that can affect subsequent organoid culture performance. To address these challenges and to facilitate the wide-spread adoption of human organoid culture we are developing reagent kits containing essential growth medium components in an individually lyophilized format for long-term storage and easy single-use preparation of organoid growth media for a variety of tissue types including human colon, mammary, and lung. We compared growth medium formulated with our kit components, with small-scale "homebrew" preparations and found equivalent or better culture performance (as calculated by doubling times) and similar morphology. Both approaches were able to generate media capable of supporting growth for at least three passages (~30 days) in 15 different organoid models. Additionally, we compared protocols and cell lines for CM generation to support organoid culture and found that an optimized, scaled-up production method could generate Wnt-3a and R-spondin1 CM with higher activity levels (up to 52%) and reduced lot-to-lot variability in comparison with commonly used small-scale protocols. We found that the optimized protocols produced CM that was stable for at least 2 months at 4°C and at least 6 months at -20°C, and was resilient against freezing and thawing. Previously frozen CM was also able to support organoid growth in culture. These results show that our tissue-specific kits and ready-to-use CM can provide a simplified, cost effective method to support the long-term propagation of human cancer organoids from multiple tissues and cancer types.

## Methods

**Organoid growth media:** For pre-prepared tissue specific growth kits, relevant growth factors were custom packaged into individual vials in a lyophilized format. Kits contained all needed growth factors (see Table 1), including a variety of recombinant proteins, small molecules and supplements needed to generate a complete growth media (excluding CM). No aliquoting or calculations were required to prepare the media using the kit. Homebrew media was prepared following public protocols and contained the same growth factors at the same final concentration as with the kit prepared media. For both kits and homebrew media, the basal media consisted of Advanced DMEM:F12 (Thermo Fisher Scientific) containing 10 mM HEPES and 2 mM L-Glutamine (ATCC® 30-2214™). Individual components were reconstituted in either basal medium or DMSO (ATCC® 4-X™). Once prepared, media was supplemented with CM if required. Once prepared, complete media was stored at 2-8°C and used within 4 weeks.

**Table 1. Growth factors in tissue-specific organoid complete growth media formulations**

Organoid	Primary growth factors
Lung	N-acetylcysteine, Nicotinamide, Noggin, A83-01, FGF-10, and FGF-7
Pancreas	N-acetylcysteine, Nicotinamide, EGF, Noggin, A83-01, FGF-10, and Gastrin
Colon	N-acetylcysteine, Nicotinamide, EGF, Noggin, A83-01, SB202190, and Gastrin
Esophagus	N-acetylcysteine, Nicotinamide, EGF, Noggin, A83-01, SB202190, and FGF-10.
Mammary	N-acetylcysteine, Nicotinamide, EGF, Noggin, A83-01, SB202190, FGF-7, FGF-10, and HRGB.

**Organoid culture:** Organoids were cultured according to previously published methods.<sup>1</sup> In brief, all organoid cultures were initiated from previously cryopreserved material, washed once in complete growth media, and then embedded in Cell Basement Membrane (ATCC® ACS-3035™). On thaw and on passage, the complete growth media was supplemented with 10 μM ROCK inhibitor (ATCC® ACS-3030™). Media changes were performed 2-3 times per week and passages occurred every ~7-20 days depending on the model.

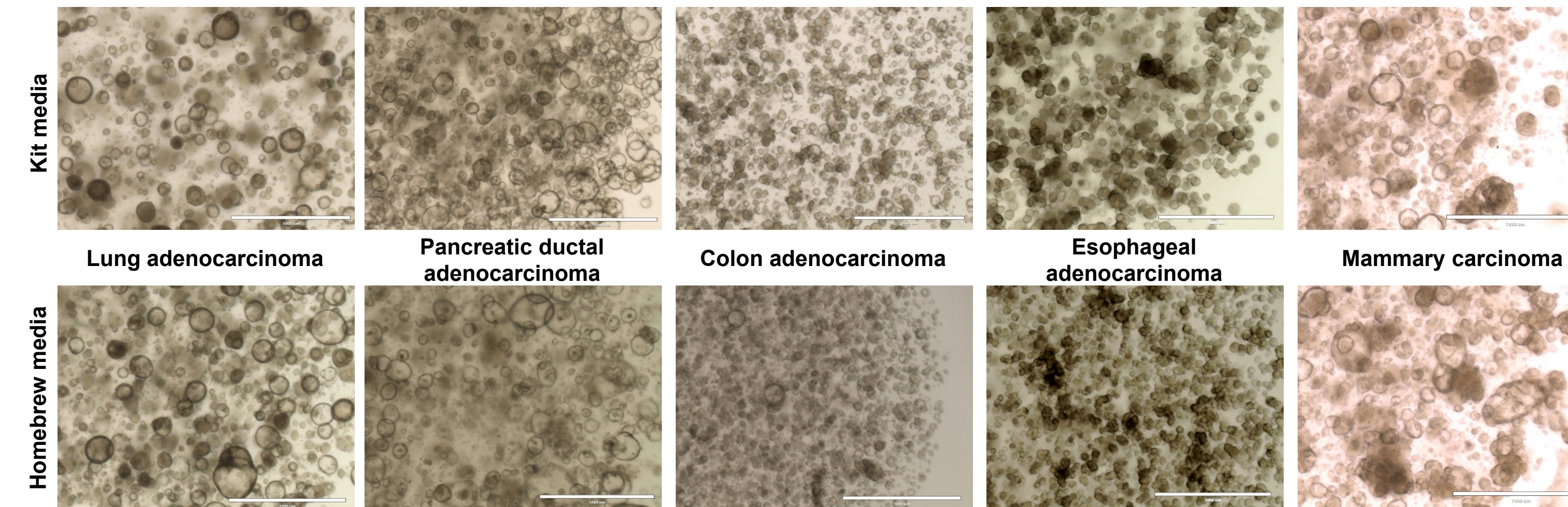
**Conditioned media generation:** Wnt-3a (WNT) CM was generated from L-WNT3A cells (ATCC® CRL-2647™) by using either published protocols<sup>2</sup> or an optimized, scaled-up protocol and made in 6-10L batches. R-spondin1 (RSPO) CM was generated from HA-R-Spondin1-Fc 293T Cells (Trevigen) using either published protocols<sup>3</sup> or an optimized, scaled-up protocol.

**WNT Activity Assay:** CM and other sources of Wnt and R-Spondin1 were tested in a standard WNT signaling assay utilizing HEK293 cells transiently expressing a construct containing TCF/LEF binding sites upstream of a luciferase reporter. Luciferase activity was measured with the Bright-Glo® Luciferase Assay System (Promega).

**Immunocytochemistry (ICC):** Organoids were fixed, paraffin embedded, and sectioned for subsequent ICC by using standard techniques. Some organoids were fixed and stained directly without sectioning.

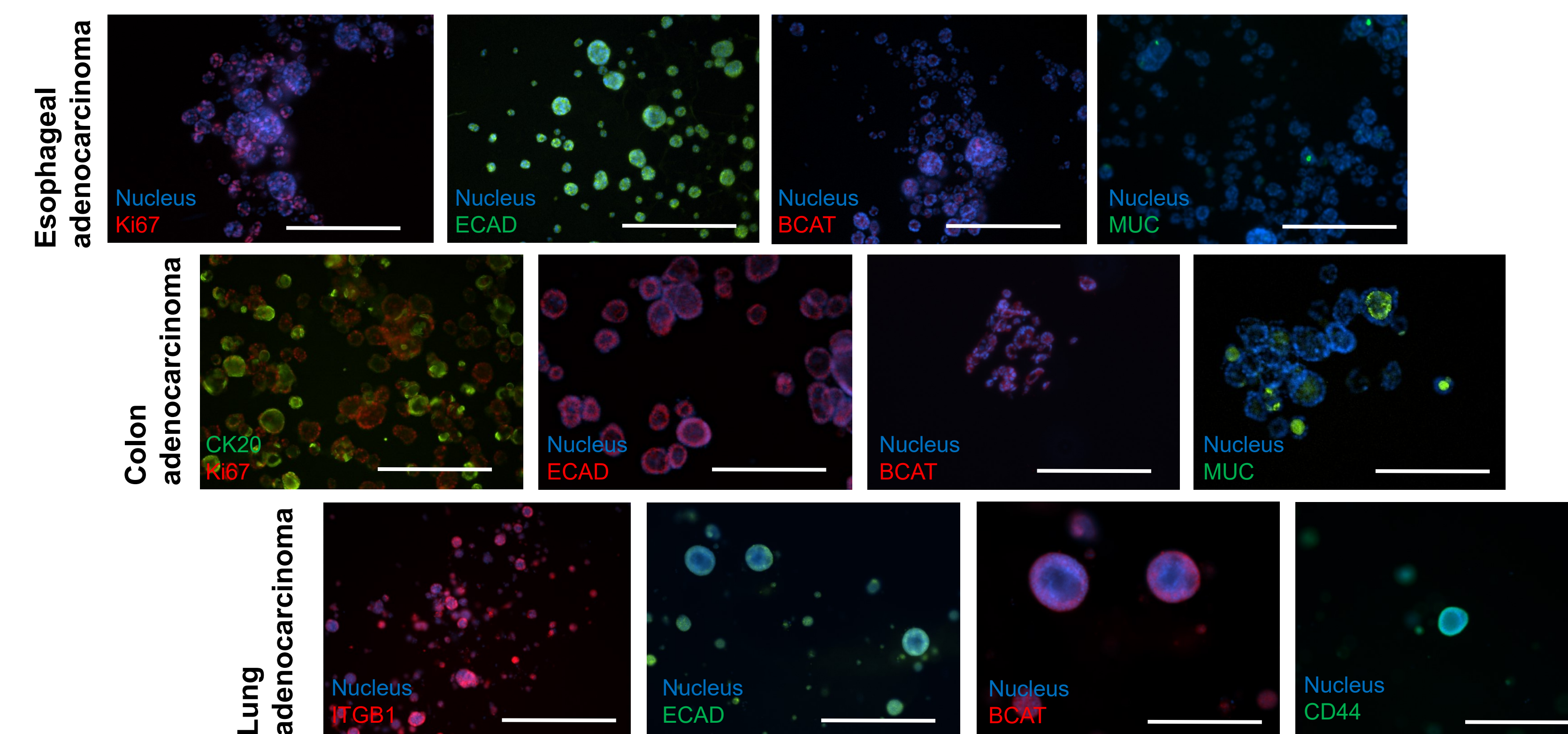
## Results

### Morphology



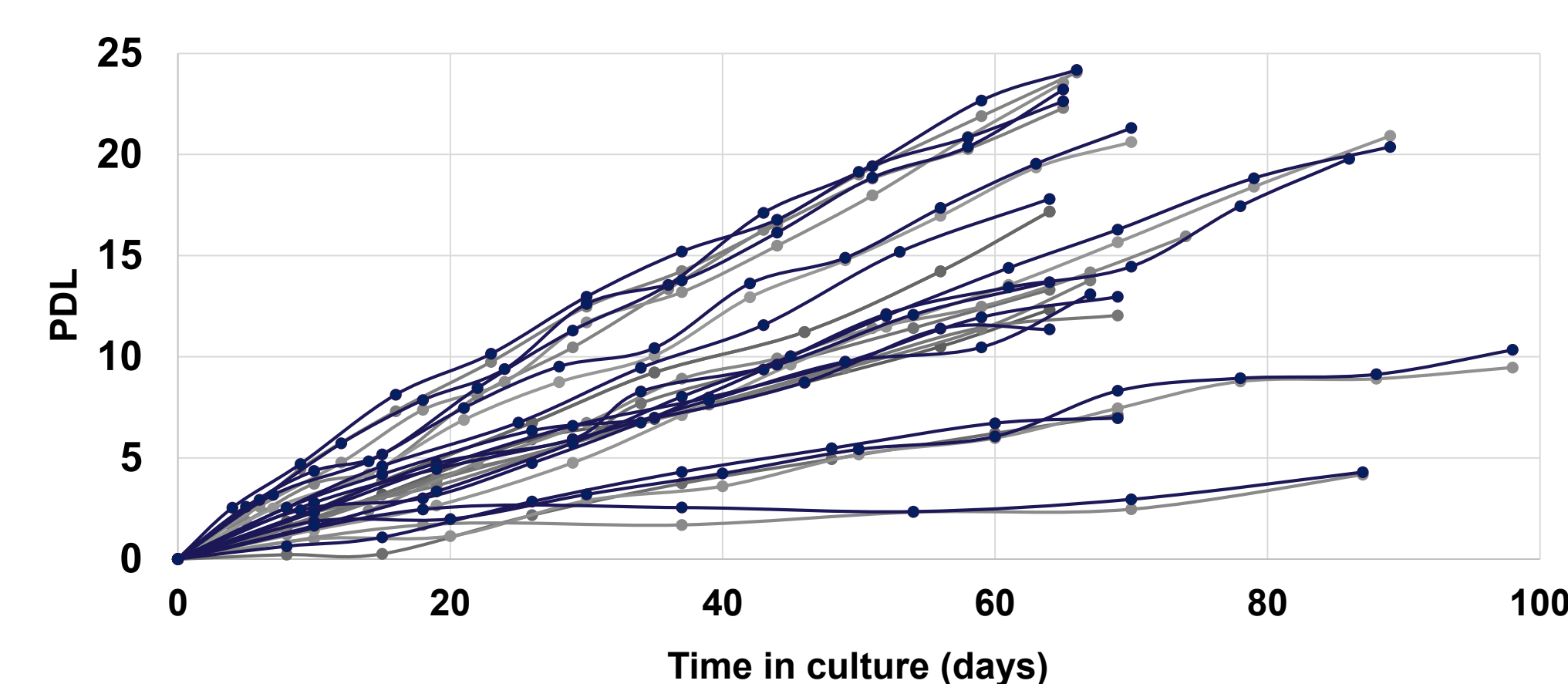
**Figure 1. Representative brightfield photomicrographs displaying culture morphology.** No differences between media preparation method were evident. 15 individual organoid models were cultured for at least 30 days (average DIV = 48) and imaged. Lung, pancreas, colon, and esophageal images were captured 7-10 days post-passage at P3. Mammary model images were taken at 25 days post-passage at P4. Images were captured with a 4x objective and the scale bars are 1000 μM.

### Immunocytochemistry



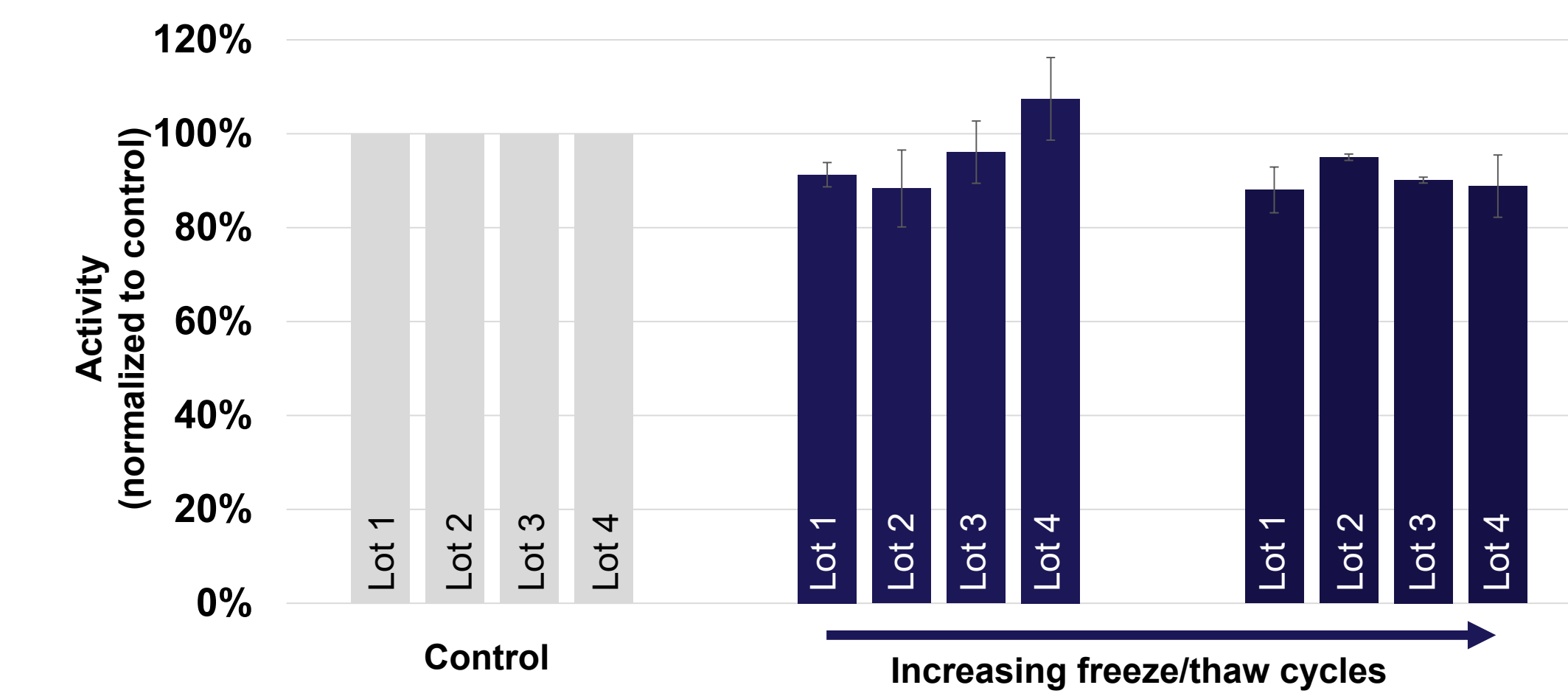
**Figure 2. Immunostaining revealed tissue- and cell-type-relevant protein expression.** Organoids showed differential expression of CK20 (cytokeratin-20; cytoskeletal), Ki67 (cell proliferation marker), ECAD (E-cadherin; epithelial marker), BCAT (Beta-catenin; WNT pathway), and MUC (Mucin; goblet cell marker) when cultured in kit media (homebrew media not shown, pancreas and mammary not shown). Neither Ki67 or MUC (1/2/5AC) expression was detected in Lung adenocarcinoma organoids but CD44 and Integrin beta-1 (ITGB1) were ubiquitous. Images were captured at 10x magnification. Scale bars are 400 μM.

### Growth rate

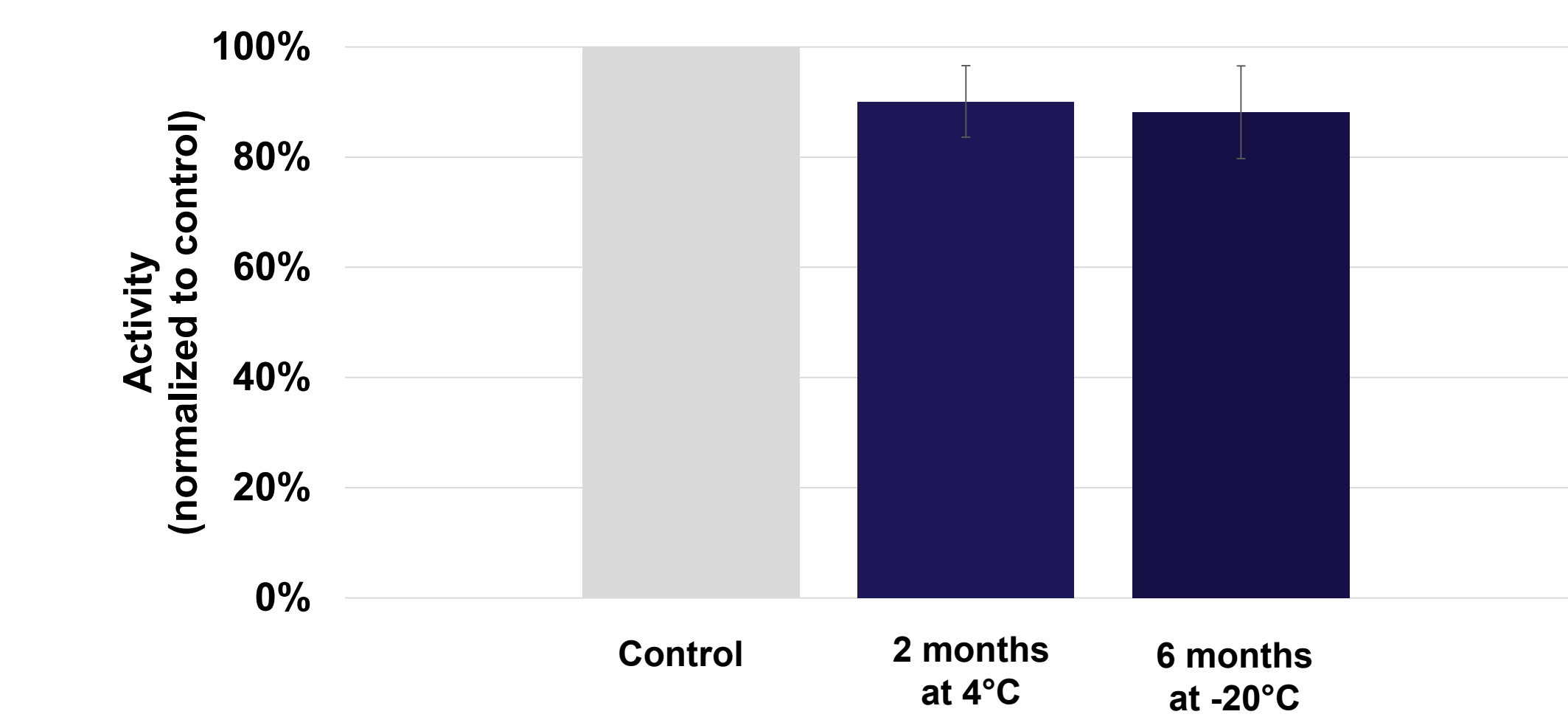


**Figure 3. Population doubling level (PDL) of fifteen different organoid models.** Organoids were cultured in homebrew media (gray) or with kit media (blue) for up to 98 days. Minor growth rate differences were seen but were highly similar at completion of culture (mean difference=0.2 PDL, n=15). Five of 15 models had a slightly lower PDL (mean difference = -0.54) with the kit media than with the homebrew equivalent. For all models, no difference between the two media preparation methods was greater than 1.0 PDL at completion of culture.

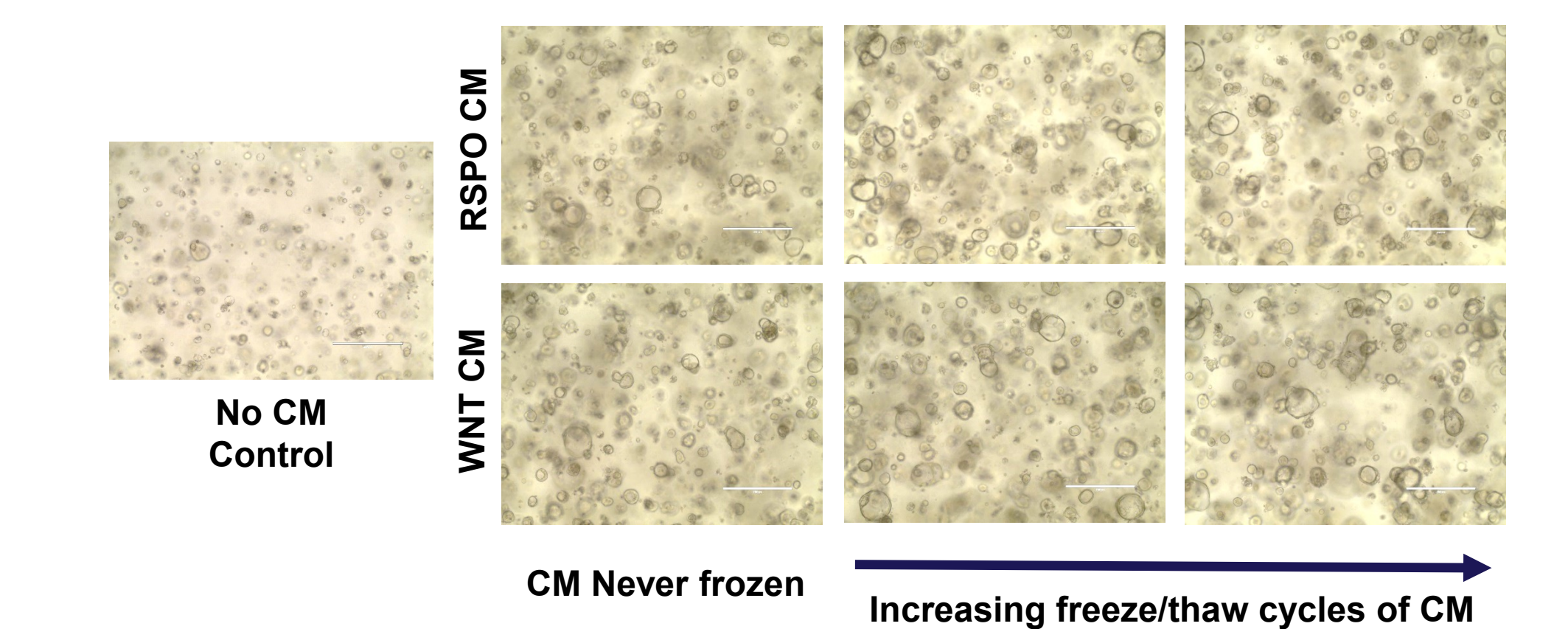
### CM activity and stability



**Figure 4. Stability of CM after freeze and thaw.** Four individual lots of CM were divided and either stored at 4°C, or subjected to repeated freeze/thaw cycles at -20°C. All samples were tested for WNT activity in triplicate. Error bars are STD. Results are normalized to pre-freeze (control) activity and set to 100%. The maximum reduction in activity seen was 12% and the mean after multiple cycles was 10%.



**Figure 5. Long-term stability of CM.** CM was divided and either stored at 4°C at -20°C. All samples were tested for WNT activity in triplicate. Error bars are STD. Results are normalized to pre-storage (control) activity and set to 100%. After 4°C or -20°C for 2 or 6 months, the peak maximum loss of activity was <10%.



**Figure 6. Previously frozen CM retains function in culture.** A WNT- and RSPO-sensitive pancreas organoid model was thawed and cultured in complete growth media with CM that had either been never frozen, or repeatedly frozen and thawed. No difference in morphology or culture performance was seen after 3 passages (~30 days) with an average PDL of 3.25. In contrast, in the absence of functional CM the model exhibited a loss of cystic morphology and expansion slowed (PDL ~1). Images were captured at 4x magnification. Scale bars are 1000 μM.

## Summary

- Organoids exhibited similar rates of growth, morphology, and cell/tissue type-specific markers regardless of method of media preparation.
- By using optimized protocols, CM generation could support large-scale organoid generation and was amenable to long-term storage while frozen, as well as multiple freeze/thaw cycles. Even after repeated freeze/thaw cycles CM was still functional in organoid culture.
- Pre-prepared tissue-specific reagent kits can simplify organoid media preparation and are suitable for use with a variety of cancer organoid models including colon, lung, pancreas, esophageal adenocarcinomas, and mammary carcinomas.

## References

- Clinton J, McWilliams-Koeppen P. Initiation, expansion, and cryopreservation of human primary tissue-derived normal and diseased organoids in embedded three-dimensional culture. *Current Protocols in Cell Biology*, 82, e66. doi: 10.1002/cpcb.66, 2019.
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