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A combinatorial culture strategy to develop pseudomyxoma peritonei organoid models

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Abstract

Background and Objectives: Few preclinical models of pseudomyxoma peritonei (PMP) have been developed, probably due to the tumor's low incidence and its peculiar characteristics of slow growth. Therefore, there is a need to develop more refined PMP models that better reflect its characteristics. The aim of the study is to develop a culture strategy to generate organoid models derived from PMP patient samples.

Methods: We followed a strategy based on combinatorial culture conditions that include the different factors essential for PMP growth and that mimic the micro-environment present in the patients.

Results: We cultured PMP samples in the presence of the various factors produced by the niche environment of PMP. We obtained 12 PMP organoid models, each of which grows under specific culture conditions. PMP-derived organoids show long-term expansion capacity and reproduce the genetic landscape and histological phenotype of the tumor of origin.

Conclusion: The organoids we developed faithfully reproduce the key features of PMP disease and will allow us to understand the biology of PMP. With them, we will be able to identify key regulatory networks that support PMP progression, providing a platform for multilevel preclinical testing, identify novel diagnostic biomarkers, and generate novel targets for patient treatments.

KEYWORDS

appendiceal tumors, mucin, organoids, pseudomyxoma peritonei, 3D models

Luca Varinelli and Marzia Di Bella contributed equally to this study.

Marcello Deraco and Manuela Gariboldi are co-last authors.

1 | INTRODUCTION

Pseudomyxoma peritonei (PMP) is a rare epithelial neoplasm that originates from a mucinous adenocarcinoma of the appendix, resulting from the neoplastic transformation of appendiceal epithelial cells that acquire a globet cell phenotype and produce mucin. Mucin accumulates intraluminally and generates an appendiceal mucocele, the perforation of which spreads the cells exfoliated by the tumor into the peritoneal cavity.[1] According to the World Health Organization (WHO) PMP is categorized into low-grade (LG) and high-grade (HG) disease. LG subtypes progress slowly, and only 20% of them have invasion ability, while the HG phenotype invades and metastasizes the peritoneal cavity in 80% of cases. Five-year survival rates are 63% and 23%, respectively.[2] The current standard treatment for PMP, based on cytoreductive surgery (CRS), in combination with hyperthermic intraperitoneal chemotherapy (HIPEC), has improved patient outcomes. This combination treatment, however, causes morbidity and mortality and is applicable to only 60%–70% of patients. In addition, a significant proportion of PMPs usually relapse. More effective and less toxic alternative treatments are therefore needed.[1] The genetic background of PMP is poorly known because of its low incidence and the difficulties associated with collecting representative tissue material. Some studies have found the characteristic alterations in *KRAS* and *GNAS* genes, along with a lower frequency of mutations in *SMAD2*, *−3* and *−4*, *TP53*, *TGFBR1* and *−2*, *PIK3CA*, and *AKT1*, while mutations in *APC* and *BRAF* are rare. Finally, activating mutations of *GNAS* can increase the expression of MUC2, through activation of cAMP-PKA.[3–5] Inflammation plays a central role in PMP by providing essential growth factors and protection from multiple factors such as hypoxia and apoptosis. Many cytokines and chemokines, including IL-6, IL-8, CXCL10, CCL2, and CCL3, are elevated in PMP. In particular, IL-6 activates several signaling pathways, such as STAT-3, Ras, MAPK, Cox-2, Wnt, and PI3K/AKT.[1,2]

At present, preclinical in vitro and in vivo models that resemble real PMP disease are lacking, because of the low incidence of PMP and its slow growth. The most commonly used models in cancer research and anticancer drug discovery are cancer cell lines, patient derived organoids (PDO) and tumor patient derived xenografts (PDX).[6] Cancer cell lines are easy to propagate and allow extensive experimentation; however, they usually loose growth potential after a few in vitro steps and permanent cell lines can be established with low efficiency. PDX has great promise as a preclinical model for human cancer, but the tumor growth success of PDX is unsatisfactory, with aggressive tumors engrafting best. In addition, they are labor intense, time consuming, and ethically problematic.[7] Few in vitro or in vivo models of PMP have been developed, and only two primary PMP cell lines, although immortalized with an SV40 T-antigen lentiviral vector, have been generated.[8] PDX models were obtained from HG tumors.[9] In a recent paper, Martinez-Quintanilla et al., developed PDX that were used to identify new druggable targets in PMP disease,[10] highlighting the potential of preclinical models in defining new therapeutic strategies in a personalized manner.

PDOs represent an intermediate model between in vitro cancer cell lines and PDX models. PDOs grow as compact irregular structures, can be expanded indefinitely, are established in a relatively short time, retain the genetic state of the original tissues, and are easy to manipulate.[7] Organoids were initially developed from normal intestinal stem cells of the crypts and from colorectal cancer,[11,12] and have now been established from tumor tissues of various organs.[7] Organoids models, derived from HG PMP patients, proved their efficacy for developing personalized targeted therapies.[10] For these reasons, PDOs could be an interesting solution to overcome the previously explained research stalemate by faithfully reproducing the complexity and heterogeneity of this disease.

Here we report the generation and characterization of organoids derived from PMP patients (PMP-PDOs). The obtained PDOs reproduced multiple histological and physiological aspects of PMP disease, providing a platform to perform preclinical tests at multiple levels, identify novel diagnostic biomarkers, and characterize the biological features of PMP. New insights into the biology and treatment of PMP will optimize patient management, improving survival and minimizing surgical morbidity.

2 | MATERIALS AND METHODS

2.1 | Human tissues

Tumor tissues were obtained from patients with PMP during CRS/HIPEC procedures. The biological samples were collected in adherence with the guidelines of the IRCCS Foundation, Istituto Nazionale dei Tumori di Milano, and the Accelerator Award Project, review board protocols (INT53/21). The study complied with the Declaration of Helsinki and was approved by the Ethics Committee of the Fondazione IRCCS Istituto Nazionale dei Tumori of Milan, Italy. Written informed consent from each patient was obtained. The specimens were placed in cold PBS 1X, supplemented with 50 ng/mL gentamicin and 50 ng/mL amphotericin B, and immediately transferred to the laboratory for the development of PMP-PDOs. A part of the specimen was frozen in liquid nitrogen for molecular analyses. Formalin-fixed-paraffin-embedded (FFPE) blocks were prepared for immunohistochemistry (IHC) analyses. Clinical information and characteristics of the patients are reported in Table 1.

2.2 | Development of PMP-derived organoids

PMP-PDOs were developed based on the protocols already established for colorectal-derived PDOs,[11,13,14] properly modified to mimic the specific PMP microenvironment. Briefly, tumor tissues were cut into small specimens, washed 10-times with cold PBS 1 X supplemented with 50 ng/mL gentamicin and then digested with 1 mg/mL collagenase type II for 1 h at 37°C. The cells were recovered, resuspended in a commercial basement membrane matrix (Matrigel) and dispensed into a 24 multi-well (40 µL/well) that was filled with

TABLE 1 The main pathological and clinical characteristics of the PMP patients from whom the PDO were developed.

Patients	PDOs	Gender	Age	Diagnosis	Grade	PCI index	Mutations	Cellularity (%)	Tissue sampling for PDO development	PDO medium
1	PMP1	M	66	Low grade appendiceal mucinous neoplasm	Low	Low	*GNAS; KRAS*	20	CRS/HIPEC	#2
2	PMP2	M	52	Low grade appendiceal mucinous neoplasm	Low	High	*GNAS; KRAS; FBXW7*	30	CRS/HIPEC	#2
3	PMP3	F	51	Low grade appendiceal mucinous neoplasm	Low	High	*ATM*	5	CRS/HIPEC	#2
4	PMP4	F	54	Low grade appendiceal mucinous neoplasm	Low	Low	-	40	CRS/HIPEC	#3
5	PMP5	F	73	Mucinous adenocarcinoma	High	High	*KRAS; TP53; FLT3*	20	CRS/HIPEC	#2
6	PMP6	M	50	Low grade appendiceal mucinous neoplasm	Low	High	-	20	CRS/HIPEC	#6
7	PMP7	M	77	Low grade appendiceal mucinous neoplasm	Low	Low	*GNAS; KRAS*	10	CRS/HIPEC	#2
8	PMP8	F	63	Low grade appendiceal mucinous neoplasm	Low	High	*GNAS; KRAS; PIK3CA*	30	CRS/HIPEC	#3
9	PMP9	M	58	Low grade appendiceal mucinous neoplasm	Low	High	-	5	CRS/HIPEC	#4
10	PMP10	F	53	Low grade appendiceal mucinous neoplasm	High	High	*KRAS*	10	CRS/HIPEC	#1
11	PMP11	F	52	Low grade appendiceal mucinous neoplasm	Low	Low	-	20	CRS/HIPEC	#5
12	PMP12	M	67	Low grade appendiceal mucinous neoplasm	Low	High	-	10	CRS/HIPEC	#2

Abbreviations: CRS, cytoreductive surgery; HIPEC, hyperthermic intraperitoneal chemotherapy; PCI, peritoneal cancer index; PDO, patient-derived organoid; PMP, pseudomyxoma peritonei.

500 µL of organoid culture medium. PDO were grown in basal cell culture medium consisting of Advanced DMEM-F12 (ThermoFisher Scientific) and supplemented with gentamicin (50 mg/mL), amphothericin B (50 mg/mL), 10 mM HEPES, and 2 mM GlutaMAX (ThermoFisher Scientific). The basal medium was supplemented with the colonic-specific niche factors to obtain the WRENAS medium already described in the literature[12] as reported in Supporting Information S1: Table S1. WRENAS medium was supplemented with inflammatory and pleiotropic cytokines (IL-1, IL-4, IL-6, IL-9, IL-13), and factors (TGFβ, TNF-α, and MIF) (Supporting Information S1: Table S2). Six different media, mimicking six different conditions of the PMP microenvironment, were produced.

- Medium #1: WRENAS only
- Medium #2: WRENAS plus IL1/IL6/TNF-α
- Medium #3: WRENAS plus IL4/IL13
- Medium #4: WRENAS plus TGFβ
- Medium #5: WRENAS plus TGFβ/IL1/IL6/TNF-α
- Medium #6: WRENAS plus MIF

All the factors and/or cytokines were added, as reported in Table 2. PMP-PDOs were embedded in Matrigel (Corning) and dispensed into a 24-multiwell (200 crypt/well; 50 µL/well), incubated at 20% of O_2 and 5% CO_2 and cultured following a combinatorial strategy (Figure 1). Optimal PDO culture medium conditions were determined separately for each PDO culture (Table 2). PDOs were split every 3–4 days as follows: They were mechanically removed from the Matrigel by pipetting, incubated in Cell Recovery Solution (Corning) for 1 h at 4°C, washed trice with ice-cold PBS and seeded as described above. PMP-PDOs were frozen or prepared for molecular and/or immunohistochemical analyses.

TABLE 2 The specific media composition for each PMP-derived PDO culture.

PDO culture	Medium composition
PMP1	WRENAS medium; IL1/IL6/TNF-α
PMP2	WRENAS medium; IL1/IL6/TNF-α
PMP3	WRENAS medium; IL1/IL6/TNF-α
PMP4	WRENAS medium; IL4/IL13
PMP5	WRENAS medium; IL1/IL6/TNF-α
PMP6	WRENAS medium; MIF
PMP7	WRENAS medium; IL1/IL6/TNF-α
PMP8	WRENAS medium; IL4/IL13
PMP9	WRENAS medium; TGFβ
PMP10	WRENAS medium only
PMP11	WRENAS medium; TGFβ/IL1/IL6/TNF-α
PMP12	WRENAS medium; IL1/IL6/TNF-α

Abbreviations: IL1, interleukin-1; IL3, interleukin-3; IL4, interleukin-4; IL13, interleukin-13; IL6, interleukin-6; MIF, macrophage migration inhibitory factor; PDO, patient-derived organoid; PMP, pseudomyxoma peritonei; TGFβ, trasfroming growth factor-beta; TNF-α, tumor necorsis factor-alpha.

2.3 | DNA extraction and sequencing

DNA from FFPE sections of the PMP-PDO models and their tissue of origin was used for mutational analysis. DNA was extracted as previously reported.[14,15] Briefly, 150–200 ng of genomic DNA were quantified with Nanodrop™ 1000 (ThermoFisher Scientific) and Qubit 2.0 fluorimeter (Thermofisher Scientific) and used for mutational analysis with the 50-gene Ion AmpliSeq Cancer Hotspot Panel v2 (Life Technologies), with the Ion-Torrent™ Personal Genome Machine platform as previously reported[14] (Life Technologies, genes analyzed: *ABL1*, *AKT*, *ALK*, *APC*, *ATM*, *BRAF*, *CDH1*, *CDKN2A*, *CSF1R*, *CTNB1*, *EGFR*, *ERBB2*, *ERBB4*, *EZH2*, *FBXW7*, *FGFR1*, *FGFR2*, *FGFR3*, *FLT3*, *GNA11*, *GNAQ*, *GNAS*, *HNF1A*, *HRAS*, *IDH1*, *IDH2*, *JAK2*, *JAK3*, *KDR (VEGFR2)*, *KIT*, *KRAS*, *MET*, *MLH1*, *MPL*, *NOTCH1*, *NPM1*, *NRAS*, *PDGFRA*, *PIK3CA*, *PTEN*, *PTPN11*, *RB1*, *RET*, *SMAD4*, *SMARCB1*, *SMO*, *SRC*, *STK11*, *TP53*, and *VHL*). Twelve PMP-PDO models (PMP1; PMP2; PMP3; PMP4; PMP5; PMP6; PMP7; PMP8; PMP9; PMP10; PMP11; PMP12) and their corresponding surgical samples were analyzed.

2.4 | Histochemistry (HC) and IHC

HC and IHC analyses on PMP-PDOs and their corresponding tissues of origins were performed as described in Varinelli et al.[14] Samples were prepared as previously described.[13,14,16] IHC was performed with the following mouse anti-human antibodies: Ki-67, CK7, CK20, CDX2, and MUC2. Antigen retrieval for the antibodies was carried out using a preheated target retrieval solution (pH 6.0) for 30 min. Tissue sections were blocked with FBS serum in PBS for 60 min and incubated overnight with primary antibodies. The antibody binding was detected using a polymer detection kit (GAM/GAR-HRP; Microtech) followed by a diaminobenzidine chromogen reaction (Peroxidase substrate kit, DAB, SK-4100; Vector Lab). All sections were counterstained with Mayer's hematoxylin. Images were acquired with a DM6000B microscope (Leica Biosystems). Dilutions and experimental conditions are listed in Supporting Information S1: Table S3. Each experiment was performed in triplicate, and a representative section for each marker was chosen.

2.5 | Medium selection, influence of stem-related factors, microenvironment-related factors, and long-term expansion ability of PMP-PDOs

To determine the best medium for their growth, PMP-PDOs were seeded in 24 multiwells and cultured using the six different combinations of media as described above. The best medium was selected based on the number of PDOs present in each well 2 weeks after

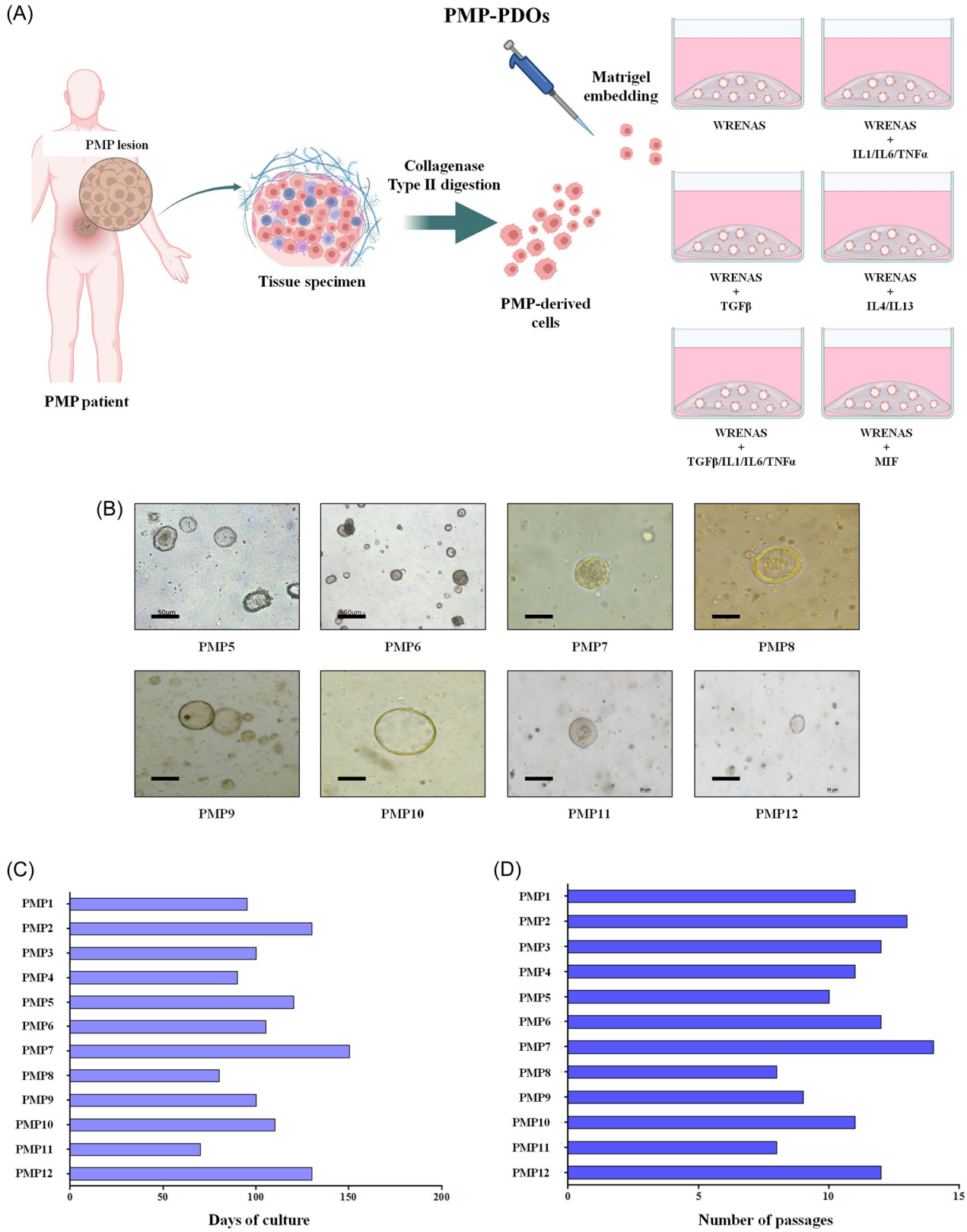

FIGURE 1 (See caption on next page).

seeding. PDOs were grown using a medium depleted of stem- and microenvironment-related factors. PDOs were counted 2 weeks after culture and the impact of these factors on PDO growth was determined by the number of PDOs present in each well. The long-term expansion ability of the PDOs was tested by seeding them as described above. PDOs were cultured using a medium that was either complete or depleted of stem- and microenvironment-related factors. For each medium, the total number of PDO expansions was determined. PDOs counting were performed using Qpath software (https://qupath.github.io, version 0.2.3). Images used for Qpath analyses were acquired using Aperio Leica ScanScope XT (Leica Biosystems). Three different fields were counted for each experiment. Each experiment was performed in triplicate.

2.6 | Statistical analyses

Statistical analyses were performed using GraphPad Prism software (version 9.4.1 [676]; GraphPad Software). Data are expressed as mean and SD. A two-tailed Student's *t*-test was used to compare paired groups. Differences among more than two groups were evaluated using two-way ANOVA. A $p < 0.05$ was considered statistically significant. The data generated and analyzed during the current study are available from the corresponding author upon reasonable request.

3 | RESULTS

3.1 | Development of PMP-derived PDO

Twelve PMP-PDO cultures (PMP1, PMP2, PMP3, PMP4, PMP5, PMP6, PMP7, PMP8, PMP9, PMP10, PMP11, and PMP12) were developed following the proposed protocol that mimics the features of the PMP microenvironment (Figure 1A), as detailed in Section 2. The main characteristics of the patients from which the PDO model were derived are summarized in Table 1. We processed a total of 30 tumor tissues from 30 different patients, with a success rate of 40%. Initially, the success rate was lower, as the tissue samples used for PDOs development had low cellularity (<5%). To increase the success rate in obtaining PMP-PDOs, each patient-derived sample was examined by a pathologist, so that only tumor areas with a cellularity of more than 10%−15% were selected. The PDO models we developed presented different morphologies, characterized by the development of cystic structures and/or tumoroid aggregates surrounded by mucin, within an average size of about 150–200 μm (Figure 1B and Supporting Information S1: Figure S1). The culture time and number of passages were recorded for each PDO model (Figure 1C,D): PDOs grew on average for 104 days, and cultures were expanded for an average of 11 passages (Figure 1D). All PMP-PDOs were successfully cryopreserved, and five of them (PMP1, PMP2, PMP3, PMP6, and PMP7) were successfully tested for their capacity to restart growth after more than four passages. PDO models secreted a variable amount of mucin, which decreased after several passages. This reduction of mucin production was correlated with a decrease in PDOs proliferation rate as previously described.[10]

3.2 | PMP-PDOs grew in MEDIA enriched in factors that recapitulate PMP microenvironment

Based on protocols for establishing PDOs from intestinal crypts, we designed six different combined culture conditions that included factors essential for the growth of PMP (see Section 2). The medium that provided the best growth conditions was selected based on the number and size of PDOs present in each well approximately 2 weeks after seeding. We found that six (PMP1, PMP2, PMP3, PMP5, PMP7, and PMP12) of the 12 PDO models depended on media enriched in proinflammatory factors, such as IL1, IL6, TNF-α, and MIF (Figure 2) two (PMP4 and PMP8) grew in the presence of media supplemented with pleiotropic factors (Figure 2), while only PMP10 grew in WRENAS medium without any supplement (Figure 2). Moreover, PDOs grown with the best medium condition also displayed an average size of 200 μM, while the size decreased to 80–100 μM when PDOs were cultured in the other media. These results are in line with several experimental evidence showing that PMP disease is characterized by the development of a proinflammatory microenvironment which favors its development (e.g., IL1, IL6, and TNFa).[17] In addition, stimulation through pleiotropic factors (e.g., IL4 and IL13) of the mucin promoter appears to be important for the growth of PMP neoplastic cells.[17,18]

3.3 | PMP-PDOs reproduce the characteristics of PMP disease

Mutational and immunohistochemical analyses of the obtained PMP-PDOs and their respective tumors of origin showed that they

FIGURE 1 Development of PMP-PDO models: (A) Workflow of the protocol used for developing PMP-PDOs. (B) Record the number of culture days for each PMP-PDO culture. PMP1, 2, 3, 5, 7, and 12 were cultured in medium #2. PMP4 and 8 were cultured in medium #3. PMP9 and 11 were cultured in medium #5. PMP6 was cultured in medium #6 (C), expansion potential of PMP-PDOs. PMP1, 2, 3, 5, 7, and 12 were cultured in medium #2. PMP4 and 8 were cultured in medium #3. PMP9 and 11 were cultured in medium #5. PMP6 was cultured in medium #6 (D) representative bright field images of PMP-PDOs from eight patients. Scale bar: 100 μm. Images were acquired 2 weeks after PDO seeding. IL1, interleukin-1; IL3, interleukin-3; IL4, interleukin-4; IL13, interleukin-13; IL6, interleukin-6; MIF, macrophage migration inhibitory factor; PDO, patient-derived organoid; PMP, pseudomyxoma peritonei; TGFβ, trasfroming growth factor-beta; TNF-α, tumor necorsis factor-alpha; WRENAS, **W**nt3a **R**-spondin-1 **E**GF **N**oggin **A**83-01 (ALK-inhibitor) **S**B20193 (Rock-inhibitor).

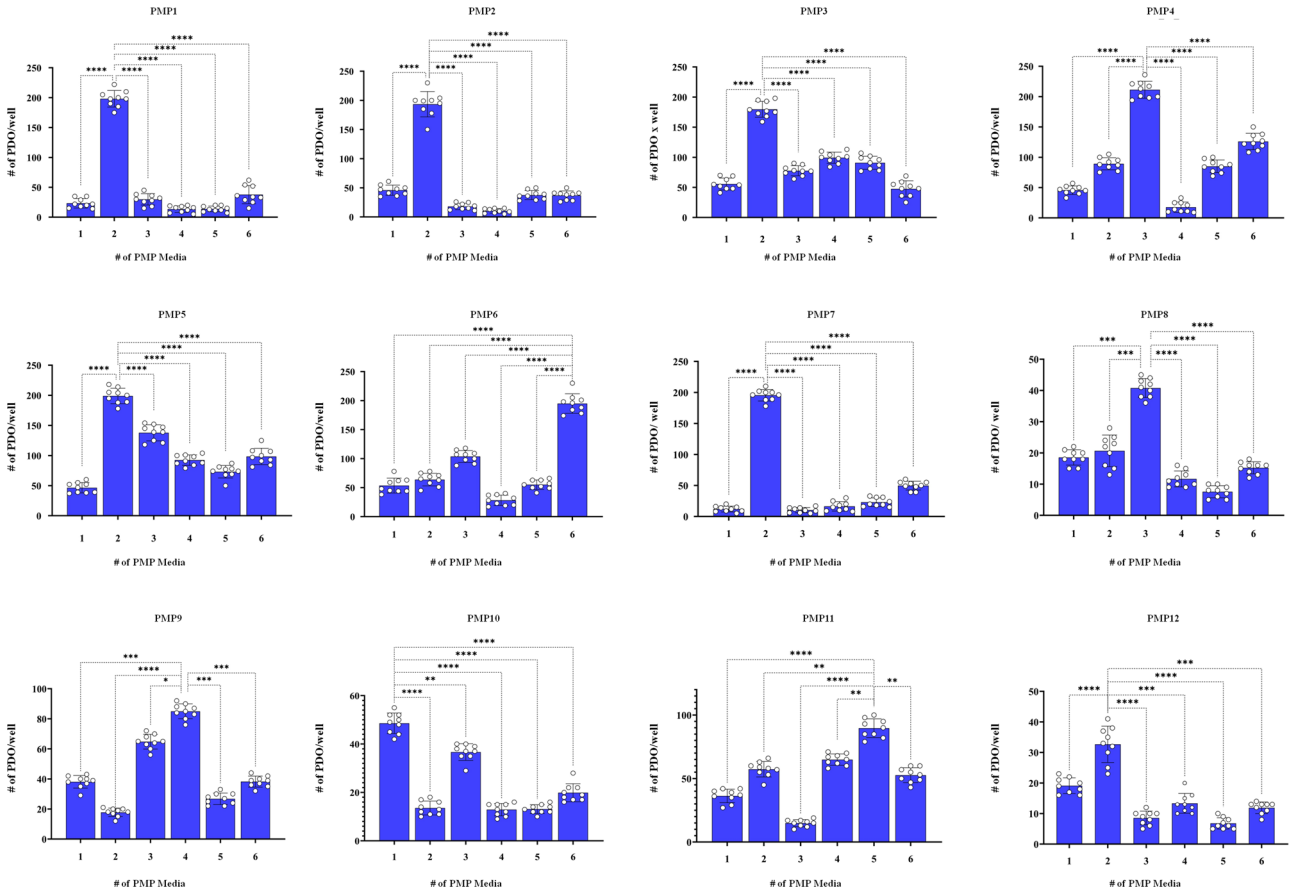

FIGURE 2 PMP-PDOs grow in MEDIA enriched in factors that recapitulate the PMP microenvironment. Number of PMP-PDOs per well cultured in six different media (#1, #2, #3, #4, #5, and #6) resembling the main features of the PMP microenvironment. The composition of the media is specified in Section 2. ****$p < 0.001$, Student's *t*-test. Data represent mean ± SD of $n = 3$ biological replicates. IL1, interleukin-1; IL3, interleukin-3; IL4, interleukin-4; IL13, interleukin-13; IL6, interleukin-6; MIF, macrophage migration inhibitory factor; PDO, patient-derived organoid; PMP, pseudomyxoma peritonei; TGFβ, trasfroming growth factor-beta; TNF-α, tumor necorsis factor-alpha; #1, medium #1, WRENAS only; #2 medium #2, WRENAS plus IL1/IL6/TNF-α; #3, medium #3, WRENAS plus IL4/IL13; #4, medium #4, WRENAS plus TGFβ; #5, medium #5, WRENAS plus TGFβ/IL1/IL6/TNF-α; #6 medium #6, WRENAS plus MIF.

recapitulate the characteristics of the human tumor tissue counterpart (Figure 3). Among the most common mutated genes in PMP, we observed *GNAS* and *KRAS* with a mutation frequency of 33.33% and 50.00%, respectively. In four PDOs, we found mutations in the *GNAS* gene at codons 608 and 201 (A608V, R201G, and R201C), with R201C being the most represented. Six PDOs carried mutations in the *KRAS* gene, at codons 12 and 13: Three PDOs were G12V, while two were G12D and one G13D. One PDO model showed a mutation in *ATM* gene. We also found mutations in *TP53*, *FLT3*, *FBXW7*, and *PIK3CA* genes (Figure 3A and Supporting Information S1: Table S4). The same mutations were found in the corresponding tumor tissue.

The expression of the typical protein markers of PMP disease was assessed on seven PMP-PDO models (PMP1; PMP2; PMP3; PMP4; PMP5; PMP6; PMP7). IHC analysis showed that they expressed the PMP-specific markers CK7 (when also expressed by the patient's tissue), CK20, and CDX2 in a similar percentage of cells as the tissue from which they originated (Figure 3B, Supporting Information S1: Figure S2). All PMP-PDOs and corresponding tissue samples were positive for MUC2, the primary molecular marker of PMP[19] (Figure 3B, Supporting Information S1: Figure S2). The percentage of MUC2-positive cells in PMP-PDOs was similar to that of the tissue of origin (Figure 3B and Supporting Information S1: Figure S2). The PMP-PDOs were also Ki-67-positive, suggesting that they were actively proliferating (Figure 3B, Supporting Information S1: Figure S2).

3.4 | Influence of PMP microenvironment-related factors on PMP-PDO growth

The protocol for the development of PMP-PDOs that we propose aims to recreate a proinflammatory microenvironment, which has been clearly shown to be involved in PMP development.[17] The presence of a massive accumulation of mucin suggests the key role of this protein in the process of PMP dissemination in the peritoneal cavity.[18] In addition, the involvement of several pleiotropic factors in

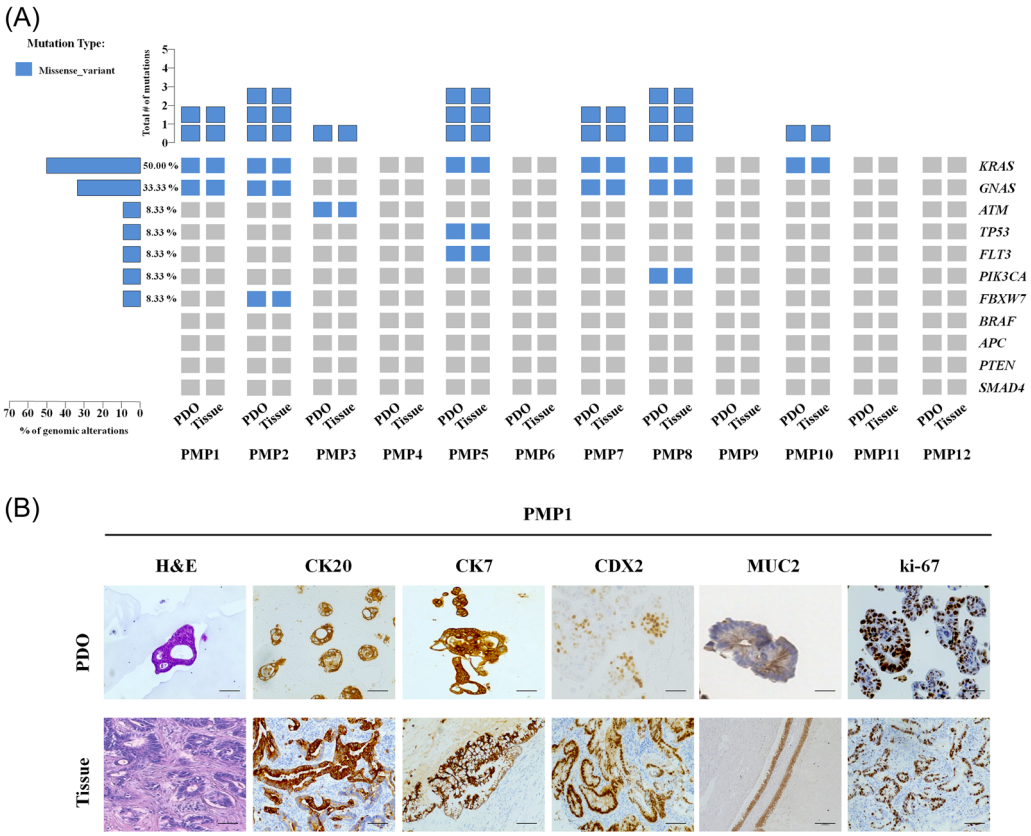

FIGURE 3 PMP-PDOs reproduce the mutational and phenotypical characteristics of PMP disease. (A) Mutational analysis showing the concordance between PDOs and their corresponding tissues. On the left, the percentage of genomic alterations detected across the samples analyzed (bottom) and the total number of mutations/sample. Passage numbers: PMP1: P5, PMP2: P6, PMP3: P3, PMP4: P4, PMP5: P5, PMP6: P3; PMP7: P5; PMP8: P3; PMP9: P4; PMP10: P5; PMP11: P4; PMP12: P5). (B) Comparative histochemistry (HC) and IHC analysis of PMP-derived PDO and their tissue of origin using the PMP-derived protein markers CK20, CK7, CDX2, MUC2, and Ki67. Total magnification: ×100. Scale bar, 100 µm. CDX2, caudal type homeobox 2 protein; CK7, cytokeratin 7; CK20, cytokeratin 20; IHC, immunohistochemistry; Ki-67, marker of proliferation Kiel 67; MUC2, mucin type 2; PDO, patient-derived organoid; PMP, pseudomyxoma peritonei.

disease progression has been widely debated.[1,18] To better understand whether the components of the culture medium are crucial for the formation and growth of PMP-PDOs, we cultured them in the absence of these factors and assessed their growth. The five PDO models (PMP1, PMP2, PMP3, PMP5, and PMP7) grown on media supplemented with proinflammatory factors (IL1, IL6, and TNF-α) grew better in the presence of all three factors (from approximately 150 to 200 PDO/well) (Figure 4A–E). Removal of one of the microenvironment factors from the growth medium caused a 35% reduction in PDOs per well, while removal of two factors resulted in a decrease of approximately 60% (Figure 4A–E). Note that the simultaneous removal of all three factors caused a drastic reduction in PDO growth capacity by 80% (Figure 4A–E). For the PMP4 model, grown in media enriched with pleiotropic cytokines (IL4 and IL13), the ability to form PDOs was reduced by approximately 50% in the absence of either cytokine (Figure 4F). Removal of both cytokines from the culture medium resulted in a reduction of the ability to develop PDOs by approximately 75%, suggesting a synergistic effect between IL4 and IL13 (Figure 4F). Finally, the PMP6 model, grown in WRENAS medium supplemented with MIF cytokine, showed a reduction in the number of PDOs per well of about 70% when cultured in WREANS medium without MIF (Figure 4G).

3.5 | Influence of stem cell-related factors on growth of PMP-PDOs

PMP disease often shows alterations in stem cell-related Wnt and Notch pathways, leading to an aberrant Goblet-like secretory phenotype characterized by massive mucin production in the peritoneal cavity.[19,20] This evidence suggests the prominent role of stem cell-related niche factors in the development of PMP-PDOs. Therefore, we determined the requirement of stem cell niche factors (Wnt3A, R-spondin1-, RSPO1-, and Noggin) in the PMP culture medium (common to all seven PMP-derived PDO models tested), for the early developmental process of PMP-PDOs. The combined presence of all three factors related to the stem component is the most efficient condition for the growth of all seven PDO models, with a median number of organoids per well ranging from 150 (PMP6) to 210 (PMP4) (Figure 5). Removal of Wnt3A alone led to a drastic decrease

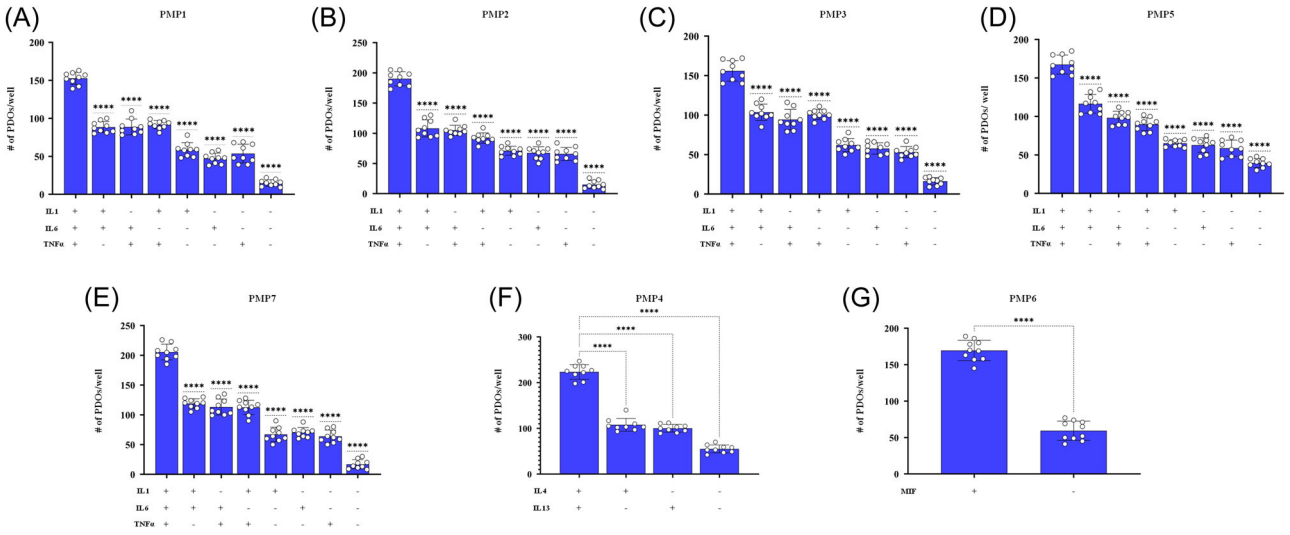

FIGURE 4 Influence of PMP microenvironment-related factors on PMP-PDOs growth: (A–E) PMP-PDO models cultured in the presence or absence of IL1, IL6, and TNF-α; (F) PMP-PDO models cultured in the presence or absence of IL4 and IL13; (G) PMP-PDO models cultured in the presence or absence of MIF. ****$p < 0.001$, Student's t-test. Data represent mean ± SD of n = 3 biological replicates. IL1, interleukin-1; IL3, interleukin-3; IL4, interleukin-4; IL13, interleukin-13; IL6, interleukin-6; MIF, macrophage migration inhibitory factor; PDO, patient-derived organoid; PMP, pseudomyxoma peritonei; TNF-α, tumor necorsis factor-alpha.

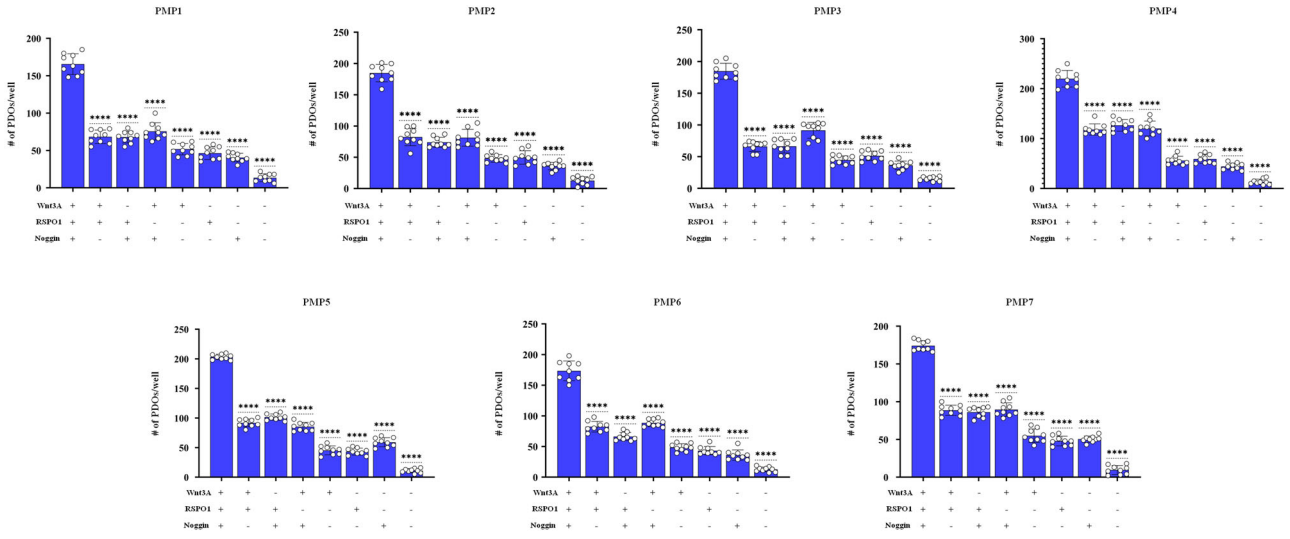

FIGURE 5 Influence of stem cell-related factors on PMP-PDOs growth. PMP-PDO models cultured in the presence or absence of Wnt3a, RSPO1, and Noggin factors. ****$p < 0.001$, Student's *t*-test. Data represent mean ± SD of $n = 3$ biological replicates. Noggin, protein noggin; PDO, patient-derived organoid; PMP, pseudomyxoma peritonei; RSPO1, protein R-spondin-1; Wnt3a, protein Wnt3A.

in PDOs development, with a reduction in the number of PDOs per well of up to 50% in all models (Figure 5). A similar trend was observed when PMP-PDOs were grown in an RSPO1- or Noggin-deprived medium (Figure 5). Furthermore, the absence of two of the three stem cell-related niche factors reduced the development of PDOs in all models tested by approximately 70% (Figure 5). Finally, the simultaneous removal of Wnt3a, RSPO1, and Noggin dramatically decreased PDO formation; resulting in less than 20 PDOs/well in all the seven models analyzed (Figure 5). These results indicated the high dependence of PMP-PDOs on stem-related factors.

3.6 | Stem-related factors are required for long-term expansion of PMP-PDOs

Finally, we evaluated the impact of factors related to the micro-environment or tostemness on the long-term expansion of PMP-PDOs growing the seven PDO models in the presence and/or absence of PMP niche factors. We observed that PDOs growing in WRENAS medium supplemented with IL1, IL6 and TNFα (PMP1, PMP2, PMP3, PMP5and PMP7), showed a reduction of about 40% in the number of passages when they were grown in the absence of IL1,

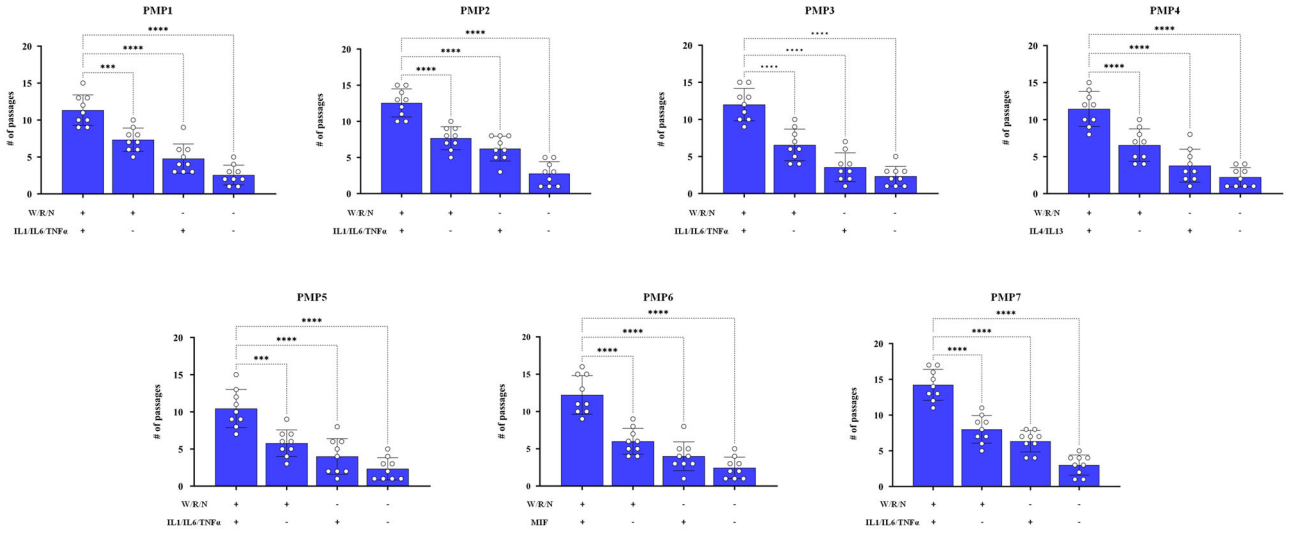

FIGURE 6 Stem-related factors are required for the long-term expansion of PMP-PDOs. PMP-PDO models in culture media enriched or depleted in stem cell-related factors (W, R, and N) and/or in PMP microenvironment-related factors (IL1, IL6, TNF-α, IL4, IL13, and MIF) as indicated. ****$p < 0.001$, Student's *t*-test. Data represent mean ± SD of $n = 3$ biological replicates. IL1, interleukin-1; IL4, interleukin-4; IL6, interleukin-6; IL13, interleukin-13; MIF, macrophage migration inhibitory factor; N; protein Noggin; PDO, patient-derived organoid; PMP, pseudomyxoma peritonei; R; protein R-spondin-1; TNF-α, tumor necorsis factor-alpha; W, protein Wnt3a.

IL6 and TNFa (Figure 6). On the other hand, absence of Wnt3A, RSPO1 and Noggin reduced their expansion of about 65–70% (Figure 6).

Similar results were also obtained with PDOs grown in WRENAS medium supplemented with either IL4 and IL13 or MIF (PMP4 and PMP6 respectively). Infact, PDOs grown in the absence of the Wnt3A, RSPO1 and Noggin factors were expanded only for a small number of passages, equal to about 2 (Figure 6). In contrast, in the absence of the microenvironment-related factors (IL4, IL13 andMIF), PDOs could grow for approximately 4 and 6 passages, respectively (Figure 6). These results suggest that the presence of stemness-related factors appears to be determinant for the long-term expansion of PMP-PDOs.

4 | DISCUSSION

PMP is still a poorly understood disease, with many fundamental questions still unresolved. In addition, the lack of appropriate research models contributes to making PMP a little studied disease.[1] We developed for the first time a novel 3 dimension (3D) culture system for the generation of PMP-PDOs, implementing previously established protocols for obtaining CRC-derived PDOs.[11–14] Different factors regulating the molecular events of PMP were added to the medium enriched in colonic-niche factors in different combinations.

PMP is characterized by a proinflammatory microenvironment that can induce overexpression of mucin (especially MUC2), thus giving neoplastic cells the ability to spread into the peritoneal cavity and resist to standard chemotherapy. As reported in the literature, inflammatory (IL-6, TNF, IL-1b) and pleiotropic (IL-4, IL-9) cytokines are known to regulate MUC2[17]; therefore, our proposed culture system included proinflammatory/pleiotropic cytokines. In agreement with this evidence, we observed the best growth conditions in proinflammatory-like media, with nine out of 12 PDO models requiring the supplementation of proinflammatory cytokines into the WRENAS medium. Furthermore, six of the seven PMP-derived PDO models harboring mutations in the in *GNAS*, *KRAS* and/or *ATM* genes grew in media enriched in proinflammatory or pleiotropic cytokines, in line with previous evidence highlighting the prominent role of *GNAS* and *KRAS* genes in promoting inflammation, thereby favoring tumor growth and tumor spreading capacity.[20,21] Two of the five PDOs that did not harbor classical mutations of PMP (*GNAS* and *KRAS*), grew in media without proinflammatory factors supplementation. Inflammation can also trigger alterations in signaling pathways that regulate cell-lineage development, such as Wnt/Notch pathways, and lead to an aberrant goblet-like secretory phenotype, suggesting that Wnt alterations may play a role in PMP.[22] Therefore, we promoted the cell-lineage pathways with the addition of the niche factors Wnt3A and R-spondin1, together with the BMP4 inhibitor Noggin, to enhance the stem pathway response.[23] We observed that supplementation of the medium with stem cell-related factors, such as Wnt3A, R-spondin1, and Noggin, was critical in sustaining the long-term expansion capacity of our PDO models. These data are in line with previously reported evidence indicating that the Wnt/β-catenin pathway is rarely genetically altered in PMP disease.[24]

Many components of the PKA and TGF-β pathways are also mutated in PMP,[24] suggesting us to test the influence of TGF-β and PKA activators on the development of PMP-PDO models. However, we have not obtained PDO models showing TGF-β dependence,

probably because of the small number of tissue samples processed. Hypothesizing that a proinflammatory microenvironment plays an important role in the development of PMP, and based on our previous studies,[16,25] we tested the effects of a pleiotropic cytokine, MIF, on culture media. MIF has proinflammatory functions and oxidoreductase activities that play a key role in cancer development and microenvironment formation in vivo. MIF can downregulate tumor suppressor genes, upregulate COX-2 and PEG2, induce the production of proinflammatory cytokines (such as IL-1, IL-6, and TNF), regulate hypoxia conditions, induce angiogenesis and enhance tumor growth,[26] thus recapitulating all the physiological characteristics of PMP. However, only one PDO model (PMP6) grew in the presence of MIF.

Although we succeeded in developing PMP-PDOs from 12 patients, their generation still remains challenging. The success rate was about 40%, in line with the previous work from the Quintilla-Martinez group,[10] but far from the 70%–80% we achieved for the development of PDOs from peritoneal metastases from CRC.[13,14,16] One of the main problems with PMP tissue is the presence of few epithelial cells, surrounded by an abundant layer of mucin, which makes their isolation difficult. To overcome this problem, surgical specimens were subjected to an extemporaneous pathological examination (EPE) by an experienced pathologist who selected regions of tissue enriched with neoplastic epithelial cells. We received tissue for PDO development from 30 patients. Seven PDOs were obtained from tissue with ≥20% tumor cellularity, five were between 6% and 10% and two were around 5%. The tumor cellularity of the 18 cases from which we did not obtain organoids was between 1% and 10%. Further implementations of the protocol to increase the success rate of PMP-PDOs development could include selective isolation of epithelial cells, using culture systems based on collagen-coated plates to expand them and increase the epithelial cell pool, which will then be cultured following the protocol for PDO development. Also, PMP grows in the peritoneal cavity in the absence of oxygen, a condition that could promote neoplastic progression. In addition, the excessive mucin accumulation and tumor growth rate in PMP could be regulated by hypoxia-inducible factor-1α (HIF-1α).[26,27] The presence of a low-oxygenation environment could contribute to an increased rate of PDO models. To mimic the physiological growth conditions of PMP, the impact of a low-oxygenation environment on the fitness of PDOs should be considered. Moreover, our model lacks the surrounding microenvironment. In fact, PDOs are representative of the pure tumor epithelial component but fail to recapitulate the entire heterogeneity of the pathology. In recent years, the role of the microenvironment in tumor development and progression has been widely demonstrated.[28] In particular, it would seem to play a key role in the response to therapeutic treatment, by making the neoplastic cells chemoresistant.[29,30] Therefore, the addition of different components of the PMP microenvironment, such as cancer-associated fibroblasts, extracellular matrix elements, and/or immune cells, could be a viable strategy to increase the success rate in obtaining PMP-derived PDOs. For all these reasons, future perspectives will be focused on the development of new ex vivo models where PDOs are grown on natural decellularized scaffolds,[13] including different cell components of the tumor microenvironment, to increase the complexity of the model, to mimic PMP disease.

5 | CONCLUSION

In conclusion, PMP is a disease with unique and often unpredictable behaviors, the biology of which is largely unexplored. The development of PMP-PDOs could enable in vitro testing of antiblastic and targeted agents and will be instrumental in designing future Phase I and II trials of systemic and/or biological therapies. Furthermore, 3D models of PMP could support innovative individualized treatment strategies based on in vitro drug sensitivity screenings.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data generated and analyzed during the current study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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