Establishing Pure Cancer Organoid Cultures: Identification, Selection and Verification of Cancer Phenotypes and Genotypes

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Abstract

Precision medicine requires *in vitro* models which will both faithfully recapitulate the features of an individual's disease and enable drug testing on a wide variety of samples covering the greatest range of phenotypes possible for a particular disease. Organoid technology has immense potential to fulfill this demand, but it will be necessary to develop robust protocols that enable the generation of organoids in a dependable manner from nearly every patient. Here we provide a user's guide, including detailed step-by-step protocols, to the establishment, isolation and verification of gastric cancer organoids. Selection strategies include omission of growth factors, addition of drugs, isolation of distinct phenotypes and generation of monoclonal lines. For confirmation of cancer identity, we use sequencing, drug selection, karyotyping and histology. While we specify these protocols for human gastric cancer organoids here, the methods described are applicable to organoids derived from other tissues as well.

Introduction

Cancer is a global health burden, with an estimated 14 million new cases every year [1]. Novel and improved drugs that are less toxic and that specifically target cancer cells are needed in the clinic. However, drug development is particularly challenging in oncology: only 1 out of 15 anti-cancer drugs reaches the market from phase I trials, compared to 1 out of 8 drugs for other disease areas [2]. One of the reasons for the inadequate rate of translation of drugs from bench-to-bedside is the lack of suitable pre-clinical model systems. Classical tumor models include cancer cell lines and patient-derived xenografts, which are both vital to drug discovery efforts but also bear inherent limitations (reviewed in Refs. [3,4]).

In the past decade, the development of organoids has given basic and translational researchers an unprecedented new model that has already had clinical impact (reviewed in Ref. [5]). This advance originated from the identification of adult somatic tissue-resident stem cells, which can both self-renew and differentiate into the cell types of the organ of origin not only *in vivo* [6] but also *in vitro* [7]. Once the adult stem cells have been seeded into an extracellular matrix, equipped with a specific cocktail of growth factors and inhibitors, they form 3D, self-organized structures – organoids – which can be propagated *in vitro* indefinitely. Organoids closely resemble the primary tissue they are derived from and thus allow the *in vitro* study of a range of processes which recapitulate *in vivo* biology (reviewed in Refs. [8–10]).

Table 1. Human gastric organoid (hgo) medium

Factor	Stock solution	Final concentration
Ad++		30%
B27	50×	1×
Primocin	50 mg/ml	100 µg/ml
WNT3A-CM		50%
RSPO1-CM		10%
NOG-CM		10%
N-acetyl-L-cysteine	500 mM	1.25 mM
EGF	500 µg/ml	50 ng/ml
FGF10	100 µg/ml	100 ng/ml
Gastrin I	100 µM	1 nM
TGF-ßi	25 mM	2 µM
ROCKi	10 mM	10 µM

In a similar manner to organoids from normal tissue, tumor organoids have been established from stomach [11], colon [12], prostate [13], pancreas [14], liver [15] and breast [16] cancers. The development of tumor organoid cultures has quickly led to the generation of "living" biobanks of tumor organoids, often also containing organoids from matched normal tissue [17–24]. The efficiency of organoid generation varies between approaches, but can—in an ideal case such as for colon cancer—reach 100% [21]. Even those tissues with lower success rates, for example, 50%–74% for gastric cancer [17,18], far exceed the 1%–10% efficiency rate for classical cancer cell lines [3]. This improved efficiency of generation, together with the predictive value of organoid assays in terms of patient outcome, suggests two ways by which tumor organoids may serve clinical need: (i) organoid biobanks can be utilized for preclinical screening of new drugs [21] and (ii) individual patient-derived organoids can predict the response of a particular patient to a drug, thus enabling personalized medicine [3,17,25]. Both applications have already been highly useful in identifying drug-responders in cystic fibrosis [26,27]. A high efficiency of generation is important in a clinical setting because (i) biobanks are more effective if they encompass all subtypes of a particular disease and (ii) patient-tailored treatment should ideally be accessible to all patients. This is already the case in cystic fibrosis, where intestinal organoids can be generated from rectal biopsies [26], but protocols for cancer organoid establishment could be improved.

During optimization of the growth conditions for various cancer organoids, it has become apparent that tumor organoids can actually be overgrown by organoids derived from contaminating normal epithelium. This may at first seem counterintuitive, but it would appear that many tissue samples, even those from within a macroscopically visible tumor, still contain some healthy cells that can thus give rise to normal organoids [4,13,15,17,18,21,22].

Therefore, it is essential to develop robust and reproducible strategies to isolate populations of cancer organoids, ideally while conserving the heterogeneity of the parental tumor. Using gastric cancer (GC) organoids as an example that can be adapted for other cultures, we provide a detailed guide to obtaining pure cancer organoid cultures and validating their identity.

Results

Generation of cancer organoids

Detailed step-by-step instructions for all procedures described here can be found in the supplementary information. The basic method for the generation of gastric cancer organoids was reported in 2015 [11]. Briefly, tumor tissue pieces of about 1 cm^2 are mechanically disrupted and enzymatically digested. Seeded in extracellular matrix and supplied with the right mixture of growth factors (Table 1), organoids will form (Fig. 1). The growth rate of tumor organoids varies tremendously between patient-derived lines. Whereas normal human gastric organoids follow a strict passaging regime of 1:6 every 14 days, we have experienced passaging rates from 1:10 every 14 days to 1:2 every 2 months for tumor organoids. It was indicated in many reports on tumor organoids that, in contrast to normal organoids, they exhibit a range of patient-specific morphologies and different cellular architectures, which recapitulate the histological features of the patient's tissue and tumor subtype. Descriptions of the organoids include the following:

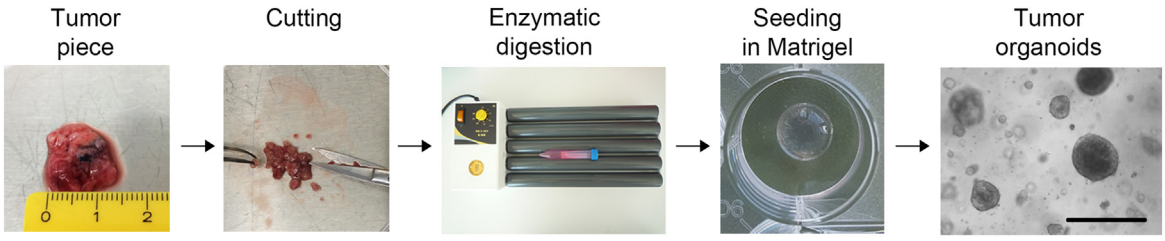

Fig. 1. Schematic summarizing the individual steps for establishment of human gastric tumor organoids from tumor tissue. The scale bar represents 400 µm.

irregular in shape, solid organoids of diverse sizes, cystic organoids with ruffled layers of cells, "grape-like" organoids, and organoids that were essentially entirely discoherent [13,15–22,24]. Consequently, tumor organoids can be grouped according to their cancer subtype as Epstein–Barr virus (EBV)-positive, intestinal-type, diffuse-type and mixed (Fig. 2). Frequently, organoids with the typical normal phenotype (spheres with clear lumens) will be initiated from the seeding material. If these normal organoids are not eliminated from the culture, they will overgrow the tumor organoids.

Selection of subpopulations of organoids by selective pressure, phenotype or clonal expansion

An elegant approach to selecting tumor-derived cultures is based on prior knowledge of mutational patterns dictating growth factor dependency of the organoid culture. For example, the vast majority of colorectal cancers (CRCs) have developed activating mutations in the Wnt signaling pathway. Therefore, the dependency of normal colonic stem cells on WNT3A can be exploited to selectively expand tumor organoids. The omission of WNT3A from the culture medium creates a selective pressure that will only allow tumor organoids to grow. In gastric cancers, however, there is no such universal set of mutations driving activity in a single pathway. A high percentage of gastric cancers exhibit mutations in the p53 pathway and can therefore be selected with the application of the small molecule Nutlin-3, which stabilizes TP53 by disrupting the binding of TP53 to its negative regulator E3 ubiquitin ligase, MDM2 [11,21]. Therefore, tumor organoids that harbor alterations in the p53 pathway can withstand selective pressure from the application of Nutlin-3, while normal organoids die (Fig. 3a). Similarly, growth of organoids from tumors harboring mutations in the EGF receptor (EGFR) signaling pathway can be selected by EGF withdrawal or EGFR inhibition [12,21]. Applying particular combinations of withdrawal of niche factors can increase the yield of different cancer subtypes [18].

Another approach for removing normal organoids from cultures is phenotype-based manual selection. If a culture displays a clear mix of populations, the cancer organoids can be manually selected, either in bulk, to preserve heterogeneity, or as single organoids. Several rounds of manual selection might be required before a pure population is obtained (Fig. 3b).

A third possibility is to use flow cytometry-based cell sorting to collect single cells from which clonal cancer organoids can be established (Fig. 3c) [28]. Since organoid growth from single cells is fairly slow and inefficient, a sufficient number of single cells are required as a reasonable starting point. We agree with the recommendations of Jager and colleagues [29] that 10,000 cells are a good starting point. If this approach is used to generate monoclonal lines of organoids, note that stringency is paramount, and it

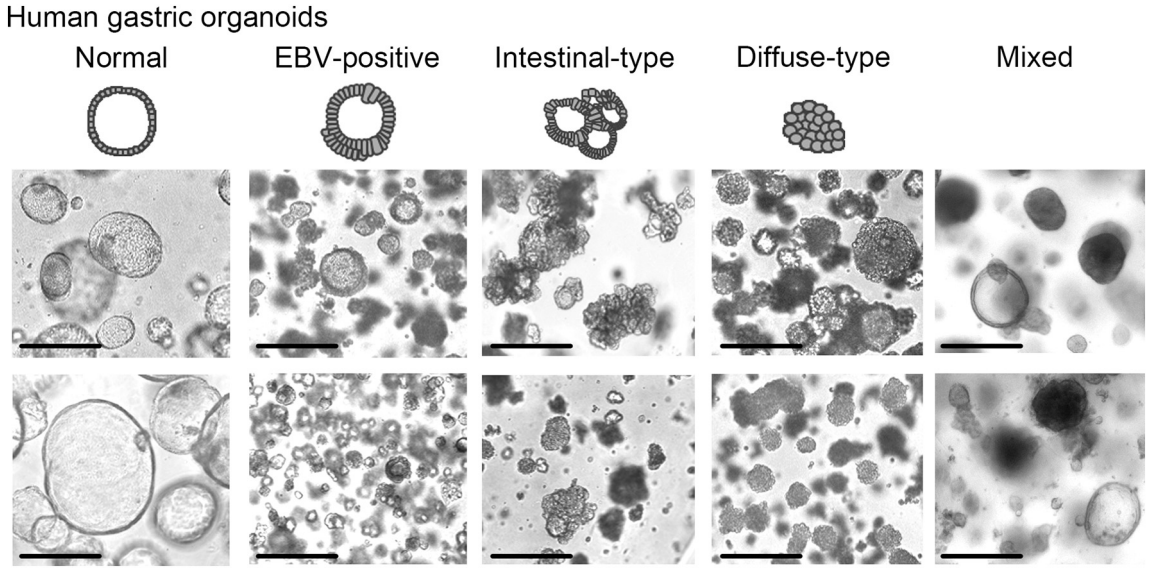

Fig. 2. Representative brightfield images of normal gastric and tumor organoids. Brightfield images for each subgroup are from two independent patients. Normal gastric organoids are cystic and spherical in shape and composed of a single layer of epithelial cells with a large lumen. Tumor organoids carrying the EBV genome are generally cystic, but ruffled with multiple layers of cells. Typical intestinal-type gastric tumor organoids are irregular in shape, with the occasional formation of a small lumen. Diffuse-type gastric tumor organoids form solid cell clusters, some with loose aggregation giving a "grape-like" appearance. Mixed populations contain normal organoids growing as spheres with a lumen and irregularly shaped cancer organoids. The scale bar represents 300 μm.

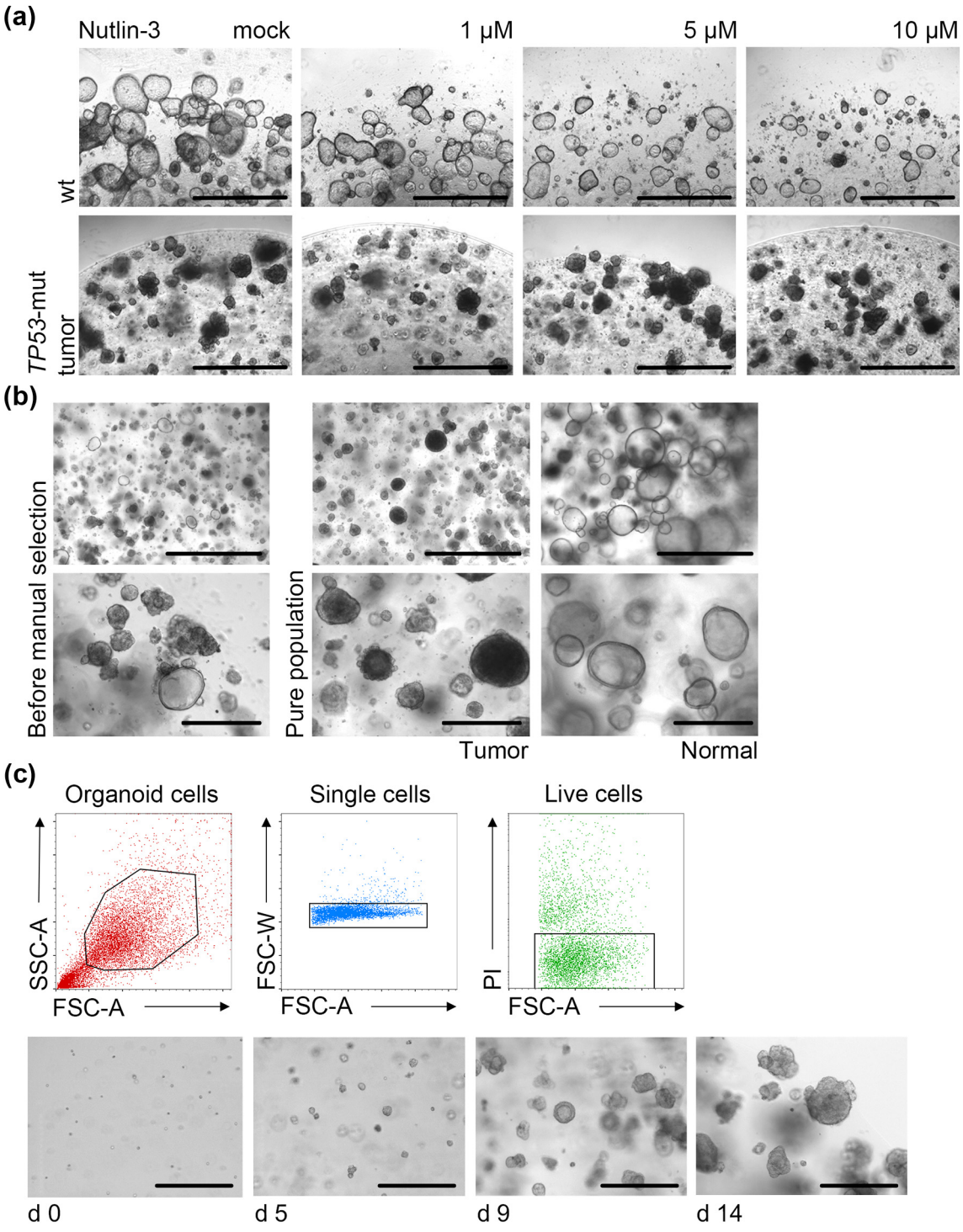

Fig. 3. Selection of subpopulations of organoids. (a) Selective pressure: brightfield images of wild-type and *TP53*-mutant organoids after 7 days of Nutlin-3 selection (increasing concentration). The scale bars represent 1 mm. (b) Manual selection *via* morphological phenotype: brightfield images of organoids before (mixed phenotypes) and after (pure populations: tumor organoids left, normal organoids right) the manual selection procedure. The scale bars represent 1 mm (upper row) and 400 µm (lower row). (c) Single-cell FACS of tumor organoids: gating strategy to exclude doublets and dead cells for single cell FACS (top) and brightfield images of tumor organoids grown from single cells (bottom). The scale bars represent 400 µm.

is preferable to sort 100 true single cells rather than 10,000 cells that may contain doublets.

All three approaches for selecting pure tumor organoids presented here can lead to a loss of cellular heterogeneity as compared to the initial cultures. Therefore, it is recommended to freeze early passages of established cultures as references for initial cell content.

Verification of subpopulations by sequencing, inhibitor testing, metaphase spread or histology

After homogeneous organoid populations have been established, it is necessary to verify tumor identity in the culture. We recommend routine verification of the identity of the organoids every few months to prevent cross-contamination between lines.

Sequencing is probably the most powerful and thorough approach for verification of identity. Whole-genome sequencing of the primary tumor sample and the corresponding tumor-derived organoids has been described extensively elsewhere [29] and is the gold standard. A more cost-effective and less labor-intensive alternative is to perform whole-exome sequencing, or to focus on a panel of well-characterized, cancer-associated genes, a technology used for cancer diagnosis and therefore well-established in many pathology departments. The advantage of whole-genome or whole-exome sequencing is the parallel generation of allele-specific copy number information, which together with data on mutation can provide comprehensive information on the tumor content and extent of normal cell contamination, as well as intra-tumoral heterogeneity [17]. After next-generation sequencing of the primary tumor, the presence of the specific mutations identified can be confirmed in the matching tumor organoids. For this, also, (next generation) panel sequencing is feasible or even conventional Sanger sequencing of single selected genes (Fig. 4a).

Once particular mutations have been determined, selection pressure can be used for additional confirmation of identity. As described above, Nutlin-3 can be used to select for tumor organoids containing mutations in the p53 pathway. The growth of normal organoids is strongly inhibited by treatment with Nutlin-3, while in contrast, tumor organoids lacking functional TP53 are insensitive to Nutlin-3 treatment. Tumor organoids harboring *PIK3CA* mutations will grow despite withdrawal of EGF or addition of a MEK inhibitor [30]. *KRAS* mutations can also be selected for *via* withdrawal of EGF [31]. Other inhibitors or selection methods can be found for a variety of well-defined cancer mutations; for example, *SMAD4* mutations can be selected for by adding TGF-ß [30] or removing NOG [31] from the culture medium (Fig. 4b).

For tumors that display chromosomal instability and aneuploidy [17,32,33], karyotyping is a valuable method for confirmation of identity. To karyotype, cells need to be arrested in metaphase before the chromosomes are spread on glass slides and examined by microscopy (Fig. 4c).

Lastly, cancer organoids retain the histopathological features of the original tumor *in vitro* and following xenotransplantation [11,15–17,19,21,24]. Comparative histology can be carried out after fixation of organoids and original tissue samples, paraffin embedding and sectioning followed by immunohistochemistry (Fig. 4d).

Discussion

Robust and efficient protocols for the generation of organoids are needed for their utility as pre-clinical models. Here we provide a stepwise workflow to obtain pure tumor organoid cultures from human gastric carcinoma and to verify the tumor identity of the resulting cultures. We and others have previously observed the outgrowth of normal organoids from tumor samples, and therefore describe strategies to rid cultures of contaminating normal organoids. We emphasize the feasibility of these approaches and provide a detailed step-by-step protocol in the supplement, aiming to contribute a useful resource, especially for groups using human tumor organoids for the first time.

The outgrowth of normal organoids from tumor tissue may be surprising at first, but this is a widely observed phenomenon [4,13,15,17,18,21,22]. It is unclear why normal organoids can outgrow tumor organoids in culture, but it has been speculated that this may be due to a higher rate of mitotic failure and subsequent cell death in tumor cells [4].

Many laboratories have selected for a particular mutation in order to acquire pure tumor organoid populations. However, there is unfortunately no universally applicable selection method for most cancer types, such as the pancreas, liver and stomach. Therefore, researchers have used the most common mutations in these cancers to select the largest possible number of tumor cells. For example, in the case of pancreatic ductal adenocarcinoma, which displays a high prevalence *of KRAS* mutations, Seino and colleagues [22] removed EGF from the cancer organoid medium to successfully enrich *KRAS* mutant organoids. In GC, 35%–50% of tumors harbor a TP53 mutation [32], allowing selection *via* treatment with Nutlin-3 [17,18]. Combinations of several selective pressures (withdrawal of growth factors or addition of small molecules) can be helpful to increase the efficiency of selection. For example, in liver cancer, the yield of cancer organoids was improved by removing RSPO1, NOG and WNT3A from the medium and adding dexamethasone and ROCKi [15]. It is evident that while these selection strategies are highly valuable,

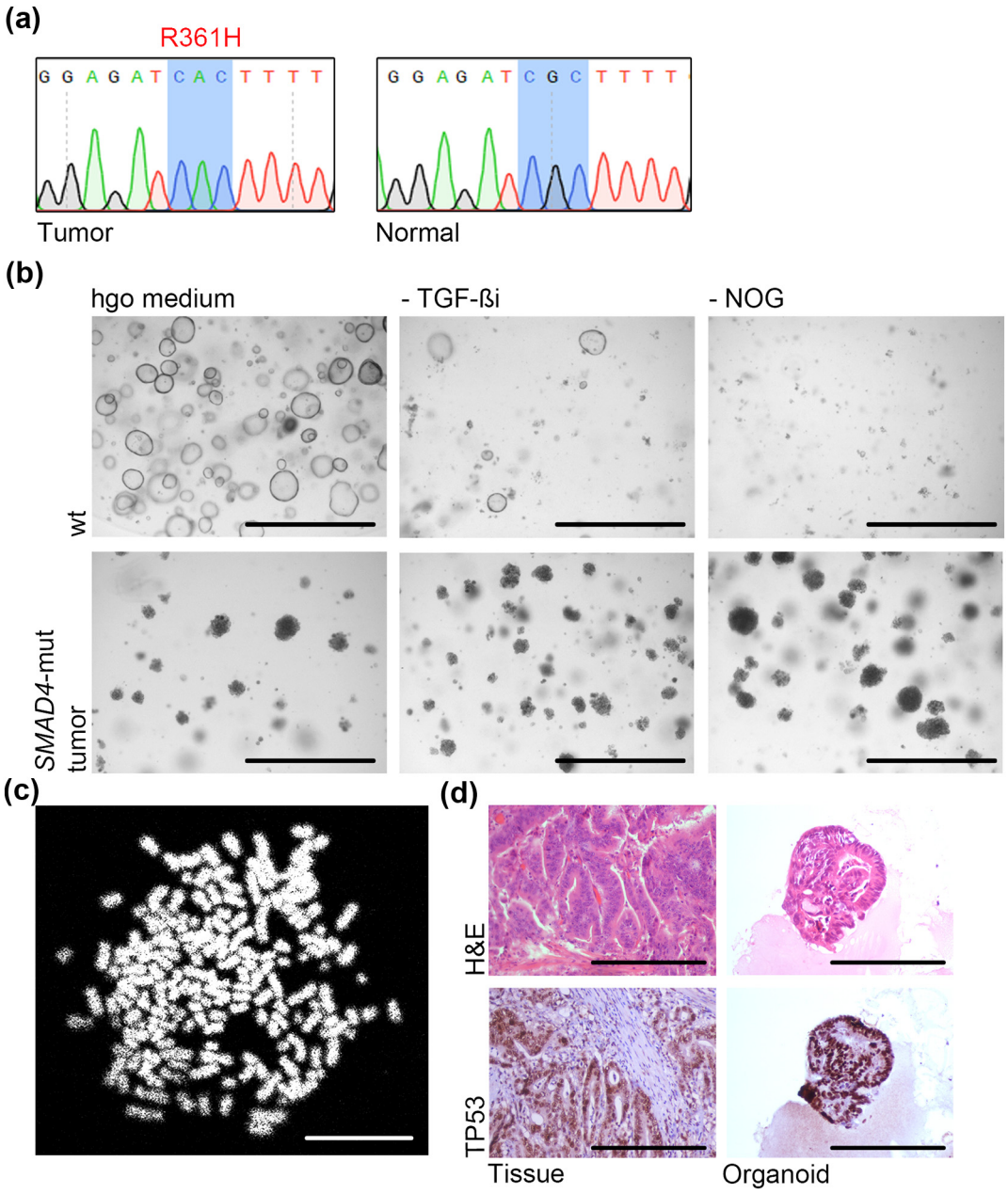

Fig. 4. Verification of tumor identity. (a) Sequencing: example chromatogram of a sequencing result checking for a specific *SMAD4* mutation (R361H) in tumor *versus* normal organoids. (b) Selection: brightfield images of wild-type and *SMAD4*-mutant organoids after selection with TGF-ß (-TGF-ßi) or NOG withdrawal (-NOG) for one passage. The scale bars represent 1 mm. (c) Metaphase spread: karyotype of tumor organoids, depicting aneuploidy. The scale bar represents 20 µm. (d) Histology: images of paraffin sections of the original tumor tissue and organoids stained by H&E and anti-TP53. The scale bars represent 250 µm.

they are not applicable to all cancers and will only serve to select specific subtypes.

The second approach we describe here isolates cancer organoids based on their growth phenotype. While normal organoids grow as a monolayer of epithelial cells surrounding a clear lumen, cancer organoids grow as compact and less organized structures (see phenotype examples in Fig. 2 and Refs. [11,15–17,19,21,24]). This irregularity is likely a result of well-known phenotypic changes in cancer cells: loss of contact inhibition, uncontrolled proliferation, defective differentiation, gradual loss of polarization and loss of overall epithelial integrity [34]. Most prominent is the difference seen in the

morphology of organoids derived from the diffuse subtype of GC; these cancer organoids seem remarkably disorganized and appear as loosely attached clumps of cells, with many single cells. It is likely that this phenotype is caused by the most frequent mutation found in this cancer subtype, the mutation of epithelial cadherin (*CDH1*), a cell–cell adhesion glycoprotein [32,35,36]. It is less obvious as to which genetic changes may result in the morphology of organoids with the EBV-positive and intestinal-type subtypes. Interestingly, sequential mutation of cancer genes in normal human intestinal organoids did not result in any particular, macroscopically visible phenotype: organoids with mutations introduced into *KRAS*, *APC*, *TP53*, *SMAD4* or a combination of these still grow as spheric, well-organized organoids with a clear lumen [30,31]. Therefore, the absence of a morphological phenotype does not necessarily indicate that the cells are normal, but the presence of a morphological phenotype most likely indicates cancerous cells.

It should be noted that the efficiency of organoid generation also depends on the quality of starting material—samples obtained before chemotherapy are more likely to yield organoids than those obtained after chemotherapy—or handling, such as the digestion time of the tumor tissue sample [15,17,19]. Differences in efficiencies between different groups might also be due to these factors, although human gastric organoids have been reported to be established with an efficiency of >50% using phenotype selection [17] and 74.6% using selection by treatment with Nutlin-3 and removal of ROCKi, TGF-ßi, EGF and FGF10 in parallel [18]. The combination of both approaches may further optimize the rate of success of cancer organoid establishment. In addition, we anticipate further general improvements to the organoid culture system through the addition of the immunological and stromal niche of the tumor [37–39], vascularization [40], usage of molecularly defined matrices [41–43] and implementation of organoids in organ-on-a-chip technology [44].

Materials and Methods

Step-by-step instructions for the entire manuscript can be found in the supplement.

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Author Contributions: N.W., C.N., M.P., H.Y. and S.Br. performed the experiments. S.Ba. conceived, designed and supervised the project. A.W. and C.T.G. contributed in the planning of the project and contributed clinical samples. K.M., S.K., S.Br. and A.R. planned, performed and supervised panel sequencing. H.Y. and S.Y.L. refined protocols. N.W. and S.Ba. wrote the manuscript. All authors provided critical comments on the project and revised the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmb.2019.05.031.

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Abbreviations used:
APC, (adenomatous polyposis coli) regulator of Wnt signaling pathway; CRC, colorectal cancer; CDH1, cadherin 1; EBV, Epstein–Barr virus; EGFR, epidermal growth factor receptor; FGF, fibroblast growth factor; GC, gastric cancer; KRAS, Kirsten rat sarcoma virus proto-oncogene; MDM2, mouse double minute 2 homolog; MEK, mitogen-activated protein kinase kinase; NOG, noggin; PIK3CA, phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha isoform; ROCKi, Rho kinase inhibitor; RSPO1, R-Spondin 1; SMAD4, SMAD family member 4; TGF-ß, transforming growth factor beta; TP53, tumor protein 53; Wnt, Wnt family member.

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