

Evaluation of the hydrogels Matrigel, Biogelx, PLTMAx and GelMA in the generation of minilungs from human embryonic stem cells (hESC)

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LETTER (BRIEF REPORT)**Evaluation of the hydrogels Matrigel, Biogelx, PLTMAx and GelMA in the generation of minilungs from human embryonic stem cells (hESC).**

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Abstract

Organoids isolated from adult cells and, especially, those synthesized from human pluripotent stem cells, both induced (hiPSCs) or embryonic stem cells, (hESCs), offer unique opportunities for disease modeling, drug testing or organ replacement (regenerative medicine). The biofabrication of functional tissue require supportive biomaterials and scaffolds generated from reliable materials. Due to the murine origin of the market choice hydrogel (Matrigel), its suitability for regenerative medicine or clinical translation is greatly limited. Thus, the development of new supportive materials for long-term cell survival and differentiation of pluripotent stem cells which are biocompatible with clinical translation and automatized synthesis, or bioprinting, is an urgent need.

In the present study, we have evaluated the suitability of four representative hydrogels, also fitting as bioinks, as supportive biomaterials for cell growth and differentiation of respiratory organoids or minilungs from hESCs. The hydrogels Biogelx, PLTMax and GelMA, were evaluated in the generation of minilungs in parallel to Matrigel. In contrast to those in Matrigel, the minilungs embedded into the hydrogels tested were smaller, less branched or fragmented. The minilungs showed the expression of the three surfactant proteins, despite exhibiting abnormal structures. The aberrant cell growth and cellular arrangement observed suggest that these promising hydrogels or bioinks tested might not be biocompatible for organoid differentiation and for establishing future strategies based on automatized synthesis or bioprinting of lung tissue.

Keywords

lung organoids, minilungs, hESCs, regenerative medicine, hydrogels, bioinks, MatrigelTM, BiogelxTM-INK, PLTMax[®], GelMA

Main text

Organoids derived from human pluripotent stem cells (hPSCs) can be used to model disease and have a great therapeutic potential including drug screening and tissue replacement therapy or regenerative medicine (1). The organoid itself, more or less complex, can be considered as the structural and functional unit for tissue replacement and therapy. The fabrication of functional tissue to replace injured or diseased tissues can be achieved by the bioassembly of the cell aggregates, supportive biomaterials and scaffolds generated from reliable materials (2). Apart from this conventional biofabrication, 3D bioprinting has emerged as an automatic technique that improves reproducibility and control over the architecture of the fabricated tissue constructs (2-4). Bioinks are essential components in any of the strategies carried during bioprinting. Bioinks are cross-linked or stabilized during or immediately after the bioprinting to generate the final shape of the fabricated tissue; their physicochemical properties such as mechanical, rheological, chemical and biological characteristics determine their suitability to function as a supportive material for bioprinting. The ideal bioink must have the following characteristics: i) must provide adequate mechanical strength while retaining the tissue mechanics, ii) adequate gelation and stabilization, iii) biocompatibility and biodegradability to emulate the native tissue homeostasis and iv) suitability for chemical modifications and the potential for large-scale production (2). An essential group of bioinks is based on hydrogels which include biomaterials such as Matrigel, gelatin, alginate, collagen, hyaluronic acid, poly (ethylene glycol), pluronic, fibrins, etc. Matrigel is a reconstituted basement membrane extracted from the mouse sarcoma extracellular matrix and has been widely used in cell and tissue cultures and 3D organoids.

Moreover, Matrigel alone or in combination with other hydrogels has served as a bioink in numerous of bioprinting studies (5-7). Biogelx (BiogelxTM-INK) hydrogels are composed of di- and tri-peptides modified at the N-terminus with aromatic structures (such as fluorenylmethosycarbonyl, Fmoc) (8, 9). These peptide mixtures can assemble to form nanostructured gels at physiological conditions through H-bonding and π -stacking interactions. Self-assemble peptides offer a printable material adequate for 3D cell culture which mimics the native extracellular matrix. These kinds of peptides have been shown to sustain pericytes viability and their differentiation to chondrocytes (8). PLTMax (PLTMax[®]) is a serum free product derived from human platelets. This platelet lysates contains more than 150 growth factors, adhesion factors, chemokines, etc. that supports the growth and viability of many cell types including fibroblasts and stem cells. PLTMax is an excellent source of nutrients for cells participating in tissue repair and also the formation of organoids (10-13). GelMA (CELLINK GelMA) is gelatin modified with methacryloil groups to crosslink the GelMA macromers with a photoinitiator or exposure to UV light (14). GelMA mimics the natural extracellular environment and shows a tissue-like elastic properties making possible cells receiving nutrients and proliferate (2, 15-17).

Many epithelial cells can grow away from the bidimensional culture and develop extensions, tubules and ducts when embedded in extracellular matrices such as Matrigel. In the case of lung bud organoids (LBOs), generated from hPSCs, it is possible to observe branching or airways and early alveolar structures as well as a proximodistal specification. Leaving aside the inherent limitations, such as the full terminal maturation and the presence of spurious mesoderm, LBOs can be used efficiently to study lung differentiation, model any respiratory disease, and as a platform to synthesize biomaterial suitable for regenerative medicine. Thus, we and others have used these respiratory

organoids or minilungs to model human respiratory diseases including fibrotic and infectious diseases (18-20). Due to the murine origin of Matrigel its suitability for regenerative medicine or clinical translation is greatly limited. Thus, the development of new supportive materials for long-term cell survival which are biocompatible with clinical translation and automatized synthesis (bioprinting) is an urgent need.

In the present study, we have evaluated the suitability of some representative hydrogels as supportive biomaterials for cell growth and differentiation of human minilungs from human embryonic stem cells (hESCs) as an initial step to establish bioprinting protocols.

Generation of minilungs from hESCs: comparison between Matrigel™ and the hydrogels Biogelx™-INK, PLTMAx® and GelMA.

The expansion and differentiation of the hESC line AND-2 was performed as previously described (19-21). The main steps of the protocol are illustrated in Figure 1A and, essentially, consists of the preparation of “good” AND-2 colonies growing along inactivated mouse embryonic fibroblasts (iMEFs) feeder cells, the depletion of the iMEFs, the induction of endoderm, anterior foregut endoderm (AFE) and, finally, the ventralization and lung formation step to generate LBOs with branching structures. Figure 1B shows representative micrographs of the checkpoints performed during the common procedure for the different hydrogels: hESCs expansion, depletion of feeder cells, differentiation to AFE and the commitment to the lung lineage. The protocol followed is common for the four conditions until day 20-25, when the organoids with folding structures are selected and embedded in the different hydrogel sandwiches according to their specific characteristics of gelation and the presence of medium and differentiation factors, as previously described for Matrigel (20). In short, the protocol for the hydrogel Matrigel is the following: at day 8, cells were briefly trypsinized into small cell clumps.

The clumps were plated onto low-attachment plates in branching medium. These three-dimensional clumps (nascent LBOs) were incubated approximately until day 20–25 of differentiation. After that, these nascent organoids were embedded into a Matrigel sandwich assembled on MW24 plates (multiwell culture plates of 24 wells). 100 μ L of Matrigel was loaded on the MW24 well and allowed to gel. Nascent organoids were picked up and divided into MW24 wells containing 50% Matrigel, diluted in branching media and immediately transferred onto the first layer of Matrigel. After gelation of this intermediate layer containing the nascent organoids, 100 μ L Matrigel was added on top. This type of manipulation and embedding of the organoids was followed with the other hydrogels according to their individual specific characteristics. Thus, the starting material was equivalent in all the conditions ensuring an equal number of nascent organoids per condition were embedded in each hydrogel. Biogelx gelation was performed following the manufacturer instructions for 3D cell culture method (9). For PLTMAx the concentration of CaCl_2 of in the medium was increased up 8 mM to promote the hydrogel gelation as indicated by the manufacturers. Finally, the gelation of the different sandwich layers of GelMA was achieved by photocrosslinking with UV 365nm following the manufacturers' instructions. Each sandwich containing various organoids was incubated with 600 μ L of branching media which was changed every 2–3 days. As previously described growing branching structures are easily visualized under the microscope after 1 or 2 weeks. Figure 2 shows representative micrographs of mature minilungs embedded in the different hydrogels evaluated. Minilungs embedded in Matrigel show the characteristics buds or extensions more or less branched growing through the hydrogel (Figure 2A, B). Buds and extensions normally enclose a lumen delineated primarily by alveolar epithelial cells type 2 (AEC-2) as we and others have reported previously (18–20). Figure 2C, D show representative brightfield micrographs of the typical epithelium

found when Matrigel is used. In contrast to those in Matrigel, minilungs embedded into the Biogelx hydrogel are smaller, less branched and fragmented (Figure 2E, F). The microscopic observation at higher magnification reveals globose extensions outlined by a poorly defined epithelium enclosing a lumen filled with cells. In addition, it is possible to observe rounded cells, probably undergoing necrosis on the outer edge of the extensions (Figure 2G, H). In the case of PLTMAx, the fragmentation of the minilungs generated are even greater than the Biogelx embedding (Figure 2I, J). However, unlike with Biogelx, the exterior of the extensions is outlined by an epithelium reminiscent of that found with Matrigel. As in the case of Biogelx, the lumens of these extensions are also filled with cells (Figure 2K, L). Finally, minilungs in GelMA hydrogel were small and dense fragmented aggregates surrounded of translucent material probably consisting of gelatin fibres (Figure 2M, N). Upon closer inspection, we can observe lumens filled with cells and high levels of cell debris and necrosis on the exterior of the organoid fragments (Figure 2O, P). To support these observations, we performed a quantitative study of the organoids in terms of some parameters: size (the longest diameter observed in each organoid; branching index (the number of branches observed in each of the organoids), lumen percentage (the percentage of those branches showing an evident lumen), and the fragmentation index as the percentage of masses below the average size of the organoid (fragments) present along the organoids. As shown in Figure 2Q-T, Matrigel-embedded minilungs exhibit the largest sizes as well as the highest levels of branching and the presence of evident lumens. Fragmentation is absent in these minilungs. By contrast, Biogelx and PLTMax minilungs show similar branching indexes and lumen percentages. Minilungs embedded in PLTMax or GelMa show the highest values in terms of fragmentation.

As previously described, the analysis by RT-qPCR of the lung lineage checkpoint at days 20 and 28 of differentiation show the typical expression profile of transcription factors *NKX2-1* and *FOXA2* of minilungs generated in Matrigel (18-24). In the cases of Biogelx and PLTMAx the expression of *NKX2-1* was undetectable at day 20 suggesting a delayed kinetics of expression with respect to the others samples (Figure 3A, B). Finally, we analyzed the expression of the surfactant by RT-qPCR as a measure of maturity at the final step of the experimentation (day 60) as previously described (19-21, 24). Despite the large structural differences of the organoids generated from the tested hydrogels, compared to Matrigel, we did not observe qualitative differences in the expression pattern of the surfactant components evaluated. Moreover, the expression levels of the surfactant were even significantly higher, in some cases, compared to those found with Matrigel (Figure 3C). Although the organoids elaborated from the different hydrogels present a similar expression pattern in the evaluated checkpoints, they show great differences in the structure of the organoids. Altogether, the results indicate that, although the new hydrogels tested can support the growth and expression of AEC-2 at distal regions of the minilungs, as happens during the typical Matrigel embedding (19-21), the structural arrangement of this cellular content is, in general, largely aberrant. This possibly makes these minilungs a worse model for studying disease and infection, where the structure of the organoids also plays a role (20). In addition, these promising hydrogels do not seem to support long term of minilungs' cells survival at the exterior of the extensions. The cytotoxicity observed might be due to the poor nutrient content of the hydrogels, the suboptimal diffusion of nutrients throughout the hydrogel sandwich, the biocompatibility of the gelation induction by calcium or UV with the correct structural differentiation, etc. It is known that the Matrigel context provides nutrients and extracellular matrix components that appear necessary to efficiently support stem cell growth. Furthermore,

when organoids are embedded in the presence of the necessary differentiation factors, Matrigel also efficiently supports the differentiation into a multitude of cell lineages. Our results also suggest that the inherent biocompatibility of the material tested is not sufficient to support cell survival and differentiation of lung progenitors to minilungs in the presence of differentiation factors. Finally, the negative effects on the cell growth and organoid structure observed, indicate that the hydrogels evaluated might not be suitable as supportive materials or scaffolds for lung organoids and, probably, for the bioprinting of lung tissue derived from hPSCs. However, further studies on these hydrogels are needed to bring them to optimal conditions in terms of their mechanical properties, viscoelasticity, nutrient supplementation and/or extracellular matrix components.

Methods

Formation of lung bud organoids

The protocols for the maintenance and expansion of the human embryonic stem cells (hESCs) and their differentiation to minilungs containing airways and alveolar lung cells have been described in detail in previous works (18, 20, 21). The specific information for the generation of lung bud organoids from the different hydrogels is the following:

The differentiation process was performed under normoxic conditions from the anteriorization stage (anterior foregut endoderm, AFE). At day 8, the cells were briefly trypsinized into small 3–10 cell clumps and the reaction was halted with stop medium (IMDM medium (BE12-722F) supplemented with 50% fetal bovine serum (FBS; F7524; Sigma-Aldrich), 2 mM Glutamax, 1% penicillin-streptomycin). Cells were then centrifuged for 5min at 850 rpm and washed carefully twice with an excess of SFD medium. The clumps were plated onto low-attachment six-well plates (cat.#3471; Corning) in branching medium (SFD medium containing 3 μ M CHIR99021, 10 ng/mL FGF10, 10 ng/mL KGF, 10 ng/mL BMP4, 50 nM all-trans retinoic acid). These three-

dimensional clumps (nascent lung bud organoids) were incubated and fed every other day for approximately 20–25 days. After that, these nascent organoids were embedded into the specific hydrogel sandwiches in MW24 wells. For Matrigel™, 100 μ L of Matrigel™ was loaded and allowed to gel. Nascent organoids were picked up with a wide mouth plastic Pasteur pipette, divided into MW96 wells containing 50% Matrigel™, diluted in branching media and immediately transferred onto the first layer of Matrigel™. After solidification of this intermediate layer containing the nascent organoids, 100 μ L Matrigel™ was added on top. Finally, each sandwich containing various organoids was incubated with 600 μ L branching medium. Medium was changed every 2–3 days. Growing branching structures were easily visualized under the microscope after 1 or 2 weeks. For Biogelx™-INK (Biogelx powder; Biogelx, UK), the hydrogel sandwich was assembled as for Matrigel following the specific manufacturer's instructions for 3D cell culture method (9). Briefly, the pre-gel solution was prepared in an incubator for 30 min until the solution reached 37°C and the sandwiches were prepared as following: 100 μ L Biogelx was pipetted on the bottom of the well and 100 μ L of medium was added carefully on the top. The addition of cell culture media containing physiological levels of calcium triggered the gelation of Biogelx. After 2h incubation in an incubator at 37°C and a humidified atmosphere of 5% CO₂, the medium on the surface was replaced by the intermediate layer contains the medium and the organoids. After gelation, 100 μ L Biogelx and 600 μ L branching media were added on top. Finally, after 2h incubation in the cell incubator 37°C and a humidified atmosphere of 5% CO₂, the medium on the surface was replaced with 600 μ L branching media. Medium was changed every 2–3 days. For PLTMAx hydrogel (PLTMAx®, ref.: PTLMax100R, Mill Creek Life Sciences, USA) the concentration of CaCl₂ in the medium was increased up to 8mM to allow gelation following manufacturers' instructions. The sandwiches were assembled as for Matrigel

with the exception that 2h incubations in the cell incubator at 37°C and a humidified atmosphere of 5% CO₂ were performed to allow gelation of the different layers. In the case of GelMA (Cellink GelMA, re.: IK-305102) the gelation was induced by radiation with 365nm UV (4 mW/cm²) following manufacturers' instructions. We employed the UV crosslinker (ref.# 215154, Boeckel Scientific). Medium on the surface was changed every 2–3 days.

Quantitative real-time RT-PCR (RT-qPCR) of minilungs

Total RNA was extracted using Trizol (cat.#15596026; Ambion) following manufacturer's instructions. cDNA was generated using the High-Capacity cDNA kit (cat.#4387406; Applied Biosystems). Real-time qPCR was performed by using the powerUpSYBR Green mix (cat.#A25742) on the Quantstudio-3 system (Applied Biosystems) following manufacturer's instructions. Absolute quantification of each gene was obtained using a standard curve of serial diluted genomic DNA (cat.#11807720, Roche) and normalized to housekeeping ribosomal gene 18S.

The genes analyzed and the sequences of the oligonucleotides employed in this study, were the following: *18S* (*RNA18S*) [Forward: 5'- GTA ACC CGT TGA ACC CCA TT, Reverse: 5'- CCA TCC AAT CGG TAG TAG CG]; *NKX2-1* (*NK2 HOMEBOX 1*) [Forward: 5'- CGGCATGAACATGAGCGGCAT, Reverse: 5'- GCCGACAGGTACTTCTGTTGCTTG]; *FOXA2* (*FORHEAD BOX A2*) [Forward: 5'- TTCAACCACCCGTTCTCCATCAAC, Reverse: 5'- TGTTTCGTAGGCCTTGAGGTCCATT], *SFTPA* (*Surfactant Protein A, marker of AEC-2 cells*) [Forward: 5'-GTGCGAAGTGAAGGACGTTTGTG, Reverse: 5'- TTTGAGACCATCTCTCCCGTCCC]; *SFTPB* (*Surfactant Protein B, marker of AEC-2 cells*) [Forward: 5'-TCTGAGTGCCACCTCTGCATGT, Reverse: 5'- TGGAGCATTGCCTGTGGTATGG]; *SFTPC* (*Surfactant Protein C, marker of AEC-2*

cells) [Forward: 5'-CCTTCTTATCGTGGTGGTGGTGGT, Reverse: 5'-TCTCCGTGTGTTTCTGGCTCATGT].

Statistical analysis

Data were subjected to the Shapiro-Wilk test and D'Agostino and Pearson omnibus test to verify their normality. Statistical significance of data was determined by applying the two-way analysis of variance followed by the Tukey or Bonferroni post-tests. $P < 0.05$ is considered significant. Significance of analysis of variance post-test is indicated in the figures as *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ and ****, $P < 0.0001$. Statistics were calculated with the GraphPad Prism 10 software. The results presented in the figures are means $\pm$ SEM. Experiments were repeated three times. Figure 2: more than 70 organoids were analyzed ($n > 14$ per condition). Figure 3: data from three experiments correspond to the analysis of 10-15 minilungs per condition.

List of abbreviations

AEC-2	Alveolar epithelial cells type 2
AFE	Anterior Foregut Endoderm
FOXA2	Forhead box A2
hESC	human embryonic stem cell
hPSC	human pluripotent stem cell
iMEFs	inactivated mouse embryo fibroblasts
LBO	Lung bud organoid
mW	milliwatts
MW24, 96	Multiwell culture plates of 24 and 96 wells.
NKX2-1	NK2 homeobox 1
nm	nanometer

RT-qPCR	Quantitative Real-Time RT-PCR
SFTPA	Surfactant Protein A
SFTPB	Surfactant Protein B
SFTPC	Surfactant Protein C
μL	microliter
μm	micrometer

Declarations

Ethics approval and consent to participate

We confirm that the patients (embryo donors) provided written informed consent for the generation of AND-2 cell line and the use of derived samples. The use of the hESC line AND-2 and the experimental procedures of this study were approved by the ISCIII Ethics Committee (CEI PI 98_2022) and the National Committee of Guarantees for the Use and Donation of human Cells and Tissues (ref. no. 642 531 1; November 07th 2023): Generation of organoids from pluripotent stem cells (hPSCs): Disease modeling and development of the molecular and cellular basis for therapy and regenerative medicine.

Consent for publication

Not applicable

Availability of data and materials

Please contact the corresponding authors for data requests. Supplemental data: additional general methods and imaging used to perform the minilung metrics shown have been archived in the public repository REPISALUD under the accession number/DOI: <https://dx.doi.org/10.4321/repisalud.27555>.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

IC-H performed experiments and analyzed the data; JS performed experiments, analyzed data and wrote paper; JY and AZ performed and designed the experiments, analyzed the data, wrote the paper and conceived the project. All authors read and approved the final manuscript.

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Figure legends

Figure 1. Summary of the generation of minilungs. A. Scheme of the differentiation process followed to generate human minilungs embedded in the different hydrogels evaluated. The differentiation of the hESCs is divided into consecutive steps detailed in the Methods section. Asterisks indicate the checkpoints evaluated in this study. Initial checkpoint: expansion and selection of “good” hESC colonies prior to the differentiation

process; second checkpoint was performed around day 20, corresponding to the lung lineage commitment (RT-qPCR analysis shown in Figure 2). Finally, the last checkpoint (maturation of the cells generated) was done at >60 days of differentiation and corresponded to the RT-qPCR analysis of both airways and lung cell markers (shown in Figure 2). At day 8 the cultures were scratched with a pipette tip and the cell aggregates were picked and transferred to ultra-low attachment plates to start the formation of nascent organoids in suspension. Between the days 20-25 the organoids were embedded in the different hydrogels. The steps performed under hypoxia are indicated in red. AFE: anterior foregut endoderm. **B.** Representative micrographs taken at various points of the differentiation process. From left to the right: a hESC colony growing along feeder cells [inactivated mouse embryo fibroblasts (iMEFs)]; a hESC colony growing on a thin layer of Matrigel in the absence of iMEFs (MEFs depletion step); culture of cells growing on human fibronectin, corresponding to the anterior foregut endoderm (AFE, day 6) and culture of cells growing on human fibronectin, corresponding to day 20 (lung lineage commitment). The micrograph at day 20 corresponds to a bidimensional culture run in parallel to the nascent organoids in order to facilitate mRNA extraction and potential immunofluorescence assays for the checkpoints of interest. Scale bars: 100 μ m.

Figure 2. Results of the generation of minilungs from the different hydrogels employed. Representative micrographs of minilungs embedded in Matrigel (A-D), Biogelx (E-H), PLTMAx (I-L) and GelMA (M-P) at day 60 of differentiation. Arrowheads key: black, typical buds of LBOs; red: lumen; yellow: epithelium probably delineated by AEC-2 cells as previously reported; green: globose extensions found in Biogelx organoids; blue: poorly defined epithelium of Biogelx organoids; magenta: epithelium found in pLTMAx organoids; brown: gelatin fibers, probably; orange: cell debris and necrosis, probably]. Panels Q-T: quantitative metrics analysis of the organoids. Size of the organoids:

measurement of the longest diameter observed in the organoids. Branching index: calculation of the number of branches observed in each of the organoids; lumen percentage: percentage of those branches showing an evident lumen; fragmentation index: the percentage of masses below the average size of the organoid (fragments) present along the organoids. More than 70 organoids were analyzed ($n > 14$ per condition). The results presented in the figures are means $\pm$ SEM.

Figure 3. RT-qPCR result corresponding to the lung lineage commitment at days 20 and 28: expression of *NKX2.1* and *FOXA2* expression at day 20 (panel A, ANOVA $P = 0.0003$) or day 28 (panel R, ANOVA $P = 0.7260$); Panel S: representative RT-qPCR result at the end of the differentiation process (~ 60 days): expression of surfactant proteins SFTPA, B and C (ANOVA $P < 0.0001$). Data from three experiments correspond to the analysis of 10-15 minilungs per condition. The results presented in the figures are means $\pm$ SEM.

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Figure 1. Chamorro-Herrero et al.

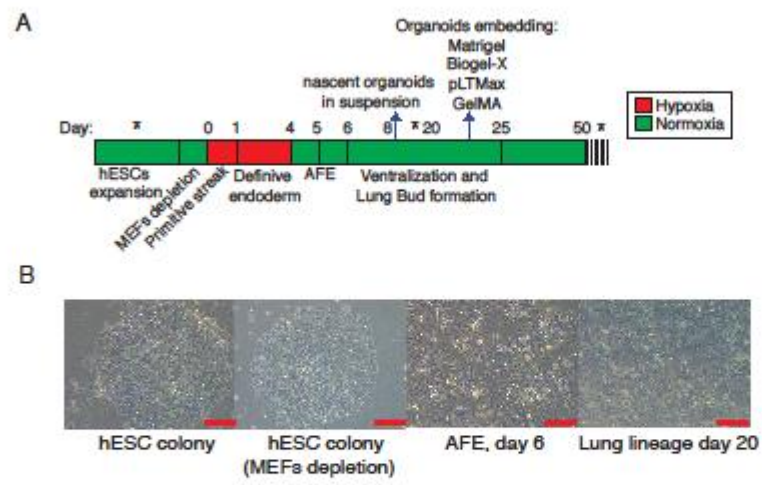


Figure 2. Chamorro-Herrero et al.

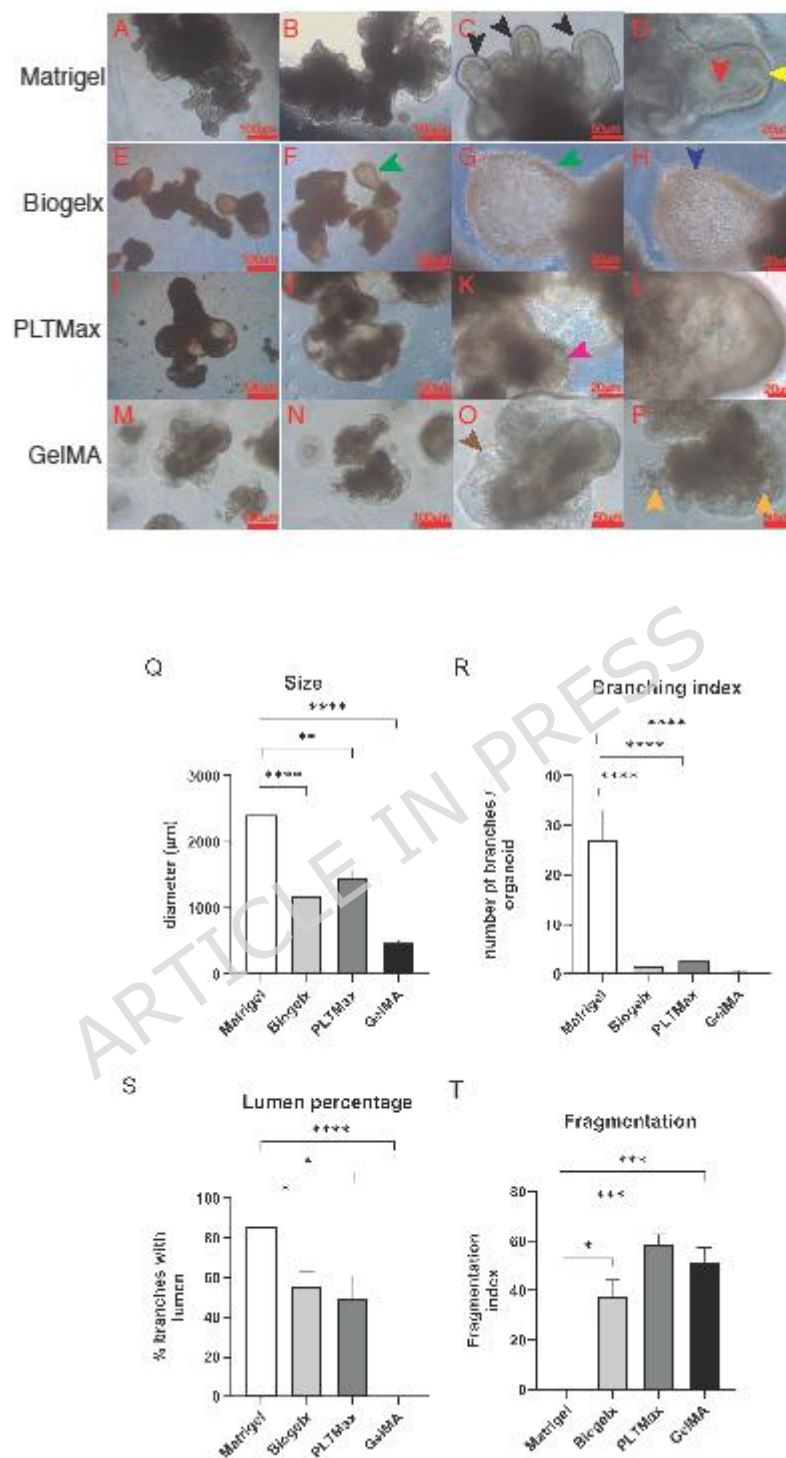


Figure 3. Chamorro-Herrero et al.

