Tissue extracellular matrix hydrogels as alternatives to Matrigel for culturing gastrointestinal organoids

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Matrigel, a mouse tumor extracellular matrix protein mixture, is an indispensable component of most organoid tissue culture. However, it has limited the utility of organoids for drug development and regenerative medicine due to its tumor-derived origin, batch-to-batch variation, high cost, and safety issues. Here, we demonstrate that gastrointestinal tissue-derived extracellular matrix hydrogels are suitable substitutes for Matrigel in gastrointestinal organoid culture. We found that the development and function of gastric or intestinal organoids grown in tissue extracellular matrix hydrogels are comparable or often superior to those in Matrigel. In addition, gastrointestinal extracellular matrix hydrogels enabled long-term subculture and transplantation of organoids by providing gastrointestinal tissue-mimetic microenvironments. Tissue-specific and age-related extracellular matrix profiles that affect organoid development were also elucidated through proteomic analysis. Together, our results suggest that extracellular matrix hydrogels derived from decellularized gastrointestinal tissues are effective alternatives to the current gold standard, Matrigel, and produce organoids suitable for gastrointestinal disease modeling, drug development, and tissue regeneration.
Three-dimensional (3D) miniature organs of the gastrointestinal (GI) tract, called GI organoids, have been established from stem cells and are grown in extracellular matrix (ECM)-embedded in vitro culture systems\(^1\text{–}^3\). Currently, the culture of most organoids, including GI organoids, relies on Matrigel, a commercialized matrix composed of various elements including laminin, collagen type IV, and growth factors. However, Matrigel has several undeniable drawbacks. As a raw material extracted from the Engelbreth–Holm–Swarm mouse sarcoma, it causes large batch-to-batch variation in cultured organoids. Given its origin, there is also a potential risk of transmission of animal pathogens\(^6\text{–}^7\); that infect macrophages and affect the immune systems (e.g., lactate dehydrogenase-elevating virus\(^8\text{,}^9\)). In addition, Matrigel is primarily composed of tumor ECM components such as laminin which is highly expressed in several types of tumors\(^10\text{,}^11\). As the matrisome, a collection of ECM and ECM-associated proteins, in tumors is substantially different from that of normal tissues\(^12\), tumor ECM-based Matrigel may not provide the tissue-specific microenvironments for GI organoids. For these reasons, many attempts have been made to develop alternatives to Matrigel\(^13\text{–}^16\). For example, synthetic poly(ethylene glycol) (PEG) hydrogels modified with ECM peptides (e.g., RGD) and protease-degradable peptides (e.g., GPQGIWGQ) or natural hydrogels, such as alginate hydrogel or fibrin gel mixed with ECM proteins (e.g., laminin-111), have been tested as artificial 3D environments for GI organoids. Despite the obvious benefits of these engineered hydrogels in culturing GI organoids, they need improvements to fully reconstitute the biochemical ECM composition of the native tissue and promote organoid development and maturation.

Here, we develop a GI organoid culture platform that mimics the native GI ECM microenvironment using decellularization techniques. We investigate whether our GI ECM hydrogels could replace Matrigel in GI organoid culture by providing cell–matrix signals optimal for GI organoid development.

**Results**

**Characterization of decellularized GI tissue-derived hydrogels.** To develop a 3D matrix that better recapitulates the biochemical ECM composition of native GI tissues and ultimately replaces the use of Matrigel in organoid culture, we prepared ECM-based hydrogels derived from decellularized pig stomach and small intestinal tissues (Fig. 1a). First, we optimized decellularization protocols by adjusting the type (non-ionic versus ionic) and treatment time of detergents for decellularization (Supplementary Fig. 1). Using our optimized decellularization protocol (Protocol 1), which is based on a non-ionic detergent (Triton X-100)\(^17\), cellular components were completely removed from stomach and intestine tissues, whereas major ECM components (e.g., glycosaminoglycans; GAG) were preserved\(^17\text{,}^18\) (Fig. 1b, c). Another decellularization protocol (Protocol 2) that uses ionic detergents (e.g., sodium deoxycholate; SDC) and has been widely used for cell removal\(^19\text{,}^20\) effectively removed cellular components but impaired ECM preservation as indicated by a decrease in the GAG content (Supplementary Fig. 1a–c). Decellularized stomach-derived ECM (termed “SEM”) and decellularized intestine-derived ECM (termed “IEM”) were lyophilized, solubilized, and then induced to form 3D hydrogels at physiological pH and temperature via the kinetics of collagen fibril assembly (Supplementary Fig. 2). Both SEM and IEM hydrogels possessed a nanofibrous ultrastructure composed of interconnected ECM fibrils similar to collagen type I hydrogels (Fig. 1d)\(^20\text{,}^21\). The hydrogels prepared with our decellularization protocol exhibited a higher elastic modulus (storage modulus measured at 1 Hz frequency) (1.6–3.3-fold) than those prepared using an ionic detergent (Supplementary Fig. 1d), indicating superior ECM preservation with our protocol. The storage modulus of hydrogel implies the ability of the hydrogel to store deformation energy, and the storage modulus is proportional to elastic energy\(^22\). In other words, higher storage modulus indicates higher elasticity and stronger mechanical property of hydrogel. Thus, ECM hydrogels with higher storage modulus may be more appropriate for the formation, growth, and long-term maintenance of organoids. As our optimized decellularization protocol allows a larger amount of ECM preservation and retains the stability of GI tissue-derived ECM hydrogels, the resultant hydrogels are more suitable for GI organoid culture.

We verified the safety of decellularized GI tissue-derived ECM hydrogels. First, we found that endotoxin levels of SEM and IEM were at 0.344 ± 0.007 EU/ml and 0.225 ± 0.016 EU/ml, respectively (Supplementary Fig. 3a). This may indicate that our protocol to decellularize GI tissues and prepare ECM hydrogels can exclude contamination of SEM and IEM hydrogels from pathogens in the GI tissues. Given that the endotoxin level acceptable for implantable medical devices is 0.5 EU/ml in the guideline of the Food and Drug Administration (FDA)\(^23\), our decellularized GI tissue-derived ECM hydrogels would be able to serve biomaterials with clinical feasibility. Next, we checked the immunogenicity of decellularized GI tissue-derived ECM hydrogels. Under the in vitro condition, the secretion of inflammatory cytokine tumor necrosis factor-α (TNF-α) from RAW 264.7 macrophages 3 and 6 h after exposure to SEM and IEM hydrogels was negligible, which was similar to that from cells with no treatment as a negative control (Supplementary Fig. 3b). Hematoxylin and eosin (H&E) staining of the tissue samples retrieved 1, 4, and 7 days after injection SEM and IEM hydrogels into subcutaneous space of mice revealed that neither tissue necrosis nor tissue damage was observed in the tissues injected with SEM and IEM hydrogels (Supplementary Fig. 3c). Toluidine staining also confirmed that excessive infiltration of inflammatory cells was not observed at the injection sites (Supplementary Fig. 3c). These results verified that decellularized GI tissue-derived ECM hydrogels are highly biocompatible and safe materials that can be considered for clinical applications. Decellularization of tissues allows manufacturing natural ECM-based materials with low immunogenicity by removing antigenic cellular components. Decellularized tissue-derived materials free from xenogeneic pathogens can be prepared through decellularization with a series of detergent treatments and sterilization processes (e.g., ethanol, ethylene oxide, ultraviolet (UV) irradiation, and supercritical carbon dioxide). Actually, Matrigel or Matrigel-derived products have never been approved by the FDA, while several commercialized products are based on decellularized tissue ECM (e.g., AlloDerm\(^\text{®}\), Meso BioMatrix\(^\text{®}\), and SynerGraft\(^\text{®}\), etc.) have been approved for clinical use by the FDA.

Intensive proteomic analysis using mass spectrometry (MS) revealed the presence of stomach- and intestine-specific matriome and non-matrisome components preserved in the SEM and IEM, which were distinct from Matrigel proteome components (Fig. 2a and Supplementary Fig. 4a, b). More core matriome and matrisome-associated proteins were detected in SEM and IEM compared to Matrigel (Supplementary Fig. 4c). In total matriome proteins, collagen subtypes and proteoglycans constituted about 67% and 13% in SEM and about 51% and 26% in IEM, respectively, whereas the portion of glycoproteins was about 67% and 13% in SEM and about 51% and 26% in IEM, respectively (Supplementary Fig. 3a). This may indicate that our decellularization protocol effectively removed extracellular matrix proteins, collagen fibrils, and ECM associated proteins from GI tissue-derived ECM hydrogels. The resultant hydrogels are more suitable for GI organoid culture.
proliferation and tissue homeostasis. Thus, SEM and IEM that contain a large quantity and variety of collagen subtypes and proteoglycans more closely reflect the in vivo GI microenvironment compared to Matrigel with only 0.4% collagen and 1% proteoglycans (Fig. 2b–d). Collagen Type VI (COL6A1, COL6A2, COL6A3, and COL6A5) and proteoglycans, such as decorin (DCN), were identified as the top 10 most abundant matrisome proteins in SEM and IEM, whereas glycoproteins, including laminin-111 (LAMA1, LAMB1, and LAMC1) and fibrinogen (FGA, FGB, and FGG), were the top 10 most abundant matrisome proteins in Matrigel (Fig. 2b–d).

Interestingly, the proteins significantly enriched in native stomach or intestine tissues were detected in much greater quantities in each tissue-specific ECM hydrogel compared to Matrigel (Supplementary Fig. 4d). Whereas Matrigel and IEM did not contain proteins known to be enriched in the native stomach. Hence, stomach or intestine tissues were detected in much greater quantities in each tissue-specific ECM hydrogel compared to Matrigel (Fig. 2b–d).

To evaluate the variation of decellularized GI tissue-derived matrices in the ECM compositions, we compared the samples under two conditions: (1) comparison of multiple sample batches derived from different locations in the same donor tissue (batch variation—porcine A1–A3), (2) comparison of multiple samples derived from different donor tissues (donor variation—porcine A–C). The compositions of matrisome proteins are quite similar in three sample batches of SEM or IEM derived from different locations in the same donor tissue (Fig. 2e, h). Interestingly, matrisome proteins in some tissues are associated with cell growth and development. Thus, we postulate that non-matrisome components in SEM and IEM hydrogels help the formation and development of GI organoids. In gene ontology biological process (GOBP) analysis of non-matrisome proteins in Matrigel, SEM, and IEM, the top ten representative GOBP terms indicated that the non-matrisome proteins of SEM and IEM were involved in cellular component biogenesis, cytoskeleton organization, and structural development, whereas those of Matrigel were more related to translation and RNA processing and metabolic process (Supplementary Fig. 5). Collectively, we speculate that SEM and IEM derived from decellularized GI tissues may serve as alternative matrices to Matrigel for GI organoid culture by better reconstituting the native GI-like microenvironment.
Fig. 2 Proteomic analysis of GI tissue-derived ECM hydrogels. a Principal component analysis (PCA) of matrisome proteins present in Matrigel, SEM, and IEM. The composition of total matrisome proteins and the most abundant top 10 matrisome proteins in b Matrigel, c SEM, and d IEM (N = 4 for Matrigel and N = 3 for SEM and IEM). Comparison of the composition of total matrisome proteins and the top ten matrisome proteins with the highest expression in e SEM samples and f IEM samples derived from different tissue batches of the same donor (porcine A1–A3) or different donor batches (porcine A–C) (N = 3). Venn diagram showing the overlap of matrisome proteins in f SEM samples and g IEM samples derived from three different donors (porcine A–C). GOBP analysis of overlapped matrisome proteins in h SEM samples and i IEM samples from different donor batches. PCA analysis and Pearson’s correlation analysis to assess the similarity of total proteins detected in k, l SEM samples, and m, n IEM samples derived from different tissue batches of the same donor (porcine A1–A3) and different donor batches (porcine A–C). Pearson’s correlation coefficient values are indicated in each box. The averaged values from three biological replicates were used for analysis in (f), (g), (i), (j), and (k–n). The data in (b–d), (e), and (h) are presented as mean ± S.D.
there was little variation in the profiles of matrisome proteins in three sample batches of SEM or IEM derived from different donor tissues (Fig. 2e, h). Moreover, the lists of the top 10 matrisome proteins with the highest expression almost overlapped between different batches from the same donor tissue or the tissue samples from different donors (eight proteins in SEM samples and nine proteins in IEM samples) (Fig. 2e, h). More than 100 matrisome proteins were overlapped in three sample batches of SEM or IEM derived from different donor tissues (SEM: 102 and IEM: 114) (Fig. 2f, i). GOBP analysis revealed that these overlapped matrisome proteins are mainly involved in the organization of ECM and extracellular structure (Fig. 2g, j). Principal component analysis (PCA) plot and heatmap of Pearson’s correlation coefficients confirmed that SEM and IEM samples derived from different tissue batches or different donor batches have high similarity in terms of protein compositions and profiles (Fig. 2k–n). Together, these proteomic analytical data demonstrate that decellularized GI tissue-derived matrices have a relatively low batch-to-batch variation in comparison to Matrigel.

To further investigate the variation in matrisome proteins and non-matrisome proteins in SEM and IEM samples, we compared the matrisome proteins and non-matrisome proteins detected in the SEM and IEM that are also expressed in native stomach and intestine tissues, respectively (Supplementary Fig. 6). Interestingly, among proteins highly enriched in the native stomach, one matrisome protein (ANXA10) and 4 non-matrisome proteins (CHIA, CLIC6, GHR1, PGC) were commonly detected in all batches of SEM samples, indicating that there is no significant variation in matrisome and non-matrisome proteins in the SEM from different tissue locations (porcine A1–A3) and donors (porcine A–C) (Supplementary Fig. 6a). In the case of proteins highly enriched in the native intestine, five matrisome proteins (ITLN2, LGAL5, LGAL5, MUC2, TINAG) were detected in all batches of IEM samples and one matrisome protein (REG4) was detected in two IEM sample batches (porcine A2, C). Total 19 non-matrisome proteins (ACTG2, ALD02, ACO1, APOA4, ARHGAP45, CASP7, EPS8L2, FABP6, GSTA1, KLC4, KRT20, KRT8, LAP1, MYH14, OLFM4, RPS6KA1, SELENBP1, SMTN, and SRI) were commonly detected in all batches of IEM samples (Supplementary Fig. 6b). Other non-matrisome proteins GSDMB and JCHAIN were detected in four (porcine A2, A3, B, C) and three batches (porcine A2, B, C) of IEM samples, respectively. Two non-matrisome proteins (BCL2L15, HSD17B11) were detected in two IEM batches (porcine B, C). Therefore, most of matrisome proteins (5/6) and non-matrisome proteins (19/23) enriched in the native intestine were overlapped in all batches of IEM samples, demonstrating again that the variation in matrisome and non-matrisome proteins in the IEM is insignificant between tissue locations and donors. Moreover, these proteins commonly detected in different batches of SEM and IEM have involved in GI tissue-specific functions such as digestion, intestinal absorption, and gastric acid secretion (Supplementary Fig. 6c, d), indicating that GI tissue-derived ECM hydrogels can provide GI organoids with native GI-like microenvironments in a highly reproducible manner.

Additionally, we analyzed ECM compositions in decellularized tissues from different parts of the GI tract. We decellularized the esophagus, an upper part of the GI tract, and compared ECM profiles of decellularized esophagus-derived ECM (EEM) with those of SEM and IEM. The types and compositions of matrisome proteins in EEM were slightly different from those in SEM and IEM (Supplementary Fig. 7a). Three matrisome proteins (COL6A1, COL6A2, COL6A3) among the most abundant top 10 matrisome proteins in EEM were overlapped with those in SEM and IEM (Supplementary Fig. 7b). Despite such a slight discrepancy, PCA plot confirmed that overall ECM profiles in different parts of the GI tract (IEM, SEM, and EEM) were close each other compared to Matrigel with quite different ECM components (Supplementary Fig. 7c). Together, we conclude that ECM profiles and compositions in decellularized tissues are varying depending on the location in the GI tract, but ECM hydrogels derived from the GI tract have high similarity in comparison to materials from non-GI tract such as Matrigel.

Utility of GI tissue-derived ECM hydrogels as a GI organoid culture matrix. To demonstrate the potential of decellularized GI tissue-derived ECM hydrogels (SEM and IEM) to substitute for Matrigel in organoid culture, we compared GI organoids generated in SEM and IEM hydrogels with those generated in Matrigel in the following four aspects: (i) organoid morphology, (ii) organoid formation efficiency, (iii) organ-specific gene expression level, and (iv) organoid functionality. Before using the hydrogels in organoid culture, the rheological properties of SEM and IEM hydrogels were characterized at various concentrations to confirm their mechanical stability for organoid culture. Consistently higher storage modulus (G') than the loss modulus (G'') in all tested concentrations indicated the stable formation of cross-linked ECM networks in the hydrogels (Supplementary Fig. 8a, b).

First, we identified each optimal concentration of SEM and IEM hydrogels that effectively supported the development of GI organoids. When mouse gastric glands were seeded in SEM hydrogels at different ECM concentrations (1, 3, 5, 7 mg ml$^{-1}$), gastric organoids formed in all tested concentrations (Fig. 3a), and the highest formation efficiency was observed at 5 mg ml$^{-1}$ (Fig. 3b). Quantitative real-time polymerase chain reaction (qPCR) demonstrated that the gene expression of a stem cell marker (Lgr5) was similar in gastric organoids grown in SEM hydrogels at concentrations of 3 and 5 mg ml$^{-1}$ and organoids grown in Matrigel. When we checked the expression levels of gastric epithelial cell markers (chief cell marker Pgc and parietal cell markers Atp4a, Atp4b) in the organoids grown in 5 mg ml$^{-1}$ SEM hydrogels, the expression of Pgc and Atp4a was comparable to that of Matrigel organoids and the expression of Atp4b was higher in the organoids grown in 5 mg ml$^{-1}$ SEM hydrogels than in the organoids in Matrigel (Fig. 3c). In general, stemness (Lgr5 and Axin2 for gastric stem cells) and gastric differentiation markers (Muc6 for mucous neck cells, Gif for parietal cells, and Pgc and Pga5 for chief cells) of gastric organoids grown in 5 mg ml$^{-1}$ SEM hydrogel were expressed at similar levels to those grown in Matrigel (Fig. 3d). Considering gastric organoid formation efficiency and gene expression levels, we selected 5 mg ml$^{-1}$ SEM hydrogel for further studies.

Similar experiments were conducted to optimize the concentration of IEM hydrogel used in intestinal organoid culture. All mouse intestinal crypts embedded in IEM hydrogels with 1–5 mg ml$^{-1}$ concentrations developed into intestinal organoids (Fig. 3g), although the formation efficiency was slightly lower than that of Matrigel (Fig. 3h). The expression levels of stem cell marker (Lgr5) and goblet cell marker (Muc2) in intestinal organoids encapsulated in IEM hydrogels were similar to or higher than those embedded in Matrigel (Fig. 3i), and the highest expression was observed at 2 mg ml$^{-1}$. All other intestinal differentiation markers (Lyz1 for Paneth cells, Chga for enteroendocrine cells, and Viil for enterocytes) of intestinal organoids grown in 2 mg ml$^{-1}$ IEM hydrogel were expressed at comparable levels to those grown in Matrigel (Fig. 3j). Given these results, we selected 2 mg ml$^{-1}$ IEM hydrogel for further studies in intestinal organoid culture. Although increased expression of stemness markers (Lgr5, Axin2) was observed in the intestinal organoids cultured in 2 mg ml$^{-1}$ IEM hydrogel, as proliferation and differentiation may occur simultaneously at the
early phase of intestinal organoid development, we speculate that differentiated cells may not be significantly less in intestinal organoids in IEM hydrogel compared to those in Matrigel. Actually, a previous study using decellularized matrices for intestinal organoid culture also showed a simultaneous increase in both stem cell markers and differentiated cell markers in the organoids grown in ECM hydrogel20, which is consistent with our current finding. Overall, GI tissue-specific stemness and differentiation signatures of the organoids in GI tissue-derived ECM hydrogels seem to be comparable to those of the organoids in Matrigel.

We further compared phenotypes and functions of organoids produced in optimized ECM hydrogels with those of organoids grown in Matrigel. Gastric organoids in SEM hydrogel (5 mg ml⁻¹) grew comparably to organoids in Matrigel in size and morphology (Supplementary Fig. 9a). Although the projected area of gastric organoids grown in SEM hydrogel was slightly smaller than that of organoids grown in Matrigel, the variation of size distribution and morphology was lower in SEM organoids compared to Matrigel organoids (Supplementary Fig. 9c, e). The coefficient of variation (CV) in size of gastric organoids was 66.7% in SEM hydrogel and 74.5% in Matrigel. Moreover, gastric organoids in both matrices
underwent similar organotypic development and contained heterogeneous populations of Ki67-positive proliferative cells and MUC5AC-, HK-, and CHGA-positive gastric cells (Fig. 3e). The areas stained positively for proliferation marker (SOX9) and major differentiation marker (MUC5AC) in the gastric organoids grown in SEM hydrogel were not statistically different from those in the organoids cultured in Matrigel (Supplementary Fig. 10a). These data indicate that the development of cellular compositions in the gastric organoids cultured in SEM hydrogel is generally similar to that in the Matrigel organoids.

The generation of functional parietal cells, one of the most important cells in the function of the stomach, is still challenging in the current culture system of gastric organoids. This limitation of the current gastric organoid culture system may be solved through recapitulation of gastric microenvironments. For example, a previous study demonstrated the co-culture of fundic organoids with stomach mesenchymal cells to maintain the function of parietal cells in gastric organoids30, in our case, fundic organoids containing parietal cells could be developed in our gastric organoid populations because we used whole stomach tissues including fundus region for the generation of gastric organoids. The presence of parietal cells in gastric organoids was confirmed by the expression of parietal cell marker genes (Atp4a, Atp4b) and protein (HK) (Fig. 3c, e). We compared the gastric function of organoids grown in SEM hydrogel and Matrigel by evaluating acid secretion using acridine orange dye. The degree of acid accumulation in organoids was similar in both groups (Fig. 3f). We also compared the gene expression of three major parietal cell markers (Gif, Atp4a, and Atp4b) in gastric organoids cultured in SEM hydrogel with that of stomach and intestine tissues (Supplementary Fig. 11a). RNA sequencing analysis revealed that the expression of those parietal cell markers in SEM organoids was significantly higher than that in intestine tissue, while significantly lower than in stomach tissue. This result suggests that gastric organoids cultured in SEM hydrogel contain parietal cells, but lacks parietal cell differentiation in comparison to native stomach tissue. We examined the gastric ultrastructure of gastric organoids cultured in SEM hydrogel using serial-section scanning electron microscopy to clarify the presence of parietal cells in gastric organoids (Supplementary Fig. 11b). Gastric parietal cells are characterized by a large number of mitochondria and intracellular canaliculi associated with acid secretion31. We found that some cells in the gastric organoids grown in SEM hydrogel have such characteristic ultrastructure of parietal cells, which directly confirmed the existence of gastric parietal cells in SEM organoids. The function of parietal cells in gastric organoids cultured in SEM hydrogel was also validated by observing acid secretion from gastric organoids treated with histamine (Supplementary Fig. 11c). However, the portion of parietal cells is much less in SEM organoids than in gastric gland tissue, and thus additional improvements would be required to further enrich parietal cells critical for the functionality of gastric organoids.

Likewise, intestinal crypts seeded in IEM hydrogel at the optimized concentration (2 mg ml−1) developed into intestinal organoids with similar shape and size as observed in Matrigel (Supplementary Fig. 9b). The variation of size distribution was lower in IEM organoids than in Matrigel organoids (CV: 35.1% in Matrigel organoids compared to Matrigel (Supplementary Fig. 9f). The variation of size distribution was lower in IEM organoids than in Matrigel organoids (CV: 35.1% in Matrigel organoids compared to Matrigel (Supplementary Fig. 9f). The variation of size distribution was lower in IEM organoids than in Matrigel organoids (CV: 35.1% in Matrigel organoids compared to Matrigel (Supplementary Fig. 9f). The variation of size distribution was lower in IEM organoids than in Matrigel organoids (CV: 35.1% in Matrigel organoids compared to Matrigel (Supplementary Fig. 9f). The variation of size distribution was lower in IEM organoids than in Matrigel organoids (CV: 35.1% in Matrigel organoids compared to Matrigel (Supplementary Fig. 9f). The variation of size distribution was lower in IEM organoids than in Matrigel organoids (CV: 35.1% in Matrigel organoids compared to Matrigel (Supplementary Fig. 9f). The variation of size distribution was lower in IEM organoids than in Matrigel organoids (CV: 35.1% in Matrigel organoids compared to Matrigel (Supplementary Fig. 9f).
mucus secretion into the lumen of GI organoids cultured in SEM and IEM hydrogels (Fig. 3e, k), but the mucus layer covering the organoids was not observed due to an insufficient number of goblet cells. Previous studies reported several methods to generate a mucus layer in GI organoids by promoting differentiation of goblet cells through depriving culture medium of Wnt3A and R-spondin1 proteins or using culture devices such as microfluidic chip33,34. Thus, a combination of GI tissue-derived ECM hydrogels with additional strategies of adjusting signaling factors or culture conditions would be required to produce functional GI organoids covered by a mucus layer.

To check the differential effect of subepithelial ECM and mucus ECM on organoid development, we prepared ECM hydrogels derived from decellularized gastric mucosa and intestinal mucosa, and compared mechanical properties and organoid formation capacity of decellularized GI mucosa-derived ECM hydrogels with those of ECM hydrogels prepared from decellularized whole stomach and intestine containing subepithelial ECM. When prepared at the same concentrations (5 mg ml⁻¹ SEM and 2 mg ml⁻¹ IEM), the elastic modulus of SEM and IEM derived from decellularized mucosal layers was significantly lower than that of SEM and IEM from decellularized whole tissues (Supplementary Fig. 12a–d). Due to poor physical and mechanical properties, mucosa-derived SEM and IEM did not support GI organoid culture. As they could not sustain the developing GI organoids, the organoids often grew attached to the bottom of the culture plate and underwent morphological deformation (Supplementary Fig. 12e, g). In particular, intestinal organoids did not retain their spherical shapes in the mucosa-derived IEM hydrogel. Consequently, the GI organoid formation efficiency was significantly lower in the mucosa-derived SEM and IEM hydrogels than in the whole tissue-derived SEM and IEM hydrogels (Supplementary Fig. 12f, h). These data demonstrate that ECM hydrogels prepared with whole GI tissues can serve as more effective culture matrices likely due to more abundant subepithelial ECM components than ECM hydrogels derived from other parts of GI tissues without subepithelial ECM.

Transcriptome profiles of GI organoids generated in GI tissue-derived ECM hydrogels. To characterize the GI organoids produced in ECM hydrogels at a transcriptomic level, we performed an RNA-sequencing analysis of GI organoids grown in GI tissue-derived ECM hydrogels in comparison to GI organoids grown in conventional Matrigel and to native GI tissues. Many differentially expressed genes (DEGs) were identified between the organoids grown in SEM and IEM hydrogels and those grown in Matrigel (Fig. 4a). Using a false-discovery rate (FDR) cutoff of <0.1 and a fold-change > 2, we identified 590 DEGs (312 upregulated genes and 278 downregulated genes) and 270 DEGs (213 upregulated genes and 57 downregulated genes) in SEM hydrogel-cultured gastric organoids and IEM hydrogel-cultured intestinal organoids, respectively. Gene ontology (GO) terms related to ECM and cell proliferation were significantly upregulated in both gastric and intestinal organoids grown in each tissue-specific hydrogel (SEM or IEM) (Fig. 4b). In comparison to Matrigel organoids, genes related to positive regulation of the developmental process, hormone activity, and calcium ion binding were also upregulated in SEM organoids, and genes related to response to wounding and cytokine activity were upregulated in IEM organoids. When the selection criterion was strengthened to genes that were upregulated at least fourfold in GI tissue-derived hydrogel groups compared to Matrigel group, most of the selected genes were involved in the extracellular region, the ECM, or the regulation of cell proliferation (Fig. 4c, d and Supplementary Fig. 13). For the GO categories related to the extracellular environment (e.g., extracellular structure organization and ECM binding) and cell–matrix interaction (e.g., cell–matrix adhesion), we compared the gene expression of GI organoids cultured in SEM or IEM with that of native GI tissues (Fig. 4e). Although many genes were downregulated in organoids compared to native tissues, the number and level of upregulated genes were much higher in organoids cultured in SEM or IEM hydrogels than in those grown in Matrigel. As the expression of ECM-related genes plays an important role in ECM remodeling and organization, which are critical for cell differentiation and organ regeneration35,36, our results suggest that GI tissue-specific hydrogels provide added benefit to GI organoid development compared to Matrigel. Based on the RNA sequencing data, we compared the expression of enteroendocrine cell subtype markers in intestinal organoids cultured in IEM hydrogel and Matrigel (Supplementary Fig. 14). Enteroendocrine cells regulate intestinal activities by producing hormones, and their subtypes are classified according to their hormone products: I cells (Gcg, Pyy), I cells (Gch), K cells (Gip), N cells (Nts), S cells (Sct), EC cells (Tph1), X cells (Ghr), G cells (Gast), and D cells (Sta)37,38. The expression levels of enteroendocrine cell subtype markers were overall similar between IEM organoids and Matrigel organoids, except for Gip and Gast with higher expression in IEM organoids and Ghr with higher expression in Matrigel organoids. These results may verify that IEM hydrogel could generate intestinal organoids containing enteroendocrine cells responsible for hormone production, which would be suitable for preclinical drug screening and translational applications.

Given that GI tissue-derived ECM hydrogels are collagen-rich matrices and collagen culture condition is known to activate Yes-associated protein (YAP) signaling39, we examined YAP signaling in the organoids cultured in IEM hydrogel and Matrigel. Immunocytochemical staining to show YAP expression and distribution in the intestinal organoids and quantification of the nuclear/cytoplasmic ratio of YAP signals revealed that YAP nuclear/cytoplasmic ratio was higher in the intestinal organoids cultured in IEM hydrogel than in the organoids cultured in Matrigel (Supplementary Fig. 15a, b), indicating YAP dephosphorylation and activation under IEM condition. RNA sequencing analysis showed that the significantly higher expression of several YAP target genes, such as Ctgf, Ankr11, Ereg, Cym61, Anxa1, and Ly6a, was observed in the IEM organoids than in the Matrigel organoids (Supplementary Fig. 15c, d). Organoid culture in 3D collagen type I hydrogel was previously reported to induce YAP/TAZ activation in intestinal organoids and conversion of cell fate to fetal-like states with regenerative capacity39,40. Yui et al. demonstrated that Wnt/collagen-based culture condition allows intestinal organoids to have genetic profiles similar to those observed during tissue regeneration. Therefore, our data from YAP analysis may suggest that IEM hydrogel comprising abundant collagens is more effective for YAP signaling activation than Matrigel and confers regenerative epithelium-like properties to intestinal organoids.

Importantly, GI organoids cultured in SEM and IEM hydrogels exhibited expression of several important genes related to GI epithelial homeostasis that more closely resembled the expression profiles of the native tissues than did organoids are grown in Matrigel (Fig. 4f, g). The expression of specific genes encoding core matrisome proteins (Pxdn and Nid1) was similar or slightly lower in gastric organoids grown in SEM hydrogel compared to the native stomach (Fig. 4f). In SEM organoids, the stomach epithelial stem cell marker Msi1, the acid-secreting parietal cell marker Dbn1, and the enteroendocrine cell marker Chgb were expressed at levels similar to the native stomach tissues. Neuregulin-1 (Nrg1) is known to promote stem cell proliferation and epithelial regeneration41 and was expressed in SEM
organoids at levels as high as those in native stomach tissues. Both SEM organoids and native stomach tissues expressed Nrg1 at significantly higher levels than Matrigel organoids. The expression of genes involved in hormone activity, such as the gastric hormone gene Pyy and the ghrelin signaling pathway-related gene Cpt1c, was also similar in SEM organoids and native stomach tissues. In intestinal organoids grown in IEM hydrogel, we found that the expression levels of core matrisome protein-encoding genes (Col4a2, Nid1, and Lama3), cytoskeleton-related genes (Flna, Gsn, and Tuba1a), and an intestinal epithelial gene involved in thiamin uptake (Tm4sf4) were comparable to those in native intestinal tissues (Fig. 4g). Compared with Matrigel organoids, IEM organoids showed significantly increased expression of genes related to wound healing, inflammation, and immune response (Procr, Mept2, Icam1, Cxcl10, Cxcl16, and Timp3), which were expressed at levels similar to those of native intestinal tissues. The expression of these genes at levels similar to the tissue may help to maintain intestinal barrier homeostasis under physiological conditions. Therefore, organoid culture using IEM hydrogel may preserve the intrinsic ability of intestinal organoids to cope with injury and to maintain tissue homeostasis. Overall, our results demonstrate that GI tissue-derived ECM hydrogels contribute to producing more mature and functional GI organoids in comparison to conventional Matrigel and enable
the generation of GI organoids which recapitulate the cellular compositions and functional properties of adult GI tissues.

Tissue-specific and age-related effects of GI tissue-derived ECM hydrogel on GI organoid development. To test the hypothesis that stomach- or intestine-specific ECM cues present in SEM or IEM hydrogels provide favorable in vivo-like microenvironments for GI organoid development, we explored the tissue-specific effects of ECM hydrogels in organoid culture. Decellularized tissue-derived ECM hydrogels were prepared from six tissue types (stomach, intestine, skin, lymph, heart, and muscle) and were tested as culture matrices for GI organoids (Fig. 5a–f and Supplementary Fig. 16). Live/Dead staining indicated that the viability of GI organoids was relatively lower in non-GI tissue ECM hydrogels (i.e., skin, lymph, heart, and muscle) than in GI ECM hydrogels (SEM and IEM) (Supplementary Fig. 16). Most cells were highly viable in GI organoids cultured in SEM and IEM hydrogels. In contrast, a larger number of dead cells was observed in GI organoids cultured in ECM hydrogels derived from decellularized skin, lymph, heart, and muscle tissues. This result clearly indicates that GI tissue-derived ECM hydrogels can provide the most favorable microenvironments for the viability and growth of GI organoids. Interestingly, gastric and intestinal organoids developed well in IEM and SEM hydrogels, respectively. The size and morphology of organoids and organoid formation efficiency were not substantially different even when they were cultured in their counterpart ECM hydrogels (Fig. 5a, b, d, e, and Supplementary Fig. 16). Combined with our observation that many important matrisome proteins were commonly present in both SEM and IEM hydrogels (Fig. 2c–e, h and Supplementary Fig. 4c), these results suggest that SEM and IEM provide quite equivalent ECM environments for GI organoids. In contrast, other types of tissue-derived ECM hydrogels (skin, lymph, heart, and muscle) did not support the development of GI organoids, and the structure of the formed organoids was immature (Supplementary Fig. 16). For example, GI organoids cultured in decellularized skin-derived ECM (SKEM) hydrogel exhibited irregular and disorganized morphology, and the generated organoids were much smaller in size and less proliferative than those grown in GI ECM hydrogels (Fig. 5a, d). Additionally, the organoid formation efficiency in SKEM hydrogels was lower than in SEM or IEM hydrogels (Fig. 5b, e), and the expression of a stemness marker was significantly lower in SKEM hydrogel organoids (Fig. 5c, f). The relative proteomic quantitation between SKEM and GI-tissue matrisomes (Fig. 5g, h) clearly demonstrated the tissue-specific effects of GI ECM hydrogels on organoid development. The quantity of the 56–59 core matrisome proteins that are critical for GI development and function was much greater in SEM and IEM hydrogels than in SKEM hydrogels. For example, the level of collagen type VI (COL6A3, COL6A5, and COL6A6) was higher in GI tissue-derived ECM than in SEM. Collagen type VI is a major component of the mechanical microenvironment of GI tissue that regulates epithelial-cell-fibroblast assembly. Although some glycoproteins, such as fibronectin (FN1) and laminin (LAMAS1, LAMB1, LAMB2, and LAMC1), were detected in SKEM, the amounts were significantly lower in SKEM hydrogel compared to native GI tissue. DEGs with 2-, 4-, and 8-fold changes are indicated by the color gradient intensity (p < 0.05, FDR < 0.1). f, g Comparison of expression values (Log2 [FPKM + 1]; FPKM, fragments per kilobase of transcript per million mapped reads) of selected genes involved in gastric or intestinal development and homeostasis in native GI tissues and in GI organoids cultured in GI tissue-derived ECM hydrogels or Matrigel (MAT versus Tissue, ****p = 0.0002 for Nid1, ****p = 0.0005 for Pdx1, ****p = 0.0097 for Ms1, ****p = 0.0001 for Dbn1, ****p = 0.0019 for Cbgb, ****p = 0.0147 for Ngl1, ****p = 0.0404 for Ppy, ****p = 0.0028 for Cpttc, SEM versus Tissue, ****p = 0.0116 for Nid1, ****p = 0.0132 for Dbn1, ****p = 0.0274 for Cbgb; N = 3) and (MAT versus Tissue, ****p < 0.0001 for Col4a2, ****p = 0.0003 for Nid1, ****p = 0.0041 for Lama3, ****p = 0.0021 for Fina, ****p = 0.0009 for Gm, ****p = 0.0002 for Tubala, ****p = 0.0006 for Tms4s4, ****p = 0.0001 for Proc, ****p = 0.0002 for Mctp2, ****p = 0.0004 for Icam1, ****p = 0.0003 for Cxcl16, ****p = 0.0009 for Timp3, IEM versus Tissue, ****p = 0.0356 for Col4a2, ****p = 0.0177 for Nid1, ****p = 0.0152 for Tubala, ****p = 0.0067 for Proc, ****p = 0.0176 for Cxcl16; N = 3). Non-significant statistical difference was indicated as n.s. (p > 0.05). The data in (f, g) are presented as mean ± S.D. Statistical significance was analyzed using one-way ANOVA with Tukey’s multiple comparisons test (f, g).
responsibility for the better ability of piglet IEM to support intestinal organoid culture. We investigated whether the addition of FN and TNC into the adult-pig IEM hydrogel improves the quality of intestinal organoids. Interestingly, the addition of FN alone into adult-pig IEM hydrogel enhanced the expression of stem cell markers (Lgr5 and Axin2) in intestinal organoids, whereas the addition of TNC alone or both FN and TNC did not increase stem cell marker expression (Supplementary Fig. 17). These results demonstrate that FN may be a more crucial factor involved in the improved ability of piglet ECM for supporting organoid culture. Here, we tested only two proteins of piglet ECM and limited concentrations (25 and 50 μg ml⁻¹), but future
Fig. 5 Tissue-specific and age-related effects of GI tissue-derived ECM hydrogels in GI organoid culture. a Brightfield images and b formation efficiency of gastric organoids cultured in decellularized tissue-derived ECM hydrogels from stomach, intestine, and skin tissues relative to gastric organoids cultured in Matrigel (MAT) at day 5 (N = 4, scale bar = 100 μm). c Comparison of mRNA expression (Lgr5) in gastric organoids in each hydrogel (SEM versus SkEM, *p = 0.0298; IEM versus SkEM, **p = 0.0045; MAT versus SkEM, *p = 0.0129; N = 4, independent experiments = 2). d Brightfield images and e formation efficiency of intestinal organoids cultured in decellularized tissue-derived ECM hydrogels from stomach, intestine, and skin tissues relative to intestinal organoids cultured in MAT at day 6 (N = 4, scale bar = 100 μm). f Comparison of mRNA expression (Lgr5) in intestinal organoids in each hydrogel (SEM versus SkEM, **p < 0.0001; IEM versus SkEM, ***p = 0.0001; MAT versus SkEM, *p = 0.0219; N = 4, independent experiments = 3). Proteomic analysis of the relative quantity of matrisome proteins contained in g SEM and h IEM compared to SkEM. Comparison of I the formation efficiency (N = 6) and j gastric marker gene expression of organoids cultured in SEM hydrogels derived from two-month-old piglets (Piglet) and 6-month-old adult pigs (Adult pig) [SEM (Adult pig) versus IEM (Adult pig) versus IEM (Piglet), **p = 0.0076 for Lgr5, *p = 0.0013 for Axin2, *p = 0.0304 for Pgc, *p = 0.0345 for Atf4α, *p = 0.0299 for Atf4β; N = 5]. Comparison of k the formation efficiency (N = 6) and I intestinal marker gene expression of intestinal organoids cultured in IEM hydrogels derived from 2-month-old piglets (Piglet) and 6-month-old adult pigs (Adult pig) [IEM (Adult pig) versus IEM (Piglet), **p = 0.0029 for Lgr5, *p = 0.0282 for Olfm4; N = 4]. Proteomic analysis of the relative quantity of matrisome proteins contained in m SEM and n IEM derived from 2-month-old piglets and 6-month-old adult pigs. Non-significant statistical difference was indicated as n.s. (p > 0.05). The data in b, c, e, f, j are presented as mean ± S.D. Statistical significance was analyzed using one-way ANOVA with Tukey’s multiple comparisons test (c, f) and unpaired, two-sided student’s t-test (g–n).

As mean ± S.D. Statistical signification are presented using one-way ANOVA with Tukey’s multiple comparisons test (c, f) and unpaired, two-sided student’s t-test (g–n).

studies to systematically identify more potent ECM molecules and optimal concentrations would be able to facilitate the development of chemically defined hydrogels for GI organoid culture.

The practicality of GI tissue ECM hydrogels for organoid culture and transplantation. So far, we have shown that GI tissue-derived ECM hydrogels have highly desirable features. Next, we sought to determine the practical applicability of these hydrogels. First, we evaluated the long-term expansion and passage of GI organoids in SEM and IEM hydrogels compared to those in Matrigel. The subculture of GI organoids in SEM and IEM hydrogels enabled stable growth and expansion of organoids persistent up to more than passages 6–8 (Fig. 6a, c). Overall, we did not observe any difference in the turnover rate and splitting ratio of passage between the organoids grown in ECM hydrogels and Matrigel. qPCR analysis of stemness markers (Lgr5, Axin2, and Olfm4) indicated that GI organoids maintained self-renewal ability after long-term expansion (days 33–45) in GI tissue-derived ECM hydrogels at comparable levels to organoids subcultured in Matrigel (Fig. 6b, d). The colon organoids-derived mouse large intestine also grew well in IEM hydrogel as similar to Matrigel. We assume that IEM could be widely used for culturing other types of GI tissue organoids with similarity to small intestinal organoids (e.g., colon organoids) (Supplementary Fig. 18a). SEM and IEM hydrogels not only supported the growth of primary tissue-derived GI organoids but also supported the growth of human pluripotent stem cell (hPSC)-derived GI organoids or GI tumoroids (Supplementary Fig. 18b–g). In Live/Dead staining to check cell viability of hPSC-derived intestinal organoids cultured in IEM hydrogel and Matrigel, the organoids grown in both matrices showed no significant difference in Live+ and Dead− areas with more than 80% viability (Supplementary Fig. 18c), indicating that the IEM hydrogel did not provoke significant impairment in the viability of human intestinal organoids. Immunocytochemical staining of human-induced pluripotent stem cell (hiPSC)-derived intestinal organoids at days 29 and 51 indicated development of human organoids expressing intestinal stem cell marker SOX9 and differentiation markers MUC2 and CHGA in IEM hydrogel (Supplementary Fig. 18d, e). These results prove their versatile utility for culturing diverse types of organoids. In the future, the applicability of ECM hydrogels needs to be fully validated for the long-term culture and development of human GI organoids.

The long-term storage potential of reconstituted ECM is important for the convenience and commercialization of hydrogel matrices. We, therefore, checked the long-term stability and bioactivity of ECM hydrogels after refrigeration or freezing. We tested SEM and IEM pre-gel solutions stored in a deep-freezer (−80 °C for 6 months) or a refrigerator (4 °C for 1 month) for GI organoid culture (Fig. 6e, h). The organoid formation efficiency in SEM and IEM hydrogels that had been frozen for 1–6 months or refrigerated for 1–4 weeks was not different from that of freshly prepared hydrogels (Fig. 6f, i). The morphology and size of gastric and intestinal organoids grown in stored SEM and IEM hydrogels were similar to those grown in fresh hydrogels (Fig. 6g, j). Finally, gene expression levels of self-renewal and differentiation markers in GI organoids were not significantly different in long-term stored ECM hydrogels and fresh ECM hydrogels, which were similar to or higher than those in Matrigel organoids (Supplementary Fig. 19).

More interestingly, ECM hydrogels could be cryopreserved together with organoids, enabling ready-to-use applications. When organoids frozen in IEM hydrogel or Matrigel were thawed, the organoids frozen in IEM hydrogel were highly viable and grew normally, but those frozen in Matrigel exhibited significantly lower viability (Supplementary Fig. 20a, b). We then quantified the live- or dead-staining areas of organoids and confirmed that GI tissue-derived ECM hydrogel protected organoids from cellular damage caused by cryopreservation better than Matrigel (Supplementary Fig. 20c). After thawing, IEM organoids preserved their original morphology before cryopreservation, whereas Matrigel organoids exhibited an unhealthy morphology (Supplementary Fig. 20d). Likewise, the expression level of the apoptotic marker Caspase-3 in intestinal organoids after thawing significantly increased in only Matrigel organoids (Supplementary Fig. 20e). Immunocytochemical staining 2 days after thawing intestinal organoid-laden IEM hydrogel or Matrigel indicated that the percentage of active (cleaved) caspase-3+ area in the whole organoid was much higher (4.8-fold) in Matrigel organoids (13.67%) than in IEM organoids (2.85%) (Supplementary Fig. 20f, g). These data suggest again that IEM hydrogel is more effective than Matrigel to reduce apoptotic signaling resulting from caspase-3 activation during freeze-thaw cycles. Previous studies demonstrated that supplementation of ECM proteins such as collagen peptides32 and hyaluronan53,54 to the freezing medium or entrapment of cells with collagen scaffolds35,36 could reduce damage and stress during the cryopreservation process. Thus, the protective effects of GI tissue-derived ECM hydrogels during cryopreservation of the organoids observed in our study may be owing to the presence of various matrisome proteins abundant in GI tissue ECM hydrogels that Matrigel does not contain (e.g., collagen subtypes, and hyaluronan). In addition to excellent storage potential, ECM hydrogels may be compatible with a wide variety of culture
systems. For example, we successfully integrated GI tissue-derived ECM hydrogels with microfluidic devices stacked on a single rocker for high-throughput dynamic culture (Supplementary Fig. 21). We anticipate that ECM-integrated microfluidic platforms will allow for mass production of GI organoids for drug screening, toxicological evaluation, and disease modeling.

Finally, we tested the feasibility of GI tissue-derived ECM hydrogels for in vivo applications. Matrigel and cell therapeutics produced using Matrigel cannot be used in human clinical applications due to safety issues arising from the tumor-derived origin of Matrigel (11,58). In contrast, ECM hydrogels derived from decellularized tissues with removal of cells containing antigenic genetic motifs can be used as safe scaffolds for clinical cell therapy with a low risk of immunological rejection and inflammatory responses (59,60).

To test whether ECM hydrogels aid efficient engraftment of transplanted GI organoids, we injected gastric or intestinal organoids encapsulated in their respective tissue-derived ECM hydrogels into the stomach or small intestine with...
acute epithelial injury using acetic acid. We first verified that the treatment of acetic acid causes epithelial damage and inflammatory reactions in the stomach and intestine tissues. H&E staining clearly showed the damaged regions in the gastrointestinal tissue treated with acetic acid and the collapse of the tissue architecture and polarization of gastric glands, which are indicative of gastric ulcer (Supplementary Fig. 22a). Likewise, the structures of villi and crypt were severely impaired in the intestinal tissue treated with acetic acid (Supplementary Fig. 22b). Tissue morphologies of mouse stomach and intestine injury models induced by acetic acid treatment were completely distinct from those of intact stomach and intestine tissues. In immunofluorescence staining for immune cell marker, F4/80+ macrophages were rarely observed in normal gastric and intestinal tissues, while F4/80+ macrophage infiltration was clearly observed in the stomach and intestine tissues of the injured models (Supplementary Fig. 22c, d). In particular, massive infiltration of F4/80+ macrophages was detected in the injured intestine tissue (Supplementary Fig. 22d). Next, we transplanted only SEM and IEM hydrogels to confirm negligible inflammatory responses following administration of SEM and IEM hydrogels. When SEM hydrogel labeled with a fluorescent dye (TAMRA-SE) was injected into the submucosal region of mouse stomach tissue, the hydrogel decomposed rapidly, and the fluorescence signals of the SEM hydrogel decreased after administration (Supplementary Fig. 23a). Infiltrated macrophages were not observed in the injection sites 1 and 4 days after SEM hydrogel injection. In the case of IEM hydrogel, the fluorescence signals of the IEM hydrogel distributed widely over the villi structures 4 h after injection into the intestinal lumen (Supplementary Fig. 23b). Similar to SEM hydrogel, macrophage infiltration was barely observed in the injected sites 1 and 4 days (Supplementary Fig. 23b). Taken together, these data demonstrate that SEM and IEM hydrogels can serve as biocompatible organoid carriers for transplantation.

For organoid instillation, GI tissue-derived hydrogels were diluted in culture medium (1:20) as described previously. We utilized fluorescently labeled GI ECM hydrogels and organoids to show specific replacement of damaged tissues by transplanted GI organoids. To trace organoid growth in vivo, we used enhanced green fluorescent protein (EGFP)+ organoids derived from EGFP transgenic mice (Fig. 6k and Supplementary Fig. 24a, b). Injection of gastric organoids using SEM-labeled SEM hydrogel allowed efficient administration of organoids into the submucosal region of the stomach with ulcers (day 0, Fig. 6f). We found that 4-, 7-, and 28-days post-transplantation EGFP+ gastric organoids were successfully engrafted into host tissues and EGFP signals of organoids were specifically detected throughout the injured sites (Fig. 6m and Supplementary Fig. 24c). Likewise, injected EGFP+ intestinal organoids with TAMRA-labeled IEM hydrogel were also engrafted into the injured intestinal tissues 4 and 7 days after transplantation (Fig. 6m and Supplementary Fig. 24d). Since diluted ECM hydrogels were applied for organoid transplantation, TAMRA fluorescent signals of IEM and SEM disappeared probably due to the rapid degradation in vivo. These data suggest that SEM and IEM hydrogels could mediate efficient engraftment of GI organoids into the injured regions for epithelium reconstruction and support the use of tissue ECM hydrogels as an effective organoid carrier for GI tissue regeneration. A long-term transplantation study would be required to check-in vivo behaviors and regenerative efficacy of transplanted GI organoids in injured GI tissues.

Discussion
In this study, we demonstrate GI tissue-mimetic matrices that can replace the use of conventional Matrigel, which is currently indispensable for GI organoid culture. Despite the effectiveness of Matrigel in culturing a wide variety of organoids, it has several critical limitations, including a lack of tissue-specific biochemical ECM factors, safety issues, and batch variation, which have prompted the development of alternative matrices for organoid culture. For example, several studies have reported synthetic or natural hydrogels (e.g., PEG, alginate, and fibrin gel) modified with ECM peptide motives (e.g., RGD) and protease-degradable peptides (e.g., GPGGWGQ) for culturing intestinal organoids. These artificial matrices are reconstituted with only a few ECM components and are not capable of mimicking the intrinsic and complex matrisome of native GI tissues. ECM hydrogels derived from small intestine tissues have been used to cultivate endodermal organoids, such as gastric, hepatic, pancreatic, and small intestinal organoids. The decellularization protocols used to produce these ECM hydrogels have historically used SDC to induce efficient removal of cellular components; however, ionic detergents may cause damage to the tissue ECM and impair the bioactivity and properties of ECM hydrogels, as observed in our analysis (Supplementary Fig. 1). Previously, we tested IEM hydrogels supplemented with three ECM proteins (laminin-111, laminin-511, and nephroectin) in various concentrations and combinations to investigate the effects of those ECM proteins on the formation and development of intestinal organoids. Because IEM hydrogel alone did not support the formation and growth of intestinal organoids due to unoptimized decellularization protocol and culture condition, our previous study did not prove the ability of IEM hydrogel comparable to Matrigel for cultivating intestinal organoids. To overcome the limited roles of existing synthetic and natural hydrogels in organoid culture, we provide evidence to support the use of ECM...
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Fig. 4c. In most mature tissues, however, the expression of matrisome proteins in Matrigel were made up of glycoproteins and lacks GI tissue-specific ECM components. More than 96% of the total matrisome proteins in Matrigel were distributed across native GI tissues and laminin-111 ranked in the top ten most abundant matrisome proteins (Fig. 2b and Supplementary Fig. 4c). In most mature tissues, however, the expression of laminin-111 is not as prominent as it is in Matrigel and different laminin isoforms, such as laminin-332 and laminin-511, are distributed across native GI tissues and in our GI tissue-derived ECM hydrogels (Supplementary Fig. 4c). Although collagen is the most abundant ECM proteins in native tissues, the total collagen content in the Matrigel matrisome is only 0.4% with only a few subtypes (~10 subtypes). In contrast, SEM and IEM retained a significantly greater amount of collagen (about 67% and 51% of total matrisome proteins in SEM and IEM, respectively) and a larger number of subtypes (19–20 subtypes) (Fig. 2c–e, 2h and Supplementary Fig. 4c), which more closely recapitulates the ECM environment of native GI tissues. When we examined proteins that are specifically enriched in the native stomach or intestine based on the Human Protein Atlas, we detected the largest number in SEM and IEM, respectively (Supplementary Figs. 4d and 6). Interestingly, we found that the majority of native GI tissue-enriched non-matrisome proteins detected in SEM and IEM were listed in the top 10 GOBP categories for non-matrisome proteins in SEM and IEM (Supplementary Fig. 5). For example, OLFM4, APOA4, and EPS8L2 are involved in the GOBP term “cellular component biogenesis,” and SMTN, PL51, and VIL1 belong to the GOBP term “organelle and cytoskeleton organization.” Based on the presence of many non-matrisome proteins that are abundant in native GI tissues, SEM and IEM hydrogels may better support cellular component biogenesis and structure development during organoid development in comparison to Matrigel. Collectively, the orchestration of GI tissue-specific core matrisome and non-matrisome proteins enables the reconstruction of native GI tissue-mimetic microenvironments to support the growth and development of GI organoids.

In this study, we demonstrate that tissue origin and donor age are critical factors to consider in the design of decellularized tissue-derived matrices for organoid culture. Additional proteomic analysis indicated that several core matrisome proteins were highly enriched in SEM and IEM in comparison to SKEM (Fig. 5g, h). These matrisome proteins, such as collagen VI, fibronectin, and laminin, are known to regulate epithelial cell behaviors, including survival and differentiation. Accordingly, SEM and IEM were more effective in the development of GI organoids than SKEM (Fig. 5a–f), further emphasizing the importance of matching organoid type to the tissue origin of decellularized matrices. Moreover, proteomic analysis of ECM hydrogels derived from the same types of tissues at different ages revealed that a variety of matrisome proteins were detected differentially in decellularized tissue ECM from piglets and adult pigs despite only a 4-month difference in age (Fig. 5m, n). Specifically, we identified fibrillin 2 and tenascin C as major matrisome components in piglet SEM and IEM, respectively, and fibronecin, a component of the GI stem cell niche, was highly expressed in both piglet SEM and IEM compared to hydrogels derived from adult pigs. Recent studies reported that enrichment of fibrillin 2 and tenascin C in neonatal lung ECM promotes tissue remodeling, and decrease of fibronectin in the aged muscle stem cell niche reduces muscle regeneration ability. Consistent with these studies, we found that age-dependent differences in tissue matrisome components affected the self-renewal ability and growth potential of GI organoids, which was indicated by increased expression of stemness markers (Lgr5, Axin2, and Olfm4) in GI organoids cultured in piglet ECM hydrogels enriched with these factors (Fig. 5j, l). Our results provide important insight into ECM remodeling during aging and suggest that age should be considered in the preparation of effective decellularized matrices for organoid development and the rejuvenation of GI epithelium.

Overall, our proteomic analytical data provide biological insight into the development of chemically-defined hydrogels for organoid culture by identifying critical matrisome elements that positively influence organoid formation and development. Hydrogels inspired by tissue-specific ECM peptide motifs not only provide 3D native tissue-mimetic matrisome complexity for organoid maturation and improved function, but also solve several inherent limitations of naturally derived materials, including variation and mass production challenges. Decorin, biglycan, and lumican were commonly present in the top ten most abundant matrisome proteins of all batches of SEM or IEM samples (Fig. 2c–e, h), which are known to play important roles in collagen fibril formation, cell proliferation, maturation, and epithelium homeostasis. Therefore, synthetic hydrogels modified with these matrisome proteins may be optimal matrices for GI organoid culture and will be explored in future work. Through in-depth proteomic analysis, we also identified several matrisome proteins that have been known to be highly upregulated in piglet ECM in comparison to adult pig ECM (e.g., FN and TNC), which can be used as the ECM components for the functionalization of chemically defined hydrogels. Actually, we found that addition of FN into adult pig GI tissue-derived ECM hydrogel could significantly improve the quality of organoids, which is similar level to those grown in piglet GI tissue-derived ECM hydrogel (Supplementary Fig. 17). This result indicates that FN may be a crucial factor involved in the ability of piglet ECM to better support organoid culture. Thus, supplementation of those crucial ECM components into synthetic polymer hydrogels such as PEG and alginate can be considered. Future studies to systematically identify more potent candidate ECM molecules and optimal ECM concentrations would be able to facilitate the development of non-xenogeneic, chemically defined hydrogels for GI organoid culture. Given a recent trend to reduce animal use in scientific research, this kind of study would be a highly valuable project.

Finally, we demonstrated the versatility and practicality of GI tissue-derived ECM hydrogels for organoid culture and transplantation. SEM and IEM hydrogels allowed for the long-term subculture of GI organoids (Fig. 6a–d) and supported long-term expansion to produce a large number of organoids, which is necessary for disease modeling and regenerative medicine. We found that intestinal organoids cultured in IEM hydrogel exhibited less budding compared to Matrigel organoids. Since small intestinal organoids grown in 3D collagen matrix were previously reported to show morphology with fewer budding, IEM hydrogel with highly enriched collagen components was expected to induce such morphology of intestinal organoids as observed in previous studies. Although intestinal organoids grown in IEM hydrogel have an architecture with smaller buds, the gene expression levels of stem cell markers and intestinal differentiation markers are comparable to those of Matrigel organoids. In addition, after several passages, we observed that the number of intestinal organoids with budding structures increased...
in IEM hydrogel (Fig. 6c). The functionality of ECM hydrogels as organoid culture matrices was retained even after long-term storage at either −80 °C (6 months in a deep-freezer) or 4 °C (1 month in a refrigerator) (Fig. 6e–j), allowing for multiple uses of pre-gel ECM solution. More interestingly, organoids could be cryopreserved within the ECM hydrogel and thawed without loss of ECM bioactivity or organoid viability (Supplementary Fig. 20), substantially increasing the off-the-shelf availability of organoid culture platforms. Furthermore, SEM and IEM hydrogels facilitated the engraftment of transplanted organoids in injured GI tissues (Fig. 6k–m and Supplementary Fig. 24c, d). Several genes involved in wound healing were upregulated in GI ECM organoids in comparison to Matrigel organoids (Fig. 4g), suggesting that organoid transplantation using GI tissue-derived ECM hydrogels has the potential to provide highly effective therapeutic strategies to address incurable GI diseases, such as inflammatory bowel disease, Crohn’s disease, and gastric ulcers as well as tissue defects following tumor resection. Our strategy to provide tissue-specific ECM hydrogels can be extended into other types of tissue organoids with limitations in Matrigel culture (e.g., liver organoids).

In summary, we demonstrate the potential of IEM and SEM hydrogels as alternatives to Matrigel for GI organoid culture. Our strategy using animal tissue-derived ECM hydrogels did not fully address the translational limitation of Matrigel due to its animal origin. Although both Matrigel and decellularized animal tissue-derived materials are prepared from xenogeneic sources, unlike Matrigel of mouse tumor origin, decellularized matrix derived from non-tumor tissues can be customized to the clinical setting through a well-controlled decellularization process. In this study, we provide experimental evidence on how our approach with decellularized GI tissue-derived ECM hydrogels can resolve the major issues of Matrigel including (1) batch-to-batch variation, (2) safety, and (3) high-cost issues. Our proteomic analysis revealed that decellularized GI tissue-derived ECMs have a relatively low batch-to-batch variation and donor variation if porcine donor conditions (e.g., age and gender) are controlled for sourcing GI tissues (Fig. 2e–n and Supplementary Fig. 6). We also confirmed that our ECM hydrogels show clinically acceptable levels of endotoxin and marginal immunogenicity both in vitro and in vivo due to the absence of xenogeneic pathogens and antigens of cellular components (Supplementary Fig. 3). Cost estimation from our calculation considering the price of porcine GI tissue indicates that decellularized GI tissue-derived ECM hydrogels are much more cost-effective for GI organoid culture than Matrigel (Supplementary Table 1). Given that decellularized animal tissues or their derivatives have been approved and commercialized for clinical applications to humans, decellularized tissue-derived ECM hydrogels that can address critical limitations of Matrigel will be available for clinically relevant organoid culture. Nevertheless, GI ECM hydrogels have potential limitations in translational applications, which need to be carefully investigated and addressed as well. Intestinal organoids cultured in IEM hydrogel exhibited less budding, compared to those cultured in Matrigel, which may indicate a rather premature state of IEM organoids. Considering that IEM organoids showed higher expression of stem cell markers than Matrigel organoids, we cannot conclude that a larger portion of precursor cells still exist in IEM organoids. Despite our data on the viability of human intestinal organoids, more in-depth studies to fully check the effects of GI ECM hydrogels on human GI organoids would be inevitable in the future.

Methods

Decellularization of porcine tissues. Porcine stomach and small intestine tissues were freshly obtained from a local market (Majang Meat Market, Seoul, Korea). The experiments using porcine tissues were conducted according to ethical principles and standard guidelines of the Institutional Animal Care and Use Committee (IACUC) of Non-Causing Tissue-Injuring/Moribund Animal (Category A; use for research purposes). The fat tissues of porcine stomach and large blood vessels were removed prior to decellularization. All layers of stomach tissue were used for the study, whereas mesentery and serosa layers were removed from intestine tissue. Stomach tissue was chopped into small pieces (<0.3 × 0.3 × 0.3 cm³), and intestine tissue was cut longitudinally and then into smaller segments (10 cm). Two protocols with different chemical treatments were tested for decellularization of GI tissues; Protocol 1 is the one we optimized, and Protocol 2 is the commonly used one. For Protocol 1, both prepared tissues were agitated in buffer solutions (500 ml) in the following order: distilled water for 3 h; Triton X-100 (Wako, Osaka, Japan) with 0.1% (v/v) papain (Sigma) hydroxide (Sigma, St. Louis, MO, USA) for 4 h; distilled water for 24 h; 1% (v/v) penicillin-streptomycin (P/S; Thermo Fisher Scientific, Waltham, MA, USA) for 2 h; and distilled water for 1 h. For both tissues, every solution was removed from the tissues before the next step, and all processes were performed at 4 °C and agitated on a rotator at 180 revolutions per minute (r.p.m.). The sterilized decellularized tissues were then lyophilized and stored at 4 °C until use. Protocol 2 was carried out as previously established by agitating prepared tissues in buffer solutions (500 ml) in the following order: 4% (v/v) SDS (Sigma) for 3 h; distilled water for 24 h with 4–6 water changes; 2000 μg/DMNase-I (Sigma) in 1 M sodium chloride (Sigma) for 3 h; and distilled water for 48 h. All processes were performed at room temperature and agitated on a rotator at 180 r.p.m. The decellularized tissues from Protocol 2 were also lyophilized and stored at 4 °C until use. The mucosal tissues of the porcine stomach and small intestine were decellularized. The mucosal layer of the stomach was separated from the rest of the stomach and chopped into small pieces (<0.3 × 0.3 × 0.3 cm³). The pieces of the mucosal layer of the stomach tissue were obtained by removing the inner layer of the stomach with a slide glass. The decellularization of mucosal tissues was conducted according to Protocol 1. Decellularization of other porcine tissues (esophagus, skin, lymph, heart, and muscle) was carried out as previously described. The harvested tissues were also cut into small pieces (<0.3 × 0.3 × 0.3 cm³) and all processes for decellularization were performed at 4 °C with agitation at 180 r.p.m. If not stated otherwise: esophagus—0.5% (v/v) trypsin/ethylenediaminetetraacetic acid (EDTA, Thermo Fisher Scientific) for 2 h, 1 M sucrose (Sigma) for 30 min, 3% (v/v) Triton X-100 (Sigma) for 48 h, and 10% (v/v) SDS (Sigma) for 4 h; skin—0.25% (v/v) trypsin/EDTA/FA for 1 h at 37 °C, 70% (v/v) ethanol (Dukusen Pure Chemicals, Ansan, Korea) for 6 h, and 1% (v/v) Triton X-100 (Sigma) with 0.1% (v/v) ammonium hydroxide for 72 h; axillary lymph node—0.1% (v/v) sodium dodecyl sulfate (SDS; Wako) for 12 h; skeletal muscle—1% (v/v) Triton X-100 (Sigma) with 0.2% (v/v) SDC for 6 h; cardiac muscle—1% (v/v) SDC for 48 h. The rest of the decellularization steps include washing with distilled water, 1% (v/v) P/S treatment for 2 h, washing with distilled water, and finally decellularized tissues were lyophilized. The decellularized tissues were lyophilized using a freeze dryer (FDU-2100, Eyela, Tokyo, Japan) under the operating conditions of a vacuum gauge of 5.5 Pa and a trap temperature of −85 °C.

Characterization of decellularized tissues. To confirm the decellularization of GI tissues, DNA assays and histological analyses were performed after decellularization. DNA content was confirmed by quantifying DNA in the native and decellularized GI tissues using a DNA extraction kit (Bioneer, Daejon, Korea). The GAG content was quantified using 1,9-dimethyl methylene blue dye solution assay (Sigma) as previously reported. Both native and decellularized tissues were digested using papain (125 µg ml⁻¹) and Sigma) and diluted with distilled water. The sample solutions were treated with 1,9-dimethyl methylene blue dye, and the absorbance of the samples was measured at 525 nm using a microplate reader (Tecan, Männedorf, Switzerland). Chondroitin sulfate A (Sigma) was used as a standard serial dilution starting from a concentration of 2 µg ml⁻¹. For histological analyses, both native and decellularized GI tissues were fixed with 4% (w/v) paraformaldehyde (Sigma) for 1 day, embedded in paraffin, and then sliced into 4-µm sections for HE staining.

Protein sample preparation for MS analysis. The lyophilized tissues were reconstituted with lysis buffer (4% SDS, 0.1 M Tris-HCL (pH 7.6), and 1% protease inhibitor (Sigma)) in 500 µl and incubated at room temperature for 10 min. Subsequently, the tissues were boiled for 15 min at 95 °C on a heat block. Tissue lysis was performed on ice by sonication using a probe sonicator (Qsonica Q125, Newtown, CT, USA) and centrifuged at 14,000 g at 4 °C for 20 min. The supernatant was collected and transferred to a new tube. Protein concentration was measured using the BCA protein assay (BCA Protein Assay Kit, Thermo Fisher Scientific). Matrigel, SEM, and IEM (100 µg) protein samples were digested using Filter Aided Sample Preparation. Proteins were reduced in SDS buffer (4% SDS in 1 M Tris-HCL, pH 7.6 and 0.1 M dithiothreitol (DTT)) at 37°C for 45 min and boiled for 10 min at 95°C on a heat block. The protein samples were then sonicated for 10 min in a bath sonicator (Daiageno, Denville, NJ, USA) followed by centrifugation at 14,000 × g at 20 °C for 5 min. The supernatant was combined with 200 µl of 8 M urea (in 0.1 M Tris-HCL, pH 8.5) and transferred to a membrane filter (YM-30, Millipore Corporation, Burlington, MA, USA). The SDS was removed by centrifugation at 14,000 × g at 20 °C for 60 min, and the protein samples remained on the membrane filter. SDS removal was repeated three times. Subsequently,
cytotoxicity was performed with 100 μl of 0.05 M iodoacetamide in 8 μl media for 25 min at room temperature in the dark, followed by centrifugation at 14,000 g at 20 °C for 30 min. The pelleted cells were then resuspended in 1 ml of 0.1% Triton X-100 (Sigma, St. Louis, MO, USA). Brieﬂy, different samples were washed three times with 0.1% Triton X-100 (Sigma) using a frequency sweep mode (0.1 Hz, 1% of 50 cm × 75 mm, 30 min. The sample was desalted with a C18 spin column (Thermo Fisher Scientiﬁc) that was packed with 2 μm C18 particles at an electric potential of 1.8 kV. Full MS data were acquired in a scanning range of 300–1600 Th at a resolution of 70,000 at m/z 200 with an automated gain control (1 × 106). The intensity-based absolute quantiﬁcation was performed against the neutral loss of 32 for higher-energy collisional dissociation.

**Pepcidate and protein identiﬁcation.** Mass spectra were processed using MaxQuant (1.6.10.43)©. MS/MS spectra were queried against the target-decoy Mouse Uniprot database released on March 2020) using the MaxQuant software with a modiﬁed database against the neutral loss of 32 for higher-energy collisional dissociation. The inferences were selected from the trap column and ionized with an EASY-spray column (50 μm × 75 μm ID, Thermo Fisher Scientiﬁc) that was packed with 2 μm C18 particles at an electric potential of 1.8 kV. Liquid chromatography with tandem MS (LC-MS/MS) analysis was performed on the peptides as follows: the Q Exactive Plus Mass Spectrometer (Thermo Fisher Scientiﬁc) was used for quantiﬁcation. The intensity based absolute quantiﬁcation (iBAQ) algorithm was used as a part of the MaxQuant analysis. The iBAQ values represent the raw intensities divided by the number of theoretical peptides. Thus, iBAQ values are proportional to the molar amounts of the proteins. The iBAQ values were converted to relative iBAQ values for comparison across different samples. Label-free quantiﬁcation (LFQ) values represent normalized peptide intensities in the spectra for unmodiﬁed and ubiquitinated proteins. The Perseus software© was used to transform LFQ values into log, data, which were then calculated for fold changes, and p-values were adjusted based on FDR less than 5%. The identiﬁed proteins were compared and categorized in reference to the Human Protein Atlas© and the Matriome Project©. A gene ontology (GO) search was performed using gProfiler software (ELIXIR, Hinxton, Cambridgeshire, UK). GOBPs analyses were performed on the commonly expressed matrisome proteins in the samples from porcine A–C, the proteins that show a signiﬁcantly elevated level of expression in stomach or intestine tissue compared to any other source© and©, and the non-matrisome proteins identiﬁed in Matrigel, SEM, and IEM. PANTHER Overrepresentation Test© was selected as the statistical algorithm and GO biological process was chosen as the dataset for the GOBP analysis. Fisher's exact test with FDR correction as calculated by the Benjamini-Hochberg procedure was performed. Biological processes with FDR < 0.05 were considered as signiﬁcant.

**3D ECM hydrogel characterization.** Solubilized decellularized GI tissues were constructed into 3D hydrogels by temperature-induced self-assembly. Decellularized GI tissues were dissolved in 10 mg/ml acetic acid, 0.1 mg/ml sodium citrate, 3 mg/ml sodium ascorbate (Invitrogen), 0.5 mg/ml sodium alginate (sigma–aldrich), and 200 μM EDTA (Life Technologies) for 24 h at 4 °C. The secondary solution was added to the primary solution at a ratio of 1:1 (v/v). Mixtures were then added to a 48-well plate. The efﬁciency of organoid formation in each hydrogel was calculated as a percentage ratio of the number of formed organoids to the number of seeded glands or crypts.

**GI organoid culture.** The culture media for both gastric and intestinal organoids were made from the same basal medium, which was composed of advanced DMEM/F12 (Dulbecco's Modified Eagle Medium/Ham's F-12) supplemented with 10 mM HEPES (N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid), 2 mM Glutamax, and 100 U/ml Penicillin and Streptomycin (all from Thermo Fisher Scientiﬁc). GI organoids were cultured on Matrigel-coated 6-well plates to deﬁne the amount of organoids that could be cultured on each hydrogel. The culture media for both gastric and intestinal organoids was refreshed every 3 days. The tissue viability assay was performed using the L-Wnt-3A cell line (CRL-2647, American Type Culture Collection (ATCC), USA) and the MTT assay (Promega, Madison, WI, USA) to determine the amount of viable cells in the organoids.

**Isolation of tissue-derived gastric organoids and intestinal organoids.** For gastric and intestinal organoid culture, 6–8-week-old C57BL/6 male mice (Nara Biotech, Seoul, Korea) were fed a mixed diet and small stomach and small intestine tissues were harvested. All mice were maintained in the housing condition with a temperature of 21 ± 2 °C, a humidity of 50% ± 10%, ventilation of 10–15/1 h, the light of 150–300 Lux, and noise of less than 60 dB. These procedures were approved by the IACUC at the Infant Research Center (ITRC, IACUC-201612-540-04 and IACUC-201807-767-03). The harvested tissues were fragmented and washed with ice-cold Dulbecco's PBS (DPBS, Sigma). The stomach and small intestinal tissue pieces were incubated and rocked in 10 mM or 2 mM EDTA solution, respectively, for 30 min at 4 °C. Additionally, the stomach and small intestinal tissue samples were washed with 20 ml EBDA solution and incubated with rocking at 4 °C for 30 min. After several washes in DPBS, the number of glands from the stomach and crypts from the intestine were counted and encapsulated in growth factor reduced Matrigel (Corning) or decellularized GI tissue matrix. The cell suspension was mixed with 10 mg/ml Matrigel hydrogel, and 2 mg/ml IEM hydrogel and was injected into the back of the mouse. Skin tissues with hydrogel injection were harvested at 1, 4, and 7 days after the injection. The retrieved tissues were ﬁxed with 4% (v/v) parafomaldehyde (Sigma) for 1 day, embedded in parafﬁn, and then sliced into 4-μm sections. H&E staining and toluidine blue staining of tissue sections was performed to check whether there are tissue damage and recruitment of inﬂammatory cells in the regions injected with ECM hydrogels.
Zeocin™ Selection Reagent for the selection of Rspo1-Fc (all from Thermo Fisher Scientific). For long-term culture of gastric organoids in SEM hydrogel, 10 nM nicotinamide, 650 nm, respectively. Quantitative analysis was performed by calculating the fluorescence ratio (\(\frac{FI}{K} + FDR < 0.1. Functional annotation for gene ontology enrichment analysis was performed by cuffdiff was used to quantify gene expression\). DEGs were selected with the following parameters: fold change > 2, p value < 0.05, and FDR < 0.1. Functional annotation for gene ontology enrichment analysis was conducted based on the DAVID program (https://david.ncifcrf.gov). DEGs were visualized with a heatmap using Cluster 3.0 and Java TreeView 1.2.0.

RNA sequencing. RNA sequencing analysis was performed to compare overall mRNA expression levels in organoids grown in Matrigel, organoids grown in GI tissue-derived hydrogels, and GI tissues. Comparisons were made using freshly isolated tissues from 6-week-old male C57BL/6 mice. RNA samples were extracted from organoids and tissues using the TaKaRa MiniBEST Universal RNA Extraction Kit (TaKaRa). The majority of the analysis was conducted by DNA Link, Inc. (Seoul, Korea) as follows. The Novaseq 6000 platform was used for sequencing, and the Illumina Truseq stranded mRNA kit (Illumina, San Diego, CA, USA) was used to prepare the sequencing libraries. For data processing, base calling software bcl2fastq (Version 2.20) with ASCII Q-score (offset 33) was used. Tophat was used to map reads, and cuffdiff was used to quantify gene expression. DEGs were selected with the following parameters: fold change > 2, p value < 0.05, and FDR < 0.1. Functional annotation for gene ontology enrichment analysis was conducted based on the DAVID program (https://david.ncifcrf.gov). DEGs were visualized with a heatmap using Cluster 3.0 and Java TreeView 1.2.0.

Microfluidic device fabrication. The microfluidic devices were fabricated using polydimethylsiloxane (PDMS) by soft lithography as previously described. Briefly, PDMS pre-polymer (Sylgard 184; Dow Corning, Midland, MI, USA) was combined with a curing agent (1:10, volume ratio, Dow Corning) and poured into photolithographed silicon wafers or Petri dishes to cast patterned PDMS layers and 50 µl of dimethylsiloxane (DMSO, Fisher Scientific, Waltham, MA, USA) was used to prepare the sequencing libraries. For data processing, base calling software bcl2fastq (Version 2.20) with ASCII Q-score (offset 33) was used. Tophat was used to map reads, and cuffdiff was used to quantify gene expression. DEGs were selected with the following parameters: fold change > 2, p value < 0.05, and FDR < 0.1. Functional annotation for gene ontology enrichment analysis was conducted based on the DAVID program (https://david.ncifcrf.gov). DEGs were visualized with a heatmap using Cluster 3.0 and Java TreeView 1.2.0.

In vivo GI injury and organoid transplantation. EGFP+ gastric and intestinal organoids were obtained from 6-week-old male C57BL/6-tg(CAG-EF1G-EF1P)-mmp9 Cre-loxP mice (Bio-Link, Inc. (Seoul, Korea) as follows. The Novaseq 6000 platform was used for sequencing, and the Illumina Truseq stranded mRNA kit (Illumina, San Diego, CA, USA) was used to prepare the sequencing libraries. For data processing, base calling software bcl2fastq (Version 2.20) with ASCII Q-score (offset 33) was used. Tophat was used to map reads, and cuffdiff was used to quantify gene expression. DEGs were selected with the following parameters: fold change > 2, p value < 0.05, and FDR < 0.1. Functional annotation for gene ontology enrichment analysis was conducted based on the DAVID program (https://david.ncifcrf.gov). DEGs were visualized with a heatmap using Cluster 3.0 and Java TreeView 1.2.0.

For in vivo transplantation, 6-week-old male BALB/c-nude (CAnnN.Cg-Foxn1nu/CrljOri) mice (Oriente Bio Inc., Seongnam, Korea) were used. For in vivo tracking of the previously described protocol. Briefly, the ECM solution prepared for 4 mg/ml of peptide-encoding hydrogel was labeled with 5(6)-carboxytetramethylrhodamine succinimidyl ester [56]-TAMRA-SE, Thermo Fisher Scientific, as follows. The Novaseq 6000 platform was used for sequencing, and the Illumina Truseq stranded mRNA kit (Illumina, San Diego, CA, USA) was used to prepare the sequencing libraries. For data processing, base calling software bcl2fastq (Version 2.20) with ASCII Q-score (offset 33) was used. Tophat was used to map reads, and cuffdiff was used to quantify gene expression. DEGs were selected with the following parameters: fold change > 2, p value < 0.05, and FDR < 0.1. Functional annotation for gene ontology enrichment analysis was conducted based on the DAVID program (https://david.ncifcrf.gov). DEGs were visualized with a heatmap using Cluster 3.0 and Java TreeView 1.2.0.

For in vivo transplantation, 6-week-old male BALB/c-nude (CAnnN.Cg-Foxn1nu/CrljOri) mice (Oriente Bio Inc.) were used and these procedures were approved by the IACUC of the YLARC (permit number: IACUC-A-201904-889-01). Briefly, the ECM solution prepared for 4 mg/ml of peptide-encoding hydrogel was labeled with 5(6)-carboxytetramethylrhodamine succinimidyl ester [56]-TAMRA-SE, Thermo Fisher Scientific, as follows. The Novaseq 6000 platform was used for sequencing, and the Illumina Truseq stranded mRNA kit (Illumina, San Diego, CA, USA) was used to prepare the sequencing libraries. For data processing, base calling software bcl2fastq (Version 2.20) with ASCII Q-score (offset 33) was used. Tophat was used to map reads, and cuffdiff was used to quantify gene expression. DEGs were selected with the following parameters: fold change > 2, p value < 0.05, and FDR < 0.1. Functional annotation for gene ontology enrichment analysis was conducted based on the DAVID program (https://david.ncifcrf.gov). DEGs were visualized with a heatmap using Cluster 3.0 and Java TreeView 1.2.0.

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Organoid structural and functional analyses. To confirm the presence of parietal cells in the gastric organoids, the ultrastructure of gastric organoids was observed using field emission scanning electron microscopy of organoids. To further validate the acid secretion of parietal cells in gastric organoids in response to histamine treatment, gastric organoids in SEM hydrogel or Matrigel were incubated with 100 µM histamine (Sigma) for 1 h. The fluorescence images of gastric organoids pretreated with acridine orange were obtained and the fluorescence intensities before and after the treatment was dilution-f free 2 h after an inverted microscope [JEVOS FL Auto Imaging System (Thermo Fisher Scientific)]. Organoid area analysis was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

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crosslinking at a body temperature (37°C). The mice were sacrificed on the day of transplantation and 4, 7, and 28 days after transplantation for histological analysis. The harvested tissues were fixed with 4% paraformaldehyde for 1 day, embedded in OCT compound (CellPath, Newtown, United Kingdom), and then sliced into 10 μm sections for fluorescence imaging. Nuclei were stained with DAPI, and images of the stained organoids were acquired by confocal microscopy.

**Generation of hPSC-derived organoids.** Human embryonic stem cells (hESCs, line WA09) were obtained from the WICell Research Institute (Madison, WI, USA). hiPSCs (line WT3 and line KYOU-DXR0109B (ACS-1023)) were obtained from the Yonsei University College of Medicine and ATCC, respectively. Studies involving these cell lines were approved by the Institutional Review Board of Yonsei University (Permit Number: 7001988-202006-ES-904-01E and 7001988-202106-1230-01E). All hiPSC lines were cultured on dishes coated with hESC-qualified Matrigel (Corning) in Essential 8 Medium (Thermo Fisher Scientific). Differentiation into intestinal organoids was induced as previously reported100-102. Briefly, single hiPSCs were seeded at a density of 100,000 cells per well in a Matrigel-coated 24-well plate. The basic differentiation medium was RPMI 1640 (Thermo Fisher Scientific). When hiPSCs reached 90% confluence, they were treated with Activin A (100 ng ml−1, R&D Systems) for 3 days, and FBS was gradually increased in the medium to 0%, 0.2%, and 2%. Next, the differentiating cells were treated with FGF-4 (500 ng ml−1, R&D Systems) and CHIR99021 (3 μM, Sigma) for 4 days with 2% FBS. When mid-hindgut spheroids developed, they were transferred into Matrigel or IEM hydrogel and cultured with human intestinal organoid medium: advanced DMEM/F12 supplemented with 2 mM L-glutamine (Thermo Fisher Scientific), HEPES, P/S, 1x B27, 50 ng ml−1 human R-Spondin1 (R&D Systems), 100 ng ml−1 human Noggin (R&D Systems), and 50 ng ml−1 human EGF (R&D Systems). The viability of the hPSC-derived intestinal organoids encapsulated in IEM hydrogel or Matrigel was determined using a Live/Dead viability/cytotoxicity kit (Thermo Fisher Scientific). Live/Dead staining was performed for 30 min, and organoids were imaged using a fluorescence microscope (IX71, Olympus, Tokyo, Japan). The expression of intestinal marker proteins in the hiPSC-derived intestinal organoids was also visualized with immunocytochemical staining of the organoids.

**Tumoroid formation.** Human colon cancer cell lines (DLD-1, HT29) and human gastric cancer cell lines (MKN-74, NCI-N87) were cultured in DMEM containing 10% (v/v) FBS and 100 U/ml P/S (all from Thermo Fisher Scientific). Cells were suspended in pre-gel solutions of Matrigel (Corning), SEM hydrogel, or IEM hydrogel at a concentration of 5 x 10^5 cells/ml. After gelation, cells were cultured until the formation of tumoroids. Immunostaining and imaging of tumoroids were conducted in the same way as GI organoid staining.

**Data availability.** Proteomics data are available at the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD023694 [proteomcentral.proteomeexchange.org/cgi/GetDataset?ID= PXD023694] and PXD037356 [proteomcentral.proteomeexchange.org/cgi/GetDataset?ID= PXD037356]. RNA-sequencing data have been deposited to the Gene Expression Omnibus (GEO) public repository under accession codes “GSE165309”.

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Author contributions

S.W.C. conceived and supervised the study. S.K. and S.M. designed and performed the experiments and analyzed the data. S.W.C., S.K., and S.M. wrote the paper with contributions from all authors. Y.S.C. prepared all decellularized tissue-derived ECMs. Y.S.C., S.H.J., J.H.J., and K.H. performed proteomic analysis. J.K. designed and fabricated the microfluidic device. S.A. prepared fluorescently labeled ECM hydrogel. Y.W.J. and Y.G.K. advised the analysis and helped data interpretation.

Competing interests

S.W.C., S.K., and Y.S.C. are co-inventors on a patent application (Korean Patent 10-2021-0019942, pending) related to decellularized intestine-derived ECM for intestinal organoid culture. S.W.C., S.M., and Y.S.C. are co-inventors on a patent application (Korean Patent 10-2021-0019945, pending) related to decellularized stomach-derived ECM for gastric organoid culture. The remaining authors declare no competing interests.

Additional information

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