Chapter

Regulatory Functions of α-Amylase in the Small Intestine Other than Starch Digestion: α-Glucosidase Activity, Glucose Absorption, Cell Proliferation, and Differentiation

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Abstract

Pancreatic α-amylase binds to the *N*-glycan of glycoproteins. Here, I will show that pancreatic α-amylase has regulatory functions in the small intestine other than starch digestion. These new functions were revealed by identification of α-amylase-binding proteins in the intestinal brush border membrane (BBM). This topic will include the following four parts: 1) identification of glycoproteins that bind pancreatic α-amylase in the small intestinal BBM; 2) interactions between pancreatic α-amylase and the binding glycoproteins, sucrose-isomaltase (α-glucosidase), and sodium/glucose co-transporter 1 (SGLT1), in which pancreatic α-amylase enhanced maltose degradation of sucrose-isomaltase under conditions including calcium and sodium, and inhibited glucose uptake of SGLT1; 3) localization of pancreatic α-amylase in the small intestine by binding to the BBM and being internalized into lysosomes through the endocytic pathway; and 4) expression of endogenous α-amylase in the duodenum: *AMY2B*, a pancreatic type α-amylase, is highly expressed in the human duodenum next to the pancreas. The α-amylase expression in the duodenum is required for proliferation and differentiation of human small intestinal epithelial cells.

Keywords: pancreatic α-amylase, small intestine, sucrase-isomaltase, SGLT1, cell proliferation, cell differentiation, endocytosis

1. Introduction

α-Amylase has been found in many organisms, including bacteria, mainly Bacilli, seeds of cereals and legumes, and digestive glands of animals such as humans and pigs. These α-amylases can be obtained as homogeneous preparations and utilized in various situations. For example, mammalian α-amylase is used as a gastrointestinal medicine and digestive agent. Microbial α-amylase is used in sugar production, food processing, clothing production, and detergents because it can be produced on an industrial scale. The uses of these amylases all focus on the ability of

the amylase to degrade polysaccharides. The α-amylase in pancreatic juice and saliva clearly functions as a digestive enzyme. It is an endo-type enzyme that randomly cleaves α-1,4 glycosidic bonds such as starch and glycogen to produce maltose and oligosaccharides [1]. Therefore, α-amylase in digestive juices is an essential enzyme for animals consuming a starch diet. α-Amylase has been found in liver [2], brain [3], blood, urine, and cancer cells [4] in humans, but its physiological significance has not been completely elucidated yet.

It has been reported that porcine pancreatic α-amylase binds to an *N*-linked glycan of glycoproteins [5]. It has been shown that the binding of porcine pancreatic α-amylase to *N*-linked glycans is different from that of the polysaccharide as a substrate. It shows that the carbohydrate-binding activities of pancreatic α-amylase are not common to all types of α-amylase. α-Amylases from barley, *Bacillus subtilis*, and mammalian saliva do not have this activity. Porcine pancreatic α-amylase binds mainly to transferrin having complex-type biantennary *N*-glycans, fetuin having complex-type triantennary *N*-glycans, and ribonuclease B having high-mannose *N*-glycans. However, it does not bind to bovine submandibular gland mucin having *O*-glycans or bovine serum albumin having no sugar chains. The carbohydrate-binding activities of porcine pancreatic α-amylase are affected by pH. Porcine pancreatic α-amylase has a high affinity for both complex type and high-mannose *N*-glycans under pH 5.5. On the other hand, the binding activity is seen with only the high-mannose *N*-glycans, and the binding to the complex *N*-glycans is reduced under neutral pH. Thus, pancreatic α-amylase selectively recognizes the structure of *N*-glycans of glycoproteins depending on pH.

The biological significance of the *N*-glycan binding found in pancreatic α-amylase in vivo is unknown. Pancreatic α-amylase is synthesized in pancreatic acinar cells, packed into acidic zymogen granules (pH 5.5), and secreted into alkaline pancreatic juice (pH 8.0) [6]. Pancreatic juice flows into the duodenum and mixes with gastric acid where it is neutralized, and pancreatic α-amylase exerts its enzymatic activity. The pH in the middle of the duodenum falls from pH 6.5 to 4.5 in a 3-hour period after a meal [7]. There are glycoproteins with *N*-glycans in pancreatic zymogen granules, its membrane, and the lumen of the small intestine [8–11]. These suggest that the *N*-glycan-binding activity of pancreatic α-amylase may be play a role in the packing of pancreatic zymogen granules, its exocytosis in the pancreas, and digestion and absorption of carbohydrates in the small intestine.

In this study, we elucidated the roles of the *N*-glycan-binding activity of pancreatic α-amylase in the small intestine by identifying the glycoproteins to which the α-amylase binds on the small intestinal brush border membrane (BBM), the luminal surface of the small intestine where many glycoproteins are located. The functions and localizations of the identified α-amylase-binding-glycoproteins and analyses of interactions between α-amylase and the glycoproteins revealed that pancreatic α-amylase has the following regulatory functions other than as a digestive enzyme: (1) enhancement of α-glucosidase, sucrase-isomaltase, (2) regulation of sodium-dependent glucose uptake, and (3) localization of pancreatic α-amylase in the small intestine. Furthermore, the new discoveries show that duodenal epithelial cells express α-amylase, which is essential for proliferation and differentiation of small intestinal epithelial cells.

2. Identification of glycoproteins that bind pancreatic α-amylase in the small intestinal BBM

In the small intestine, epithelial cells in the luminal villi have a brush border on the surface, and alkaline phosphatase and other membrane-type digestive enzymes

are localized on the BBM. The BBM is covered with a glycocalyx composed of glycoproteins and proteoglycans [10, 12]. Lectin staining of rat small intestine tissue revealed that rat small intestinal BBM expresses glycoproteins having complex, high-mannose *N*-glycans, and core 1 *O*-glycans including NeuAcα2-6Gal/GalNAc, and Galβ1-3Gal [9]. It is reported that *N*-glycans of glycoproteins on the surface of porcine intestine have more complex types than those on the rats [13].

Previous reports have shown that pancreatic α-amylase binds to the *N*-glycans of purified glycoproteins [5], but it has not been confirmed whether or not pancreatic α-amylase binds to glycoproteins on small intestinal BBM. Furthermore, which glycoproteins bind to the pancreatic α-amylase and what role they play in interactions with the α-amylase were unknown. This study demonstrated the binding of α-amylase to pig small intestinal BBM by immunostaining, and identified the glycoproteins that bind α-amylase in the pig small intestinal BBM [14].

2.1 Binding of pancreatic α-amylase to small intestinal BBM

Tissue sections of pig duodenum were immunostained with an anti-pancreatic α-amylase antibody. Entire duodenal tissue sections were stained with the pancreatic α-amylase antibody when there was a large amount of food in the stomach (during non-fasting), but hardly stained when there was little in the stomach (during fasting) [14]. The localization of exogenous pancreatic α-amylase was examined by staining a 1-cm section of the duodenum of a fasted pig with the pancreatic α-amylase antibody. Tissue sections fixed in formalin and embedded in paraffin were immunostained to detect pancreatic α-amylase. The duodenal sections were agitated in pancreatic α-amylase solution at 4°C. As a result, pancreatic α-amylase bound to the BBM, which is the upper end of the duodenal epithelium (**Figure 1**), and α-amylase staining increased in a time-dependent manner with agitation. Mannan inhibited the binding of pancreatic α-amylase to BBM duodenum sections incubated in α-amylase solution for 30 min. On the other hand, galactan and colominic acid inhibited binding of α-amylase to BBM after 10 min of agitation, but did not inhibit binding of α-amylase to BBM agitated for 30 min (data not shown) [14]. Pancreatic α-amylase bound to duodenum BBM, and its binding was inhibited by

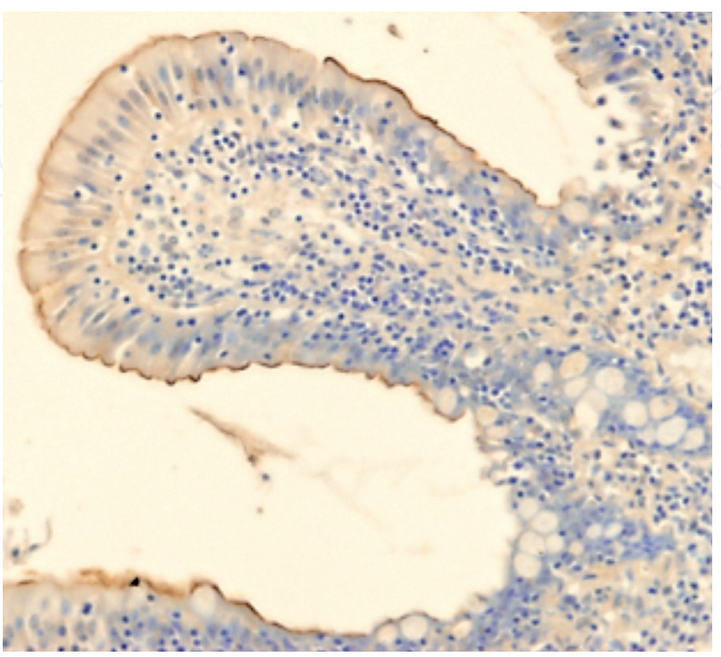

Figure 1.
Binding of pancreatic α-amylase to pig duodenum BBM.

mannan, indicating that α-amylase binds to duodenal BBM in a mannose-specific manner, even at the tissue level.

One-centimeter duodenum sections from fasted pigs were incubated with pancreatic α-amylase (10 μM) in PBS (pH 7.2) including phenylmethylsulfonyl fluoride (final concentratation 1 mM) at 4°C for 30 min, then fixed and paraffin-embedded. The paraffin sections were immunostained with rabbit anti-α-amylase IgGs-HRP. The color was developed with DAB/H_2O_2 and then counterstained with hematoxylin.

2.2 Separation and identification of glycoproteins from the small intestinal BBM that bind pancreatic α-amylase

It was shown that pig duodenal BBM contains glycoproteins that bind to pancreatic α-amylase having high mannose-type and complex-type *N*-glycans. Therefore, we separated and identified the glycoproteins binding α-amylase from fasted pig duodenal BBM [14]. The binding glycoproteins were separated from BBM solubilized with 1% TritonX-100 by using affinity chromatography with an α-amylase-Sepharose 6B column. The α-amylase-binding fractions were eluted by methyl-D-mannopyranoside and separated by molecular weight using SDS-PAGE. Seven main bands stained by SYPRO Ruby protein staining were cut out, treated with pepsin, and analyzed by LC–MS/MS for identification of the proteins in the bands. The major α-amylase-binding glycoproteins identified are shown in **Table 1**. The binding glycoproteins were grouped by functions and localizations. Group 1 consisted of membrane glycoproteins involved in α-glucosidase and sugar absorption, which function in glucose assimilation after starch digestion by α-amylase in the small intestine. Groups 2 and 3 consisted of membrane glycoproteins involved in transcytosis and proteolysis, respectively.

All seven bands were stained by lectins with ConA, GNA, and L-PHA, indicating the presence of *N*-glycans. ConA (concanavalin A), GNA (snowdrop lectin), and L-PHA (*Phaseolus vulgaris* lectin type L) bind specifically to *N*-glycans, high-mannose structures, and complex-type *N*-glycans, respectively. The identification of these α-amylase-binding proteins indicates that the *N*-glycan-binding activity

Identified proteins			
MW (kDa)	**Group 1**	**Group 2**	**Group 3**
202	SGLT1, SI		
144–156		VLA-2	Aminopeptidases, Enterokinase
110–122	SGLT1	DPP-IV, Integrin β1, ACE2	DPP-IV
99			Aminopeptidases
78	SGLT1	Transferrin, Transferrin receptor	
50–57	SGLT1	Na^+/K^+-ATPase β1-subunit	Enterokinase Amine oxidase B
46	Epoxide hydrase	CD Man-6-P receptor, Aminopeptidase N	Aminopeptidase N

SGLT1, sodium glucose co-transporter 1; SI, sucrose-isomaltase; VLA-2, integrin very late antigen-2; DPP-IV, dipeptidylpeptidase IV; ACE 2, angiotensin-converting enzyme 2.

Table 1.
Identification of α-amylase-binding proteins in intestinal BBM.

of pancreatic α-amylase in the duodenum may play a regulatory role involved in α-glucosidase/sugar absorption, transcytosis, and proteolysis.

3. Interaction between pancreatic α-amylase and binding glycoproteins in small intestinal BBM

First, effects of pancreatic α-amylase on the activities of glycoproteins in Group 1 were investigated. Group 1 consists of SI and SGLT1. SI, a membrane glycoprotein, digests sucrose, isomaltose, and maltose on the membrane as α-glucosidases. SGLT1 is also a membrane glycoprotein and plays an essential role in glucose absorption in the small intestine.

3.1 Effects of pancreatic α-amylase on α-glucosidase activity

SI plays an important role in the end digestion of starch because SI breaks down the maltose produced by α-amylase from starch into glucose as an α-glucosidase on the small intestinal membrane. SI is essential for the end digestion of starch, and its

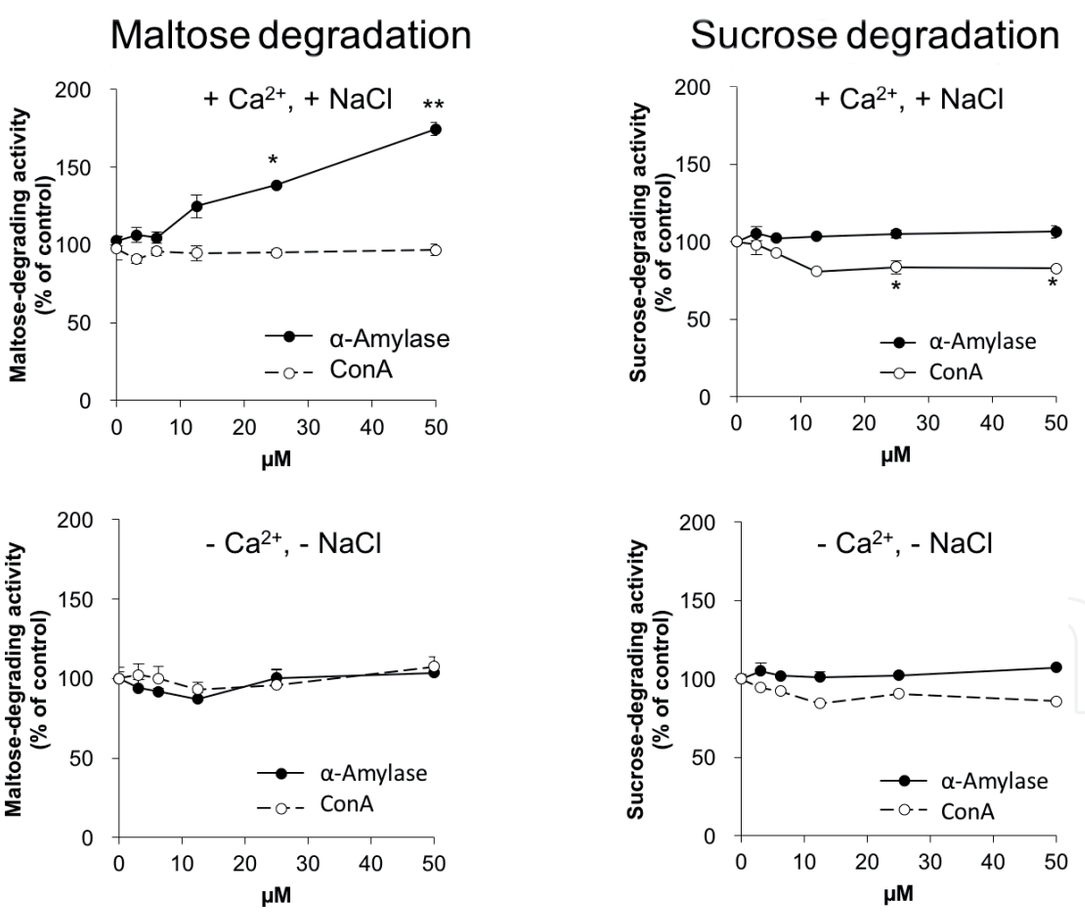

Figure 2.
*Effects of pancreatic α-amylase and ConA on maltose- and sucrose-degrading activity of pig duodenum BBM vesicles. A BBM vesicle suspension (50 μl) was pre-incubated with various concentrations of pancreatic α-amylase or ConA (0–50 μM) for 15 min at 37°C, and incubated with 0.056 M maltose (left) or 0.056 M sucrose (right) as substrate (50 μl) for 30 min (left) or 60 min (right) at 37°C. The maltose or sucrose hydrolysis was measured in the presence of 5 mM $CaCl_2$ and 0.15 M NaCl (upper) or in the absence of $CaCl_2$ and NaCl (lower). Phlorizin (0.5 mM), a glucose transporter inhibitor, was included in the substrate solution. The degrading activity by the production of glucose was measured using a glucose C-II test Wako (Wako, FUJIFILM). ●: α-amylase, ○: ConA. Results are given as means±SE; n = 6. *p < 0.05; **p < 0.01 compared with the absence of α-amylase or ConA by Student's t-test [14].*

deficiency and remarkable fluctuations in enzymatic activity are thought to have a significant effect on starch digestion and glucose absorption. In this study, a method was established for measuring SI activity using BBM that evaluates the effects of pancreatic α-amylase on SI activity as an α-glucosidase [14].

The effect of pancreatic α-amylase on SI activity was investigated with and without $CaCl_2$ and NaCl. As a comparison with α-amylase, the effects of concanavalin A (ConA), a lectin that recognizes α-mannose and α-glucose, were also measured because α-amylase shows a high affinity to α-mannose. In maltose degradation activity by SI, the α-amylase showed enhanced activity only in the presence of 5 mM $CaCl_2$ and 0.15 M NaCl, while no effect of α-amylase was shown in the absence of $CaCl_2$ and NaCl (**Figure 2**, left). ConA had no effect on the maltose degradation activity by SI. On the other hand, α-amylase did not affect the sucrose degradation activity, and ConA inhibited it by about 20% in the presence and absence of 5 mM $CaCl_2$ and 0.15 M NaCl, respectively (**Figure 2**, right).

3.2 Effects of pancreatic α-amylase on SGLT1 activity

SGLT1 is a glycoprotein having a complex-type *N*-glycan and a sodium-dependent glucose transporter indispensable for glucose absorption in the small intestine. The effect on the SGLT1 activity means that it directly affects the blood glucose concentration. This study shows the results of examining whether pancreatic α-amylase affects SGLT1 activity.

First, the timing of the addition of pancreatic α-amylase, where the effect of the α-amylase on SGLT1 activity is most frequently observed, was examined.

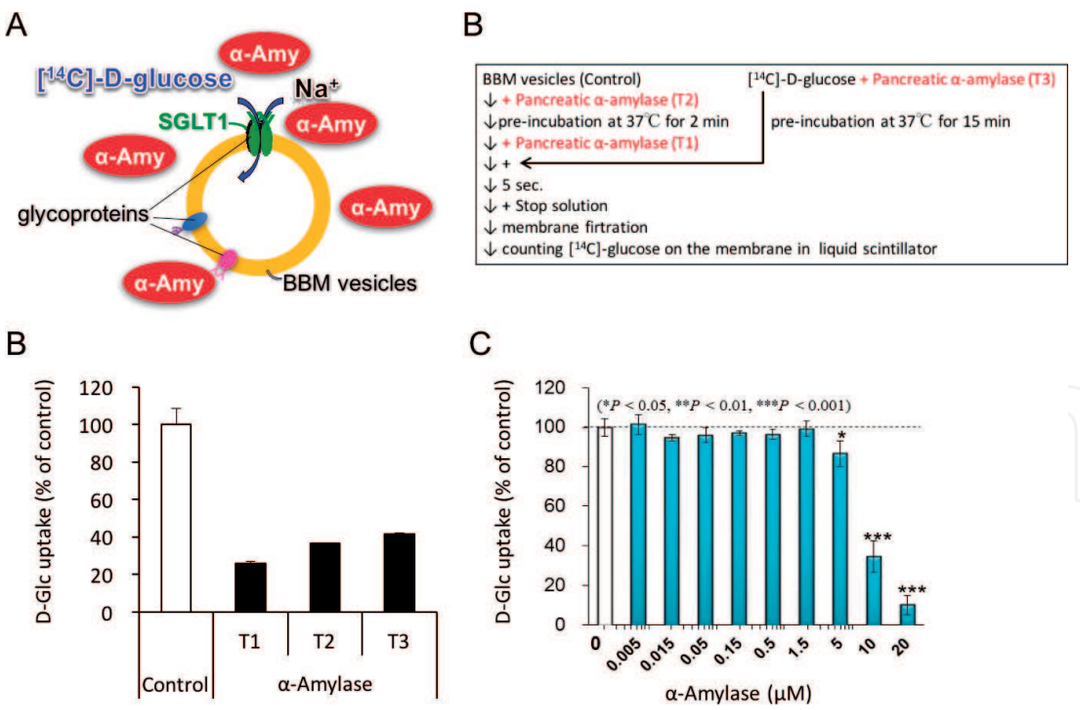

Figure 3.
*Effects of pancreaitc α-amylase on Na^+-dependent glucose uptake. SGLT1 activity was assayed as Na^+-dependent [^{14}C]-D-glucose uptake (0.2 mM) in BBM vesicles prepared from pig duodenum. (A) Schematic illustration of the SGLT1 activity measurement system using BBM vesicles. (B) Timing of the α-amylase addition to the SGLT1 activity measurement system. T1: α-amylase was added to BBM vesicles prior to [^{14}C]-D-glucose; T2: α-amylase was pre-incubated with BBM vesicles 2 min before addition of [^{14}C]-D-glucose; T3: α-amylase was pre-incubated for 15 min with [^{14}C]-D-glucose. □: Without pancreatic α-amylase (control), ■: With pancreatic α-amylase (10 μM). (C) Effects of pancreatic α-amylase on SGLT1 acrtivity under the three conditions. (D) Dose dependency of D-glucose (D-Glc) uptake on final concentrations of added α-amylase (μM). Pancreatic α-amylase was added at T1. Results are given as means ±SE; n = 6. *p < 0.05, ***p < 0.001 compared with the absence of polysaccharide by Student's t-test [14].*

T1: Pancreatic α-amylase was added just before the [^{14}C]-D-glucose substrate solution without preincubation. T2: The α-amylase was pre-incubated with BBM vesicle solution at 37°C for 2 min. T3: The α-amylase was pre-incubated with [^{14}C]-D-glucose substrate solution at 37°C for 15 min (**Figure 3A** and **B**). Inhibition of SGLT1 acitivity by pancreatic α-amylase was shown at all tested additions of the α-amylase, and T1 was the most efficient for SGLT1 inhibition (**Figure 3C**). The concentration-dependent effects of α-amylase on SGLT1 activity were examined under the T1 condition. SGLT1 activity was reduced from 5 μM of α-amylase, and inhibited to 34% at 10 μM and 10% at 20 μM (**Figure 3D**). The 50% inhibitory concentration (IC50) value was 8.1 μM.

Pancreatic α-amylase concentrations in pancreatic juice have been reported to be 4.2 mg/ml in pigs and 5–16 mg/ml in cows [15, 16]. Humans secrete 1–3 L of pancreatic juice containing several to several tens of grams of protein per day. Therefore, pancreatic α-amylase is probably present in the order of mg/ml because the total concentration of α-amylase in pancreatic juice protein is 26.5% [17]. In this study, it was found that SGLT1 activity was inhibited by pancreatic α-amylase at more than 10 μM (≒ 0.56 mg/ml).

4. Localization of pancreatic α-amylase in the small intestine

α-Amylase synthesized in the pancreas and salivary glands is mostly secreted into the gastrointestinal tract where it digests starch. Part of the α-amylase enters the blood, one-quarter of which is excreted from the kidneys into the urine, and the remaining α-amylase is degraded (inactivated) by an unknown pathway. The α-amylase in the blood is maintained at a constant level by supply from the pancreas and salivary glands, excretion outside the body, and decomposition in the body. Therefore, the blood α-amylase activity is used for diagnosing pancreatitis and other diseases. The proportion of pancreatic α-amylase (unglycosylated 54 kDa) and saliva α-amylase (unglycosylated 54 kDa and glycosylated 57 kDa) is same when the concentration of α-amylase in human blood is examined by electrophoresis. When the pancrea is completely removed due to pancreatic cancer, the blood α-amylase activity temporarily decreases, but it returns to a normal level because the α-amylase in salivary glands increases. It is reported that fluorescently labeled α-amylase injected into rat small intestine was detected in intestinal epithelium and blood, indicating that the pancreatic α-amylase was transported into small intestine tissue (endocytosis) and blood (exocytosis) [18].

The pancreatic α-amylase-binding glycoproteins identified as Group 2 in Section 2.2 contain membrane glycoproteins that have an endocytic function. Transferrin receptor (TfR) binds to iron-bound transferrin and endocytoses iron-bound transferrin into enterocytes [19]. Similarly, aminopeptidase N, ACE2, and VLA- 2 bind to human coronavirus [20, 21], severe acute respiratory syndrome (SARS) virus [22], and matrix glycoproteins [23], respectively. Further, these ligands including viruses are able to endocytose into enterocytes. Mannose 6-phosphate receptor (Man-6-P-receptor) transports binding proteins to the lysosomal system [24]. DPP-IV does not stay in the BBM, and is transported into cells via the same pathway as aminopeptidase N and transferrin (51). This study demonstrated an endocytic pathway for α-amylase secreted into the duodenum from the pancreas [25].

4.1 Endocytosis of pancreatic α-amylase into the small intestine

Two kinds of experiments were performed using pig duodenum tissues and Caco-2 human intestinal epithelial cells that had differentiated into small

intestine-like cells by culture in Transwells for 3 weeks. In the experiment using pig duodenum tissue, fasted duodenum with no stomach contents was cut into 1-cm pieces and agitated in a pig pancreatic α-amylase solution at 37°C. After agitation for various periods of time, the tissues were fixed with formalin, and paraffin-embedded sections were prepared. The sections were immunofluorescently stained with an anti-pancreatic α-amylase antibody to clarify the localization of the α-amylase. No staining was detected in the tissue sections after incubation for 0 min. The green fluorescence with anti-pancreatic α-amylase antibody was detected at the upper end of the duodenum corresponding to the BBM in the sections incubated with α-amylase for 10 min, and α-amylase was detected in the entire duodenal tissue incubation for 30 min (**Figure 4A**). In the experiment using differentiated Caco-2 cells, AlexaFluor488-labeled human pancreatic α-amylase was added to the culture medium. After incubation for 30 min at 4°C, the α-amylase in the medium was washed out, and chased for 0–60 min at 37°C in fresh medium without α-amylase. The cells were fixed with formalin, and AlexaFluor488-labeled α-amylase was detected by confocal microscopy. Green fluorescence indicating the localization of α-amylase was detected on the cell membrane only after incubation at 4°C for 30 min. In the subsequent 37°C chase, the α-amylase was detected as punctates in the cells and had decreased at 60 min (**Figure 4B**). These results

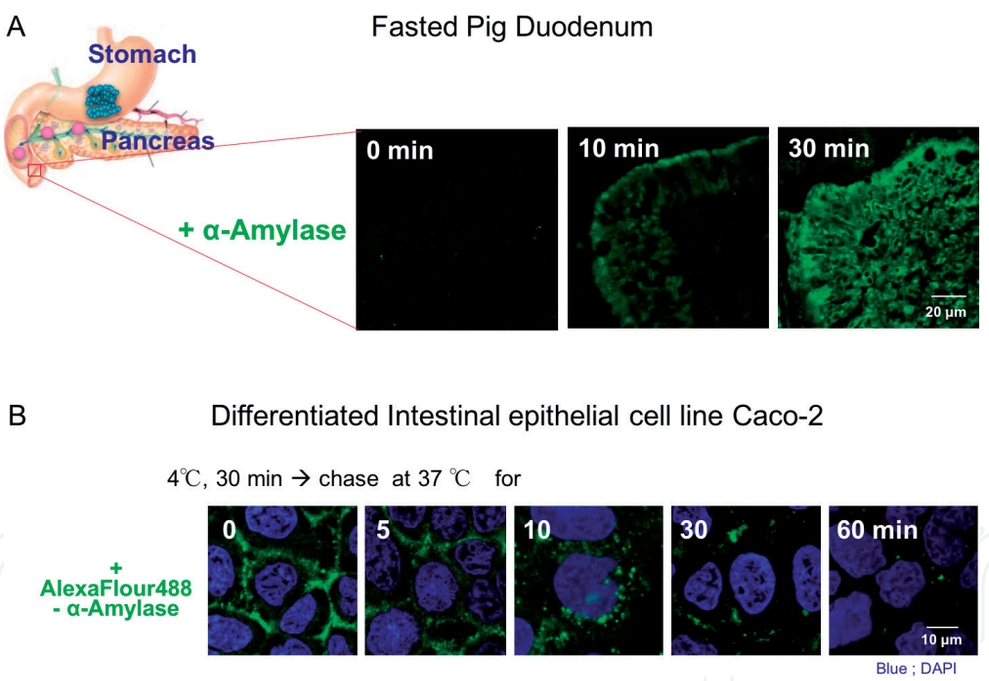

Figure 4.
Incorporation of pancreatic α-amylase into pig duodenum tissue (A) and differentiated human epithelial cells Caco-2 (B). (A) One-centimeter duodenum sections from fasted pigs were incubated with pig pancreatic α-amylase (10 μM) in PBS (pH 7.2) including phenylmethylsulfonyl fluoride (final 0.1 mM) at 37°C for 0, 10, or 30 min. The duodenum sections were fixed and paraffin-embedded. The paraffin sections were immunostained with rabbit anti-pancreatic α-amylase antibody as a first antibody and Alexa Fluor 488-goat anti-rabbit antibody. The green fluorescence was detected by a microscope (Olympus FSX100). (B) Caco-2 cells were seeded at 2.6 × 10^5 cells/cm^2 on polyester Transwell permeable supports (0.4-μm pores, 12-mm diameter), and cultured for about 3 weeks in minimal essential medium (DMEM, Sigma) supplemented with 20% heat-inactivated (56°C, 30 min) fetal bovine serum (FBS, Gibco) and 0.1 mM non-essential amino acids (NEAA, Gibco) under a 95% air and 5% CO_2 atmosphere. The cells were starved in a DMEM-NEAA medium without FBS for 18 h. After being washed with Dulbecco's Phosphate-Buffered Saline (DPBS) with Ca^{2+} and Mg^{2+}, Alexa Fluor 488-human pancreatic α-amylase (20 μg/ml) in a DMEM-NEAA medium without FBS was added and incubated at 4°C for 30 min. The α-amylase was washed out, and cultured in DMEM including in 20% FBS and 1 mM NEAA at 37°C for 0–60 min. The cells were fixed, and Alexa Fluor 488-human pancreatic α-amylase was detected by confocal microscopy (ZEISS, LSM710). The nucleus was stained by 4′,6-diamidino-2-phenylindole (DAPI) [25].

indicate that pancreatic α-amylase is incorporated into duodenal tissue and small intestinal epithelial cells in a time- and temperature-dependent manner.

4.2 Endocytosis pathway of pancreatic α-amylase to lysosomes

The results in **Figure 4B** showed that pancreatic α-amylase was internalized into the epithelial cells and disappeared. The cause of this disappearance is expected to be proteolysis. Among the α-amylase-binding proteins identified in Section 2.2, the membrane glycoprotein proteins classified into Group 3 are involved in protein degradation. In this study, co-localization with intracellular localization marker proteins was investigated to elucidate the endocytic pathway of the pancreatic α-amylase. Transferrin (Tf), early endosome antigen-1 (EEA1), and lysosomal-associated membrane protein 1 (LAMP1) were used as localization marker proteins of the cell membrane, early endosomes, and lysosomes, respectively, and were stained with an Alexa Fluor 594-labeled secondary antibody. Alexa Fluor 488-human pancreatic α-amylase (AF488-α-amylase) was mainly co-localized with Tf by chasing for 0–5 min at 37°C. Subsequently, the AF488-α-amylase was co-localized with EEA1 after chasing for 5–10 min, and then the α-amylase was finally co-localized with LAMP1 after a 30–60 min chase, followed by its disappearance (**Figure 5**). These results suggested that pancreatic α-amylase

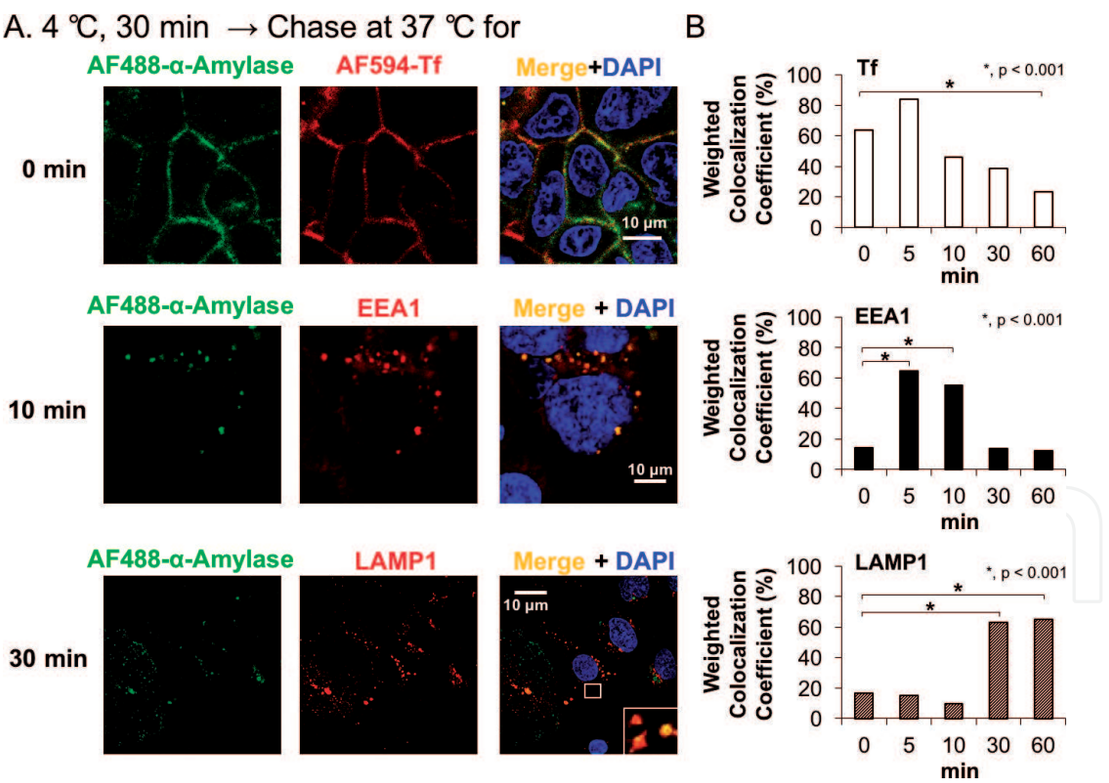

Figure 5.
Co-localization between human pancreatic α-amylase and localization marker proteins. Caco-2 cells were seeded at 2.6 × 10^5 cells/cm^2 on polyester Transwell permeable supports and cultured for about 3 weeks in DMEM supplemented with 20% heat-inactivated FBS and 0.1 mM NEAA under a 95% air and 5% CO_2 atmosphere. The cells were starved in a DMEM-NEAA medium without FBS for 18 h. After being washed with DPBS containing Ca^{2+} and Mg^{2+}, AF488-human pancreatic α-amylase (20 μg/ml) and/or Alexa Fluor 594 (AF594)-Tf in a DMEM-NEAA medium without FBS were added, and cells were incubated at 4°C for 30 min. The α-amylase was washed out, and cells were cultured in DMEM including 20% FBS and 1 mM NEAA at 37°C for 0–60 min. After the cells were fixed, they were stained with an anti-EEA1 antibody or anti-LAMP1 antibody as the first antibody, and followed by AF594-labeled secondary antibodies. The nucleus was stained by DAPI. The three kinds of fluorescence were detected by confocal microscopy (ZEISS, LSM710) (A) [25]. These weighted colocalizations were calculated as described previously [25] (B).

binds to the cell membrane and is transported into lysosomes though early endosomes and the α-amylase undergo degradation in the lysosome. The disappearance of AF488-amylases endocytosed into the cells was suppressed by chloroquine, which is an inhibitor of lysosome proteolysis (data not shown) [25]. In another experiment using pig duodenum tissue sections, α-amylase in the duodenum was well co-localized with LAMP1, and its degraded fragments were detected (data not shown) [25]. These results indicate that pancreatic α-amylase internalized by the endocytic pathway is undergoing proteolysis in the lysosome. Degraded α-amylase may be used as a source for rapid turnover in duodenal epithelial cells.

4.3 Biological significances of endocytosis of pancreatic α-amylase

Based on the SGLT1 inhibition of pancreatic α-amylase described in Section 3.2, the biological significance of endocytosis of the pancreatic α-amylase in Sections 4.1 and 4.2 is discussed as follows. **Figure 6** shows a hypothetical scheme of the biological function of α-amylase internalization [25]. (i) Within 30 min after food intake, pancreatic α-amylase has begun to be secreted from the pancreas into the duodenum, but its amount is not high. Therefore, the α-amylase on the surface of the duodenal BBM interacts with the SI on the BBM to promote starch digestion by both the α-amylase and SI. (ii) Approximately 30 min after food intake, the concentration of pancreatic α-amylase in the small intestinal lumen reaches a maximum, and the α-amylase binds to the glycoproteins in the BBM. At this time, the glucose uptake of SGLT1 is inhibited by the α-amylase, whereby rapid glucose uptake is suppressed, and postprandial hyperglycemia is corrected. (iii) At 30–60 min after food intake, the α-amylase bound to the BBM is internalized into early endosomes in the epithelial cells.

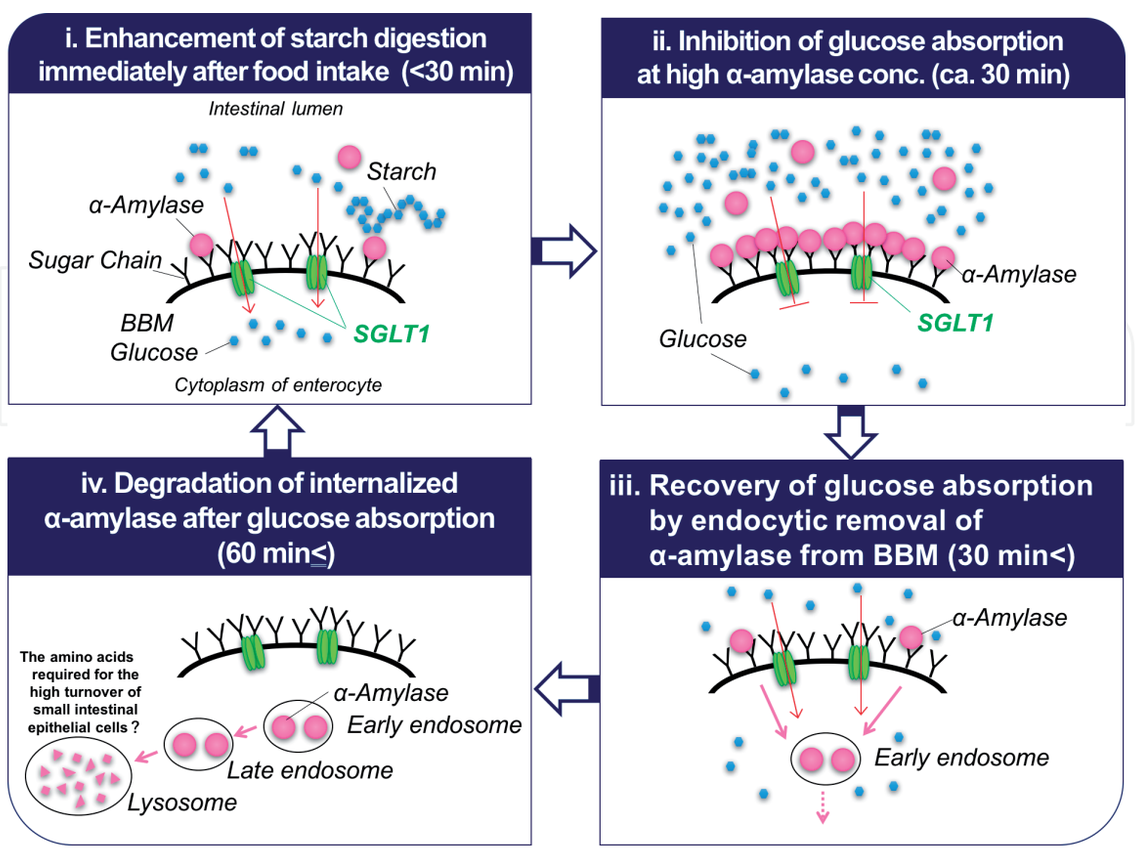

Figure 6.
A hypothetical schematic of the biological function of pancreatic α-amylase internalization.

That is, the inhibition of glucose uptake of SGLT1 in BBM by α-amylase is released, which means the blood glucose level can be maintained even when the blood glucose level drops during fasting. (iv) At 60 min or more after food ingestion, the α-amylase internalized into cells is transported to lysosomes and undergoes degradation.

5. Expression of endogenous α-amylase in the human duodenum

α-Amylase is abundantly expressed in the pancreas and salivary glands, and has been detected in liver [26], thyroid [27], parotid gland [28], white blood cells [29], lung carcinoma tissues [30], and brain [3] in humans. There are five isoforms of α-amylase including three salivary α-amylases (isoforms *AMY1A*, *AMY1B*, and *AMY1C*) and two pancreatic α-amylases (isoforms *AMY2A* and *AMY 2B*) [31]. The α-amylases in liver, white blood cells, and lung carcinoma tissues are encoded by *AMY2B* [2, 29, 30]. The brain amylases are encoded by *AMY1A* and *AMY2B* [3]. The parotid gland amylase is encoded by *AMY1C* [28]. It is reported that α-amylase activity is detected in small intestinal tissues [32]. It is not clear whether this α-amylase is an exogenous one that endocytoses pancreatic α-amylase into small intestinal tissues or an endogenous α-amylase that is expressed in the small intestine itself. An endogenous α-amylase was stained in differentiated Caco-2 cells by immunostaining with an anti-α-amylase antibody during the experiments on internalization using fluorescence-labeled α-amylase in the cells described in Section 4. In this study, we demonstrated the expression of endogenous α-amylase in human duodenal epithelial cells and identified the isoform of the α-amylase expressed in the duodenal epithelial cells. Furthermore, the biological significances of α-amylase expression in the duodenum are shown [33].

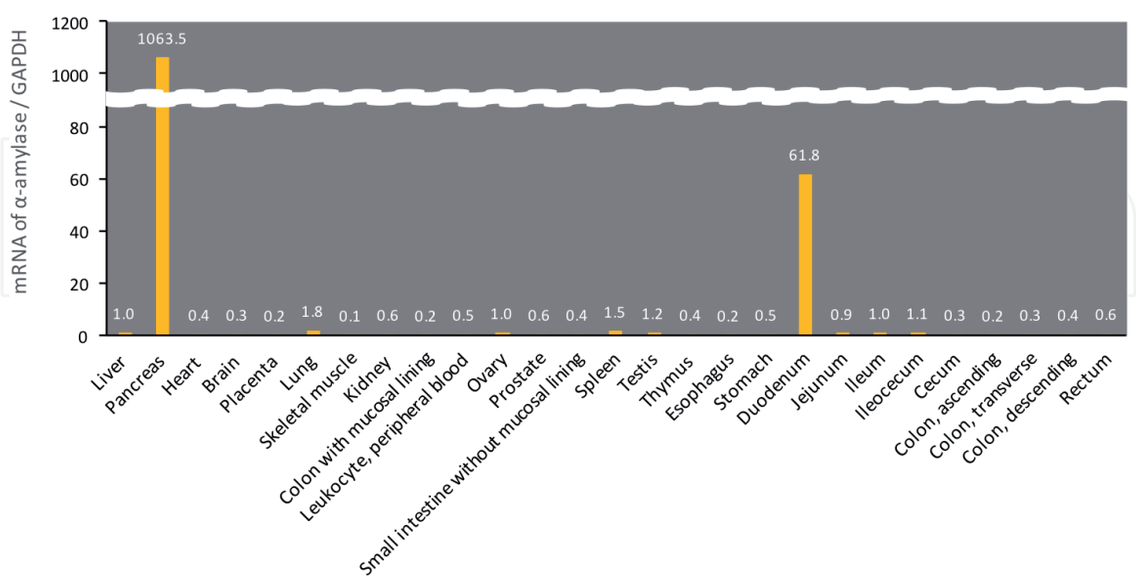

Figure 7.
Expression of α-amylase in human tissues. A reaction mixture was prepared by mixing Human MTC panel I, II or Digestive system MTC panel (Clontech) as a cDNA (5 μl), Power SYBER Green PCR Master mix (6.25 μl), 50 μM forward and reverse primers for AMY2B (0.2 μl each), and putting it in the 96-well reaction plate. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal standard. The sequences of primer sets for AMY2B and GAPDH were described previously [33]. Normalized data by GAPDH are shown with liver expression as 1.

5.1 Expression of *AMY2B* in human duodenum tissues

The mRNA expression of α-amylase in human tissues was measured by real-time PCR using human Multiple Tissue cDNA (MTC) Panels. The α-amylase was shown to be expressed most in the pancreas and then in the duodenum (**Figure 7**). The results mean that the α-amylase detected in the duodenum consists of both the endogenous α-amylase expressed in the duodenum and the exogenous α-amylase expressed by the pancreas and internalized into the duodenum. For further investigation, mRNA levels, protein levels, enzyme activities, and localization of the α-amylases were clarified using differentiated Caco-2 cells [33]. Caco-2 cells were originally human intestinal epithelial cells derived from the colon, but are known to gradually develop small intestine-like properties when differentiated by long-term culture for about 3 weeks. Therefore, we focused on the change in α-amylase expression in Caco-2 cells during differentiation into small intestine-like cells. The expression of α-amylase in Caco-2 cells increased with differentiation into small intestine-like cells in its mRNA level, protein level, enzyme activity, and immunostaining (**Figure 8**). Activity of α-amylase was detected in the cell extract but not in the culture medium, suggesting that the α-amylase was not secreted by the cells

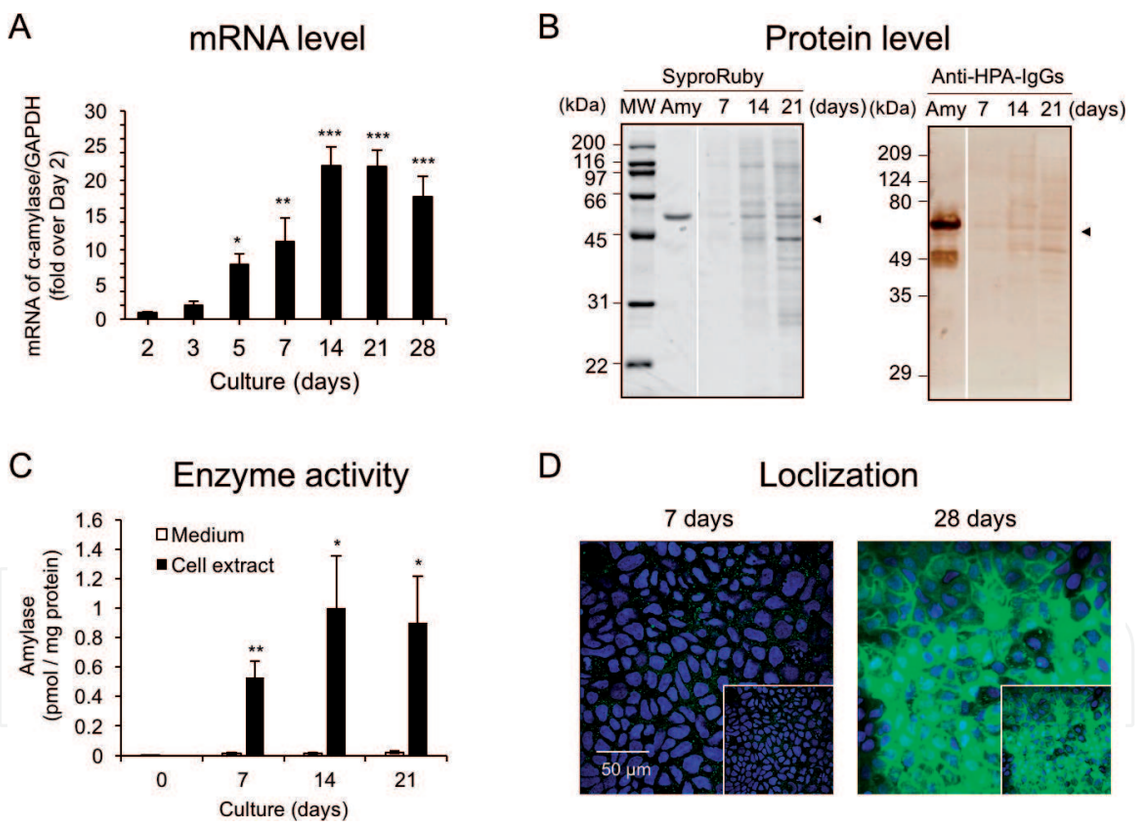

Figure 8.
Expression of α-amylase in differentiated human epithelial cells. Caco-2 cells were seeded at 5 × 10^4 cells/cm^2 in the wells and cultured for 3 weeks in DMEM supplemented with 20% heat-inactivated FBS and 0.1 mM NEAA under a 95% air and 5% CO_2 atmosphere. The cells were washed with DPBS and were prepared for each measurement as described previously [33]. (A) mRNA expression of α-amylase in the cells was measured by quantitative real-time PCR using a 7500 Real-Time PCR System (Applied Biosystems). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. Day 2 by one-way ANOVA with Dunnett's posthoc test. (B) Protein levels of α-amylase in the cell extract were detected by Western blotting using anti-human pancreatic α-amylase antibody (HPA IgGs) as a first antibody and HRP-labeled secondary antibodies. The staining was developed by 3,3′-diaminobenzidine (DAB) including H_2O_2. (C) Specific enzymatic activities of α-amylase in the culture medium and cell extract. $^*p < 0.05$, $^{**}p < 0.01$ vs. medium by paired t-test (D) Immunofluorescence staining of the permeabilized cells with anti-HPA IgGs as a first antibody and AF488-labeled secondary antibodies. The nucleus was stained by DAPI. The fluorescence was detected by confocal microscopy (ZEISS, LSM710). The lower right is an enlarged view [33].

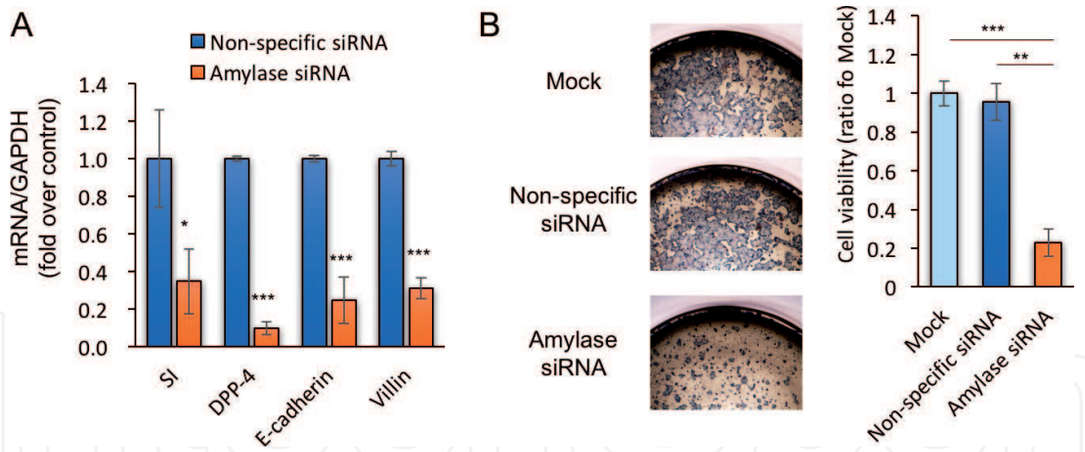

Figure 9.
Effects of α-amylase suppression by siRNA on cell differentiation and proliferation. Nonspecific siRNA as a control or the siRNA targeting α-amylase was transfected to Caco-2 cells and these cells were seeded at 2 × 10^5 cells/cm^2 (A) or 1 × 10^5 cells/cm^2 (B) in the wells. The cells were cultured for 3 days in DMEM supplemented with 20% heat-inactivated FBS and 0.1 mM NEAA under a 95% air and 5% CO_2 atmosphere. (A) The cells were washed with DPBS and were prepared for mRNA expression as described previously [33]. mRNA expressions of α-amylase differentiation marker proteins (SI, DPP-4, E-cadherin, villin) in the cells were measured by quantitative real-time PCR using a 7500 Real-Time PCR System (Applied Biosystems). (B) The cells in the 96-well plates were stained with MTT, and the cell viability was measured by an MTT assay. Mock, the cells transfected with the transfection reagent only without siRNA. Data are shown as mean ± SE, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, one-way ANOVA with Tukey's posthoc test [33].

(**Figure 8C**). Immunostaining showed that the α-amylase in the cytoplasm was localized in a dotted pattern (**Figure 8D**). The isotype of the α-amylase expressed in differentiated Caco-2 cells was identified as pancreatic type *AMY2B* by a combination of PCR and restriction enzyme treatment (data not shown) [33].

5.2 Biological significances of α-amylase expressed in the duodenum

α-Amylase has been shown to be expressed by tissues other than the pancreas and salivary glands, but the biological significance has not been elucidated. The increased α-amylase expression in obese mouse liver suggests that liver α-amylase may be a biomarker for obesity [34]. It has been hypothesized that α-amylase expressed in the brain may be an energy source in Alzheimer's disease [3]. Here, it was shown that the expression of α-amylase by Caco-2 cells was suppressed by RNA interference (RNAi), and affected cell proliferation and differentiation [33]. The expression of α-amylase was suppressed to about 30% by siRNA (small interfering RNA), and four kinds of cell differentiation markers were quantified by real-time PCR. All four differentiation markers were reduced in cells transfected with α-amylase siRNA compared to cells transfected with control siRNA (**Figure 9A**). This result indicates that α-amylase expression is required for cell differentiation. Furthermore, it was shown that the cell proliferation of cells transfected with α-amylase siRNA was dramatically reduced depending on the cell-seeding concentration (**Figure 9B**).

6. Conclusion

This study showed new functions of both exogenous and endogenous pancreatic α-amylase other than starch digestion in the small intestine. Exogenous α-amylase synthesized by the pancreas and secreted into the small intestinal tract enhances α-glucosidase activity and inhibits glucose uptake by SGLT1 in the small

intestine BBM. The exogenous α-amylase was internalized into lysosomes and degraded in intestinal epithelial cells. On the other hand, endogenous α-amylase is highly expressed in the duodenum, where it enhances cell proliferation and differentiation.

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