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# Nesfatin-1 stimulates glucagon-like peptide-1 and glucose-dependent insulinotropic polypeptide secretion from STC-1 cells *in vitro*

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ABSTRACT

Nesfatin-1 is an 82 amino acid peptide encoded in a secreted precursor, nucleobindin 2. It is an anorexigenic and insulinotropic peptide found abundantly in the hypothalamus, pancreas and gastric oxyntic mucosa. NUCB2 mRNA expression is 10 fold higher in the gastric mucosa than in brain, suggesting gastrointestinal tract as a main source of nesfatin-1. Meal responsive insulin secretion is regulated by incretins glucagon-like peptide-1 (GLP-1) and glucose dependent insulinotropic polypeptide (GIP). Since both nesfatin-1 and incretins modulate insulin secretion, we hypothesized that nesfatin-1 is present in the enteroendocrine cells, and that it regulates incretin secretion. RT-PCR analysis found NUCB2 mRNA expression, and immunofluorescence microscopy determined nesfatin-1 immunoreactivity in STC-1, an enteroendocrine cell line. NUCB2/nesfatin-1 is co-localized with GLP-1 and GIP in mouse small intestinal cells. Static incubation of STC-1 cells with nesfatin-1 upregulated preproglucagon (GLP-1 precursor) mRNA (0.01, 0.1, 1 and 10 nM) and GLP-1 secretion (0.1, 1 and 10 nM). Nesfatin-1 also enhanced GIP mRNA (0.1, 1 and 10 nM) and GIP secretion (1 and 10 nM). Together, our data support the hypothesis that nesfatin-1 is present in enteroendocrine cells and that it stimulates incretin secretion. Future studies should aim for nesfatin-1 and incretin interactions *in vivo*.



## 1. Introduction

Gastrointestinal (GI) hormones contribute to the regulation of insulin secretion and glucose homeostasis. Intestinal hormones glucagon-like peptide (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are secreted from L and K cells of the distal ileum and upper small intestine, respectively. Both GLP-1 and GIP, collectively named as incretins, stimulate insulin secretion from rat pancreas and human islets [1–3]. Under atypical physiological conditions including type 2 diabetes (T2D), the secretion of GLP-1 is impaired. However its insulinotropic and glucagonostatic activity are preserved [4]. Unlike GLP-1, GIP secretion is intact in patients with T2D, however its insulinotropic action is impaired [4]. GLP-1 analogue based drugs, for example, Byetta™ are already available as therapeutic agents for T2D [5]. The crucial role of GIP and its receptor in T2D were demonstrated in a variety of studies [2,6–8]. In addition, both GLP-1 and GIP have been shown to attenuate glucagon stimulated hepatic glucose production in both humans and rodents [9,10]. Together, incretins are important insulinotropic and glucoregulatory hormones with significant roles in energy homeostasis. In 2006, another peptide, nesfatin-1, with metabolic actions was reported [11].

Nesfatin-1 (NEFA/nucleobindin-2-Encoded Satiety and Fat-Influencing proteiN-1) is an 82 amino acid anorexigenic peptide encoded in the N-terminal region of its precursor, nucelobindin-2 (NUCB2) [11]. Administration of full length nesfatin-1 (1-82 amino acids) or its mid-segment (23-53, 30 amino acids), considered to be its bioactive core reduces food intake and fat mass [11,12] in rodents and fish [13]. Plasma nesfatin-1 concentrations are inversely correlated with glucose levels in rats and diabetic humans [14,15]. Our research group showed for the first time that nesfatin-1 and insulin are co-localized in the β cells of pancreatic islets [16], and that nesfatin-1 is insulinotropic [17]. Nesfatin-1 increases glucose stimulated insulin secretion (GSIS) by direct action involving $Ca^{2+}$ influx through L-type calcium channels [18]. Studies conducted thereon have confirmed NUCB2 expression in gastric mucosa [19]. The expression of NUCB2 mRNA is 10-fold higher in gastric mucosa of rats than in brain, suggesting stomach as the

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major source of circulating nesfatin-1. In addition, it crosses the blood-brain barrier via a non-saturable mechanism, providing the possibility that nesfatin-1 secreted peripherally may act centrally [20]. Overall, nesfatin-1 is now emerging as a multifunctional peptide [21] with reproductive [22], cardiac [23], and endocrine functions [24]. What tissues contribute to the post-meal increase in NUCB2/nesfatin-1 release? It has been shown that the stomach and pancreas are two major sources of endogenous NUCB2/nesfatin-1 [16,25]. We recently reported that NUCB2/nesfatin-1 mRNA and protein expression in the small and large intestines of C57BL/6 mice [26]. These results are in agreement with previous reports [27,28] that showed NUCB2/nesfatin-1 immunoreactivity (IR) in the duodenal submucosal layer of Sprague Dawley (SD) rats and in Institute of Cancer Research (ICR) mice.

While the above studies determined nesfatin-1 in tissues, the identity of NUCB2/nesfatin-1 expressing cells within the intestine is not known. Considering that nesfatin-1 is insulinotropic, and incretins, which are predominant insulinotropins are primarily secreted from the intestine, it warrants further research to determine whether nesfatin-1 is present in enteroendocrine cells. We hypothesized that nesfatin-1 is present in intestinal endocrine cells, especially those producing incretins, and that nesfatin-1 stimulates incretin secretion. This article presents our novel results on nesfatin-1 colocalization of GLP-1 and GIP in mice intestine. We also report the discovery that nesfatin-1 stimulates both the expression of mRNAs encoding GLP-1 (proglucagon) and GIP, and incretin secretion from STC-1 cells.

## 2. Materials and methods

### 2.1. Cell culture, RT-PCR and immunohistochemistry

STC-1 cells derived from intestinal enteroendocrine tumors were a kind gift from Dr. Timothy Kieffer (University of British Columbia, Vancouver, Canada). The cells were previously shown to secrete both GLP-1 [29] and GIP [30,31]. Cells were cultured as previously described [32]. Total RNA was extracted using TRIzol™ RNA isolation regent (Invitrogen, Catalog #15596-026) and cDNAs were synthesized using iScript™ reverse transcription supermix (Bio-Rad, Catalog #170-8840). Mouse NUCB2 (NM_001130479.2) [sense primer, 5′−CCAGTGGAAAATGCAAGGAT−3′ and antisense primer, 5′−GCTCATCCAGTCTCGTCCTC−3'; PCR conditions: denaturation: 95 °C (10 s), annealing: 61 °C (30 s) and elongation: 73 °C (30 s), 35 cycles, amplicon size: 202 bp] and mouse beta actin (NM_007393.3) [sense primer, 5′−CCACTGCCGCATCCTCTTCC−3′ and antisense primer, 5′−CTCGTTGCCAATAGTGATGAC−3'; PCR conditions: denaturation: 95 °C (10 s), annealing: 60 °C (30 s) and elongation: 73 °C (30 s), 35 cycles, amplicon size: 77 bp] mRNAs were detected using RT-PCR. The primers were validated for high primer efficiency and annealing temperatures. Gel electrophoresis (1.5% agarose) was conducted and images were captured using a gel imager (Gel Doc™ EZ system, Bio-Rad).

For immunohistochemistry, STC-1 cells were cultured in a Lab-Tek™ chamber slide system (Nalge Nunc, New York). Cells were washed with Dulbecco's phosphate buffered saline (DPBS, Life Technologies, Catalog #14190-250). The cells were then fixed in 4% paraformaldehyde for 15 min at 4 °C, permeabilized using 0.3% Triton-X (Bioshop, Catalog #TRX-777) and then washed with DPBS. For immunohistochemical studies small intestinal section were collected from *ad libitum* fed male C57BL/6J mice (Charles River, Quebec, Canada) cared under the Canadian Council of Animal Care guidelines, as approved by the University of Saskatchewan Animal Care Committee. Mice were euthanized by cervical dislocation. The intestinal sections were collected and fixed in 4% paraformaldehyde overnight at 4 °C and were processed and sectioned (4 μm thickness). These sections were deparaffinized with xylene (incubated twice in 100% xylene; 5 min at 25 °C) and rehydrated in graded ethanol series (incubated twice in 100% ethanol, once in each 95% ethanol, 70% and 50% ethanol, 2 min each at 25 °C). The sections were then incubated with 3% hydrogen peroxide in distilled water to block endogenous peroxidase activity (30 min at 25 °C). Sections were blocked in Antibody blocking buffer (10% protein block; DAKO, Cat#S0809, 3%BSA; Sigma A7906, and 100 uL of Triton-X 20%) for 10 min and permeabilized in 1% PBS-Triton-X for another 10 min. Following this, sections were incubated with primary antibodies overnight at room temperature followed by incubation with secondary antibody for 1 h at 37 °C. Primary antibodies used were: rabbit polyclonal anti-NUCB2 (custom antibody, Pacific Immunology, Catalog #1312-PAC-01, 1:200) for STC-1 cells, intestine (1:500) and mouse monoclonal anti-GLP-1 (Abcam, Catalog #ab26278, 1:500) for GLP-1 sections, mouse polyclonal NUCB2/nesfatin-1 (ENZO Life Sciences, Catalog #ALX-804-854-C100, 1:100) and rabbit polyclonal anti-GIP (Abcam, Catalog #ab22624; 1:500) for GIP sections. The respective secondary antibodies used were goat polyclonal anti-rabbit Texas Red® IgG (Red − NUCB2/nesfatin-1; Vector Laboratories, Catalog #T1-1000, 1:200), goat polyclonal anti-mouse FITC (Green − GLP-1; Abcam, Catalog #A-11034, 1:500) goat polyclonal anti-mouse Alexa Flour-594 (Red − Nesfatin-1; Invitrogen, Catalog # ab150108, 1:500) and goat polyclonal anti-rabbit Alexa Flour 488 (Green − GIP; Invitrogen, Catalog #A-11037, 1:500). Primary antibody pre-absorbed in 10 μg synthetic nesfatin-1 overnight was used as pre-absorption controls for STC-1 cells to confirm the antibody specificity. The slides were washed in 1X PBS and mounted using Vectashield® medium containing the nuclear dye DAPI (Blue; Vector Laboratories). Tissue and cells were analyzed under Nikon Eclipse-Ti inverted fluorescence microscope (Nikon, Canada), images were captured using a Nikon DS-Qi1 MC camera. Images were analyzed using NiS Elements basic research software on a Lenovo ThinkPad workstation. Since the nesfatin-1 antibody used here detects both nesfatin-1 and NUCB2, NUCB2/nesfatin-1 like immunoreactivity is used as the term to identify any immunostaining using this reagent.

### 2.2. Nesfatin-1 effects on preproglucagon/GIP mRNA expression and incretin secretion

For static incubation studies, STC-1 cells at $2 \times 10^5$ cells/well density were seeded in 1 mL DMEM (25 mM glucose) in 24-well plates. On the day of study, medium was removed and cells were washed twice with DPBS. The cells were then treated for 1 h with 1 mL of DMEM containing 0, 0.001, 0.01, 0.1, 1 or 10 nM synthetic rat full length nesfatin-1 (Abgent Technologies, USA, >95% purity; 17). Media samples were then collected and the levels of GLP-1 and GIP secreted into the media were measured using multi-species GLP-1 total ELISA kit (Millipore Inc., Catalog #EZGLP1T-36K) and rat/mouse GIP (total) ELISA kit (Millipore Inc., Catalog #EZRMGIP-55K) respectively. Cells were collected and quantitative PCRs (qPCR) were conducted using primers mouse preproglucagon (AF276754.1) [sense primer 5′−AATCTTGCCACCAGGGACTT−3′ and antisense primer 5′−AGTGACTGGCACGAGATGTT−3′, PCR conditions: denaturation: 95 °C (10 s), annealing: 56.3 °C (30 s) and elongation: 72 °C (30 s), 35 cycles, amplicon size: 112 bp] and mouse GIP (NM_008119.2) [sense primer 5′− ACAAAGAGGCACAGGAGAGC−3′ and antisense primer 5′−AGCCAAGCAAGCTAAGGTCA−3′, PCR conditions: denaturation: 95 °C (10 s), annealing: 60 °C (30 s) and elongation: 72 °C (30 s), 35 cycles, amplicon size: 180 bp]. Mouse beta actin (internal control) qPCR was conducted employing the primers and conditions described earlier. Amplification and detection of respective genes were performed in duplicates in each

experimental group for relative mRNA expression using the iQ™ SYBR® Green Supermix (Bio-Rad, Catalog #170-8880) on a CFX-Connect™ Real-Time PCR detection system (Bio-Rad, Canada). A melting curve analysis was carried out at 65 °C to 95 °C and the absence of any dimer formation or artifacts was confirmed. The PCR efficiency was 97% and relative gene expression data were obtained after normalizing the data using the Livak method [33].

### 2.3. Statistical analysis

Statistical analysis of RT-qPCR and immunoassay data were conducted using one-way ANOVA followed by Tukey's multiple comparison test. IBM SPSS™ version 21 (IBM., USA) was used for statistical analysis and GraphPad Prism version 5 (GraphPad Inc., USA) was used for generation of graphs. Significance was considered at $P < 0.05$. Data are expressed as mean + SEM (standard error of mean).

## 3. Results

### 3.1. NUCB2 mRNA expression in STC-1 cells

A band of approximately 200 base pairs was detected (Fig. 1A) in STC-1 cells. The internal control beta actin was also incorporated in the gel resulting in a band of approximately 100 base pairs. The no template negative control (NTC) reaction did not result in any bands.

### 3.2. NUCB2/nesfatin-1 immunoreactivity in the cytoplasm of STC-1 cells

Fluorescence microscopy and imaging showed NUCB2/nesfatin-1 immunoreactivity in the cytoplasm of STC-1 cells (**Red;** Fig. 1B; B–C). Nuclei are stained in DAPI (**Blue;** Fig. 1B; A and C). The no primary antibody negative control (Fig. 1B; E) and preabsorption control (Fig. 1B; D) showed no fluorescence confirming the specificity of the antibody used.

### 3.3. NUCB2/nesfatin-1 Co-localizes with GLP-1 and GIP immunoreactive cells in male C57BL/6J mice intestine

NUCB2/nesfatin-1 (**Red;** Fig. 2B and F), GLP-1 (**Green;** Fig. 2A) and GIP (Green; Fig. 2E) immunopositive cells were detected in small intestinal sections of male C57BL/6J mice. In addition, nesfatin-1 was found to be co-localized with GLP-1 and GIP immunopositive cells in small intestine (**Yellow;** Fig. 2C and G). Nuclei were stained with DAPI (**Blue;** Fig. 2A–H). The no primary antibody negative control (Fig. 2D and H) showed no fluorescence.

### 3.4. Nesfatin-1 stimulates GLP-1 and GIP release from STC-1 cells

Nesfatin-1 (0.01, 0.1, 1 and 10 nM) stimulated preproglucagon mRNA expression (Fig. 3A) and GLP-1 secretion (0.1, 1 and 10 nM) (Fig. 3B) from STC-1 cells at 1 h post-incubation. No effects were detected for other doses tested (Fig. 3A–B). Nesfatin-1 (0.1, 1 and 10 nM) also stimulated GIP mRNA expression (Fig. 3C) and GIP secretion (1 and 10 nM) (Fig. 3D) from STC-1 cells. No effects were detected for other doses tested (Fig. 3C–D).

## 4. Discussion

This research reports two main discoveries: 1. co-localization of NUCB2/nesfatin-1 and incretins GLP-1 and GIP in the intestinal enteroendocrine cells, and 2. nesfatin-1 stimulation of both GIP and GLP-1 mRNA expression and secretion from intestinal cells. Nesfatin-1 is an anorexigenic peptide that was found in several tissues including the hypothalamic feeding centers, pancreas and stomach of mice [11,16,19]. Presence of NUCB2 mRNA and protein expression was also reported in non-mammals including fish [22,34] and frogs [35]. More recently, the presence of NUCB2/nesfatin-1 in rat intestine was characterized by PCR, Western blot and/or immunohistochemistry [27]. NUCB2/nesfatin-1 IR cells were localized in the lower third and middle portion of the gastric mucosal gland and the submucous layer in the duodenum of SD rats and ICR mice [21]. Western blot analysis showed higher NUCB2 protein expression in pancreas, stomach and duodenum. Recent findings from our lab also showed NUCB2 mRNA and protein expression in the small and large intestines of male C57BL/6J mice [26]. The findings from current research reiterate and extend these findings. We confirm that NUCB2/nesfatin-1 is indeed present in the intestine and that it is localized to the enteroendocrine cells.

First, we showed that NUCB2 mRNA is expressed in intestinal endocrine STC-1 cells and found NUCB2/nesfatin-1 immunoreactivity in its cytoplasm. These results are highly suggestive that intestinal enteroendocrine cells could be a source of endogenous nesfatin-1 *in vivo*. Second, immunohistochemical staining of intestinal sections from male C57BL/6J mice showed NUCB2/nesfatin-1 immunoreactivity, confirming the findings using STC-1 cells. Nesfatin-1 immunopositive cells were observed in the mucosal crypts of the intestinal villi and in the submucosa, concentrated in the Brunner's glands. This is consistent with previous observations in the rat intestine [27]. The presence of nesfatin-1 IR here suggests that it may be involved in enzyme activation, nutrient absorption and preservation of intestinal walls which warrants further consideration [27]. Intestine is a major endocrine tissue, giving rise to a spectrum of hormones including incretins that has important functions on feed intake and energy metabolism. Since we found nesfatin-1 IR in small intestinal sections and since our previous findings clearly shows its protein and mRNA expression in the intestine [26], we hypothesized that nesfatin-1 could be colocalized with incretins. To address this, immunohistochemical analysis of intestinal sections from *ad libitum* fed male C57BL/6J mice were conducted. The main new finding here was that NUCB2/nesfatin-1 is co-localized with GLP-1 and GIP in small intestine. The co-localization was found distributed throughout the mucosa and submucosa, especially in the crypts of the villi. However, not all cells observed under a given area of the section showed co-localization. There were some cells that were immunopositive for NUCB2/nesfatin-1, but not for incretins. The NUCB2/nesfatin-1 immunopositive enteroendocrine cells were largely present in the base of the villi and in the crypts, and exhibited the rounded or flask shaped morphology typically exhibited by the intestinal hormone producing cells. The colocalization of nesfatin-1 with intestinal hormones suggests possible local (autocrine/paracrine) or endocrine actions of nesfatin-1 on these peptides. Our next aim was to determine whether nesfatin-1 modulates incretins.

*In vitro* static incubation studies found a dose-dependent stimulatory effect for nesfatin-1 on GLP-1 (proglucagon) and GIP mRNA expression, and GLP-1 and GIP secretion into media. Nesfatin-1 effect on GLP-1 expression and secretion is highly dose-dependent, with the upregulation quite apparent between 0.1 and 10 nM when compared to controls. One common concern regarding the RT-qPCR data on the relative mRNA expression of proglucagon is the specificity of the primer used, as proglucagon encodes multiple peptides including GLP-1, GLP-2, oxyntomodulin and glicentin in intestinal L cells *in vivo*. The primers used in this study are highly specific for the GLP-1 coding region of proglucagon mRNA. The dose-dependent effects on GIP expression and secretion are also clear, with nesfatin-1 having a stimulatory effect at 1 and 10 nM. Nesfatin-1 at an effective dose of 0.1 and 1 nM was reported to upregulate preproinsulin mRNA expression and glucose-dependent insulin release from MIN6 cells [31]. It also stimulated

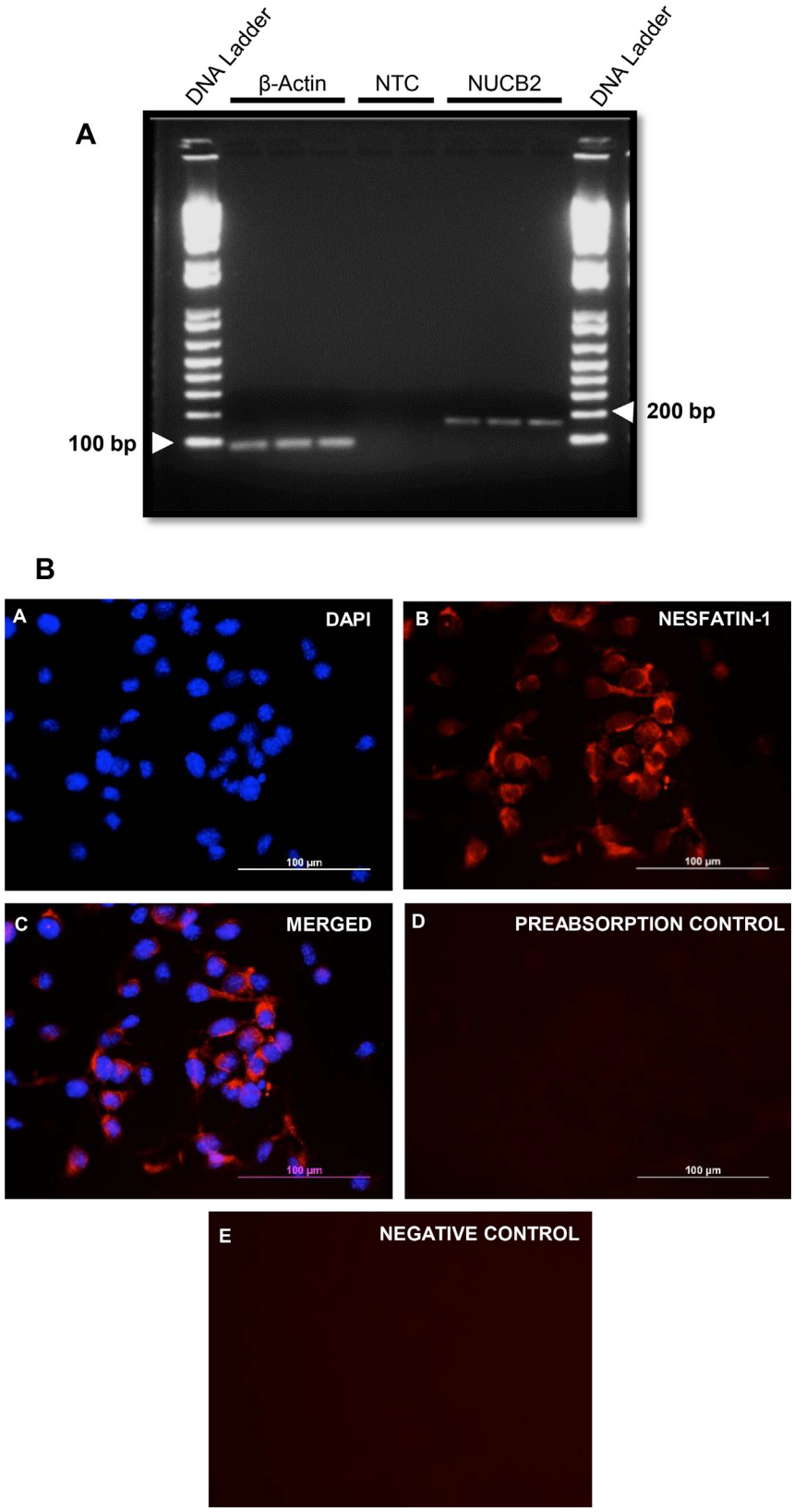


**Fig. 1. A**. Gel electrophoresis image showing a band of approximately 200 basepairs representing NUCB2 in STC-1 cells. No amplicon was detected in the negative control PCR reaction without the template cDNA (NTC). **B**. Photomicrographs showing nesfatin-1 (**Red; B and C**) and nuclear stain DAPI (**Blue; A and C**) in STC-1 cells. The preabsorption control (**D**) and no-primary antibody negative control (**E**) showed no immunofluorescence confirming the specificity of the antibody used. Scale bar = 100 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

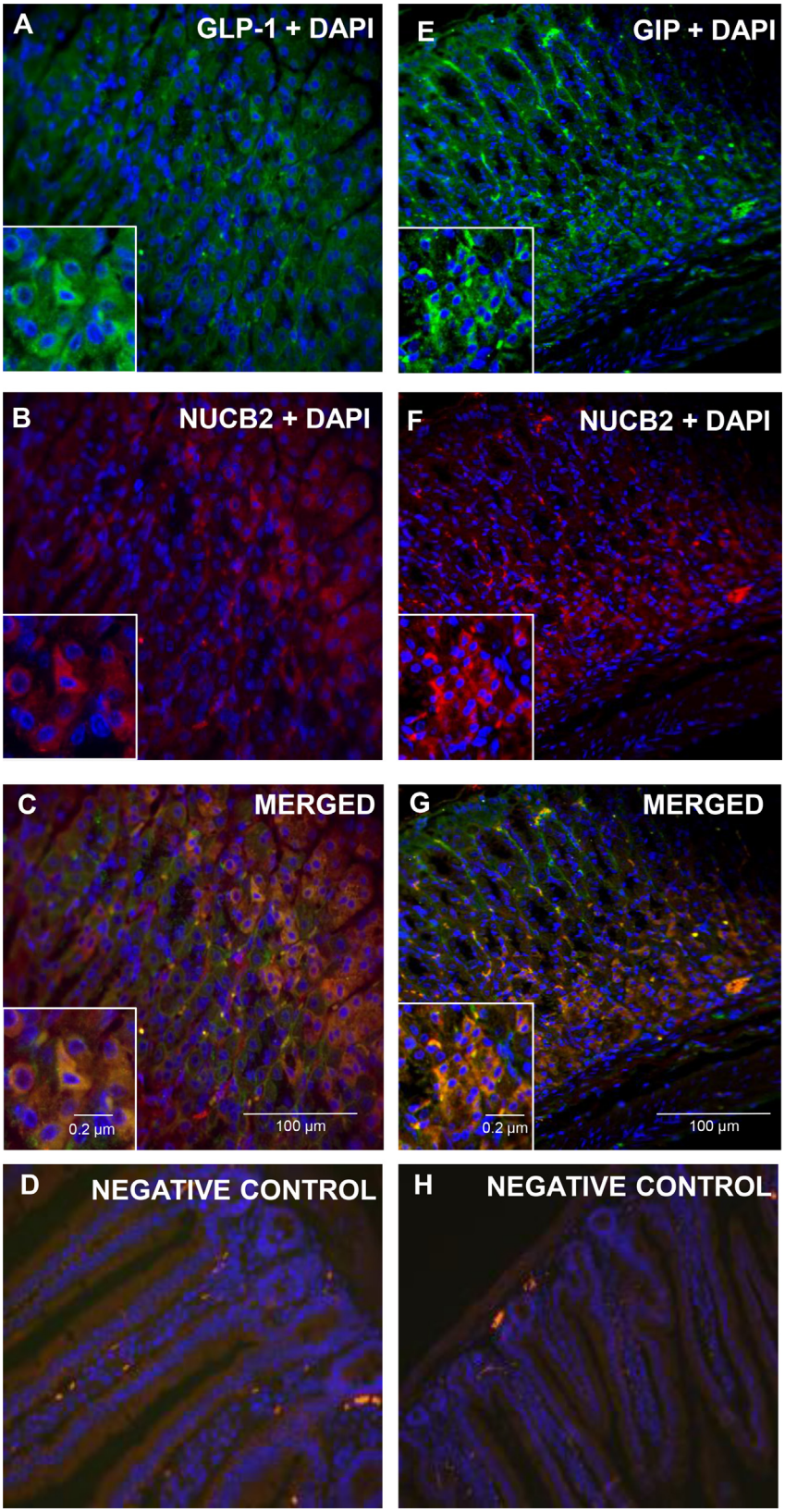


**Fig. 2.** Photomicrographs showing NUCB2/nesfatin-1 IR (**Red; B and F**), GLP-1 (**Green; A**), GIP (**Green; E**) and co-localization of NUCB2 IR with GLP-1 and GIP IR (**Yellow; C and F**) in mice small intestine. Nuclei were stained with DAPI (**Blue; A-H**). No NUCB2/nesfatin-1 and/or GLP-1, GIP staining were detected in secondary antibody alone controls **(D and H)**. Scale bar = 100 µm, scale bar of inset = 0.2 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

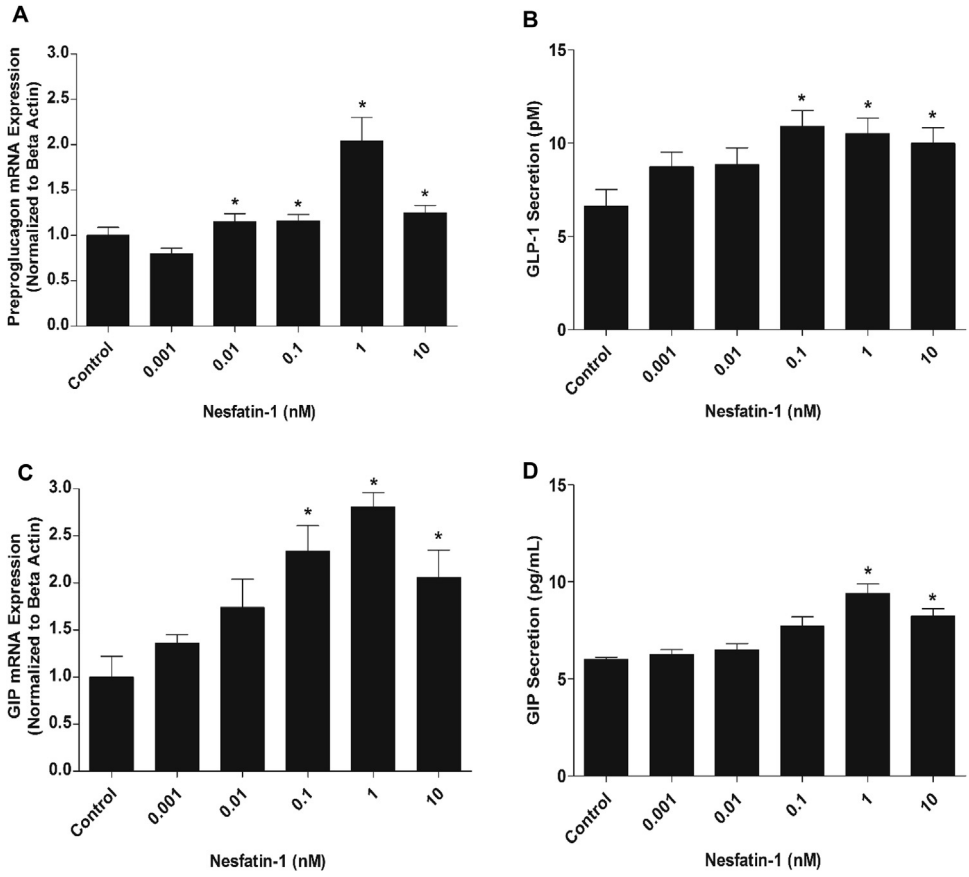


**Fig. 3.** Nesfatin-1 enhances preproglucagon (GLP-1 precursor) mRNA expression (0.01, 0.1, 1 and 10 nM) (**A**) and stimulates GLP-1 secretion (0.1, 1, 10 nM) (**B**) ($p < 0.05$, significance denoted by *) from STC-1 cells compared to no treatment controls and other doses tested. It also enhances GIP mRNA expression (0.1, 1, 10 nM) (**C**) and GIP secretion (1 and 10 nM) (**D**) ($p < 0.05$, significance denoted by *) from STC-1 cells compared to no treatment controls and other doses tested. One-way ANOVA followed by Tukey's multiple comparison test was used for statistical analysis. Data are presented as mean + SEM. n = 8 wells/treatment pooled from two different studies.

insulin release from isolated mouse islets corresponding to 0.01, 0.1 and 1 nM doses [17]. Our data on nesfatin-1 stimulation of incretins from STC-1 cells were detected at comparable doses. It is currently unknown whether the stimulatory effects of nesfatin-1 on incretins are also seen *in vivo*, and this aspect requires further research. GLP-1 and GIP are metabolic and insulinotropic peptides [1,2,36]. Our discovery that nesfatin-1 directly stimulates the expression and secretion of incretin hormones provides new insights into nesfatin-1 action. Whether GLP-1 and/or GIP are critical for mediating the metabolic and insulinotropic effects of nesfatin-1 require additional studies.

The current research provides strong evidence for NUCB2/nesfatin-1 expression in intestine thus strengthening previous observations [26,27]. It also for the first time reports the co-localization of nesfatin-1 with GLP-1 and GIP immunopositive cells in mouse intestine. The studies carried out also characterized STC-1 cells as NUCB2/nesfatin-1 expressing cells and that nesfatin-1 dose-dependently modulates hormone secretion from this cell line. This highlights STC-1 cells as useful *in vitro* models for studying nesfatin-1 biology, besides the secretion kinetics of intestinal hormones. Nesfatin-1 has been primarily researched for its role in insulin secretion and satiety [11,17,37]. It is becoming increasingly apparent that nesfatin-1 has additional unknown functions, and the discovery that nesfatin-1 stimulates incretin secretion is a major contribution in line with this notion. The results that nesfatin-1 is co-expressed with incretins and that it modulates GLP-1 and GIP secretion provide a new biological action for nesfatin-1. Future studies should focus on nesfatin-1 regulation of incretins *in vivo*, and the mechanism by which nesfatin-1 regulates incretins.

## Conflict of interest

None.

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## Transparency document

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