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A Luminescent Sensor for Investigating Serotonin Metabolism in Neuroendocrine Cancer

Dane H. Tow[1], **Carlos H.F. Chan**[1,2], **James R. Howe V**[1,2], **Po Hien Ear**[1,2,*]

[1]Department of Surgery, University of Iowa Carver College of Medicine, Iowa City, IA

[2]Holden Comprehensive Cancer Center, University of Iowa Carver College of Medicine, Iowa City, IA

Abstract

Background: Serotonin is emerging as a promising therapeutic target in tryptophan hydroxylase 1 (Tph1)-positive tumors, but further mechanistic studies are needed to effectively target dysregulated serotonin metabolism. One challenge is a lack of methods for studying the dynamic nature of serotonin metabolism. Here we report the development of a genetically encoded luminescent biosensor, termed iSero-Rluc, for the real-time detection of serotonin in live cells.

Methods: The engineered serotonin binding domain (iSero) and *Renilla* luciferase (Rluc) reporter were cloned into yeast and mammalian expression vectors to create a fusion protein that could act as a biosensor to detect endogenous serotonin levels in live cells. The iSero-Rluc biosensor was stably expressed in the BON cell line and luciferase assays, mass spectroscopy, immunofluorescence, and western blotting were used to study serotonin metabolism under different cell culture conditions.

Results: The iSero-Rluc sensor detected exogenous serotonin in a yeast model. When stably expressed in the BON cell line, iSero-Rluc revealed that serotonin biosynthesis is increased in an anchorage-independent growth state and is induced upon serum starvation.

Conclusions: The iSero-Rluc biosensor is a powerful tool in the study of tumor serotonin metabolism. It enabled real-time detection of alterations in serotonin synthesis in living cells under various growth conditions and has the potential to provide greater insight into serotonin metabolism in different stages of tumor progression and to identify therapeutic strategies to target cancer metastases and carcinoid crisis.

[*]Correspondence should be addressed to Po Hien Ear, Po Hien Ear, PhD, Research Assistant Professor, Department of Surgery, University of Iowa, 375 Newton Road, MERF 4235, Iowa City, IA 52242, Phone: (319) 335-8983, pohien-ear@uiowa.edu.

We describe the development of a luminescent serotonin sensor that can detect dynamic changes of serotonin levels in neuroendocrine cancer cells under different growth conditions. This work provides valuable contributions to the understanding of neuroendocrine tumors and the identification of effective therapies for metastatic disease and carcinoid syndrome.

Declaration of interest

We declare no conflict of interest.

Keywords

serotonin; luminescent biosensor; neuroendocrine tumor; different growing conditions; TPH1

INTRODUCTION

Peripheral serotonin biosynthesis is catalyzed by the rate-limiting enzyme tryptophan hydroxylase 1 (Tph1). Dysregulated serotonin metabolism is theorized to contribute to the progression of multiple solid tumors including cholangiocarcinoma, pancreatic, breast, prostate, lung, colon, and neuroendocrine, with most data derived from *in vitro* studies [1]. An increasing number of *in vivo* studies have shown anti-tumor effects of serotonin synthesis inhibition in Tph1-positive tumors including cholangiocarcinoma, pancreatic adenocarcinoma, and colon cancer models [2] [3]. Additionally, our lab has recently demonstrated that inhibition of Tph1 reduces tumor growth in a neuroendocrine cancer model *in vivo*, a cancer type frequently associated with Tph1 overexpression and aberrant serotonin metabolism [4]. In our study, reduction of serotonin production via Tph1 inhibition leads to a reduction of tumor vascular density [4]. These results suggest that serotonin produced by the neuroendocrine cancer cells acts in an exocrine fashion on tumor-associated stromal cells, alters the tumor microenvironment (TME), and possibly primes the metastatic niche.

Similar to serotonin's functions as a neurotransmitter in the central nervous system [5], the mechanism of serotonin synthesis, release, and action is likely highly regulated and dynamic within the TME but is understudied. One of the major barriers for advancing the knowledge of serotonin system in cancer biology is the rapid dynamics of serotonin metabolism and the difficulty in the spatial and temporal resolution of cellular and tumor serotonin [6]. Mass spectroscopy is the current gold-standard technique for serotonin measurement yet is limited to end-point experiments, requires a large tissue sample, and does not allow *in situ* analysis. Neither ELISA nor immunohistochemistry techniques can easily capture time dynamics, and both are limited in sensitivity and specificity by the quality of commercial anti-serotonin antibodies.

To address the lack of innovative methods for detecting serotonin in real-time, Unger et al. engineered a serotonin binding protein (iSero) with high specificity to serotonin coupled with a green fluorescent protein as a fluorescence-based serotonin sensor (iSeroSnFR), enabling real-time detection of neuronal serotonin *in vitro* and for brain tissue sections [7]. We aimed to leverage this technology for our own studies in neuroendocrine cancer models; however, a fluorescent reporter is not ideal for *in vivo* tumor biology research. Biological tissues and many pharmaceutical compounds exhibit auto-fluorescence, masking the signal from the sensor. Therefore, our objective in this study was to generate a luminescent serotonin sensor by constructing a fusion protein of the iSero domain and the *Renilla* luciferase (Rluc) reporter (iSero-RLuc). We hypothesized that different growth conditions found in the TME and during distinctive stages of metastasis affect serotonin biosynthesis in neuroendocrine tumor cells and the iSero-Rluc sensor can be used to study these processes.

MATERIALS AND METHODS

Reagents and equipment

Reagents used included Ampicillin (Sigma; cat #A9393), Puromycin (AdipoGen; cat #AGCN20078), Serotonin (Sigma; cat #H9523), Resazurin (MedChemExpress; cat #HY-111391), insulin-like growth factor I (IGF-1), hepatocyte growth factor (HGF), epidermal growth factor (EGF), and basic fibroblast growth factor (FGF-2) (PeproTech; cat #100–11, #100–39, #AF-100–15, #100–18B). A 200 Infinite Microplate reader (TECAN, cat #30190085) was used for luminescent intensity, optical density, fluorescent intensity, and absorbance measurements. A CKX53 microscope, DP74 camera, and cellSens software (Olympus) were used for fluorescent microscopy.

Antibodies

Primary antibodies and dilutions: anti-*Renilla* luciferase (Sigma; cat #MAB4410) at 1:2000; anti-actin (Sigma; cat #A2066) at 1:2000, anti-serotonin (Sigma, cat #S5545) at 1:1000 for IF; anti-TPH1 (Sino Biological; cat# 11931-R145) at 1:500; anti-pTPH1-S58 (R&D Systems; cat #PPS039) at 1:1000. Secondary antibodies and dilutions: anti-mouse HRP-conjugated IgG (Cell Signaling Technology; cat #7076) at 1:3000; anti-rabbit HRP-conjugated IgG (Jackson Labs; cat #111–035-003) at 1:10000; anti-rabbit Fluorescein-conjugated IgG (Jackson Labs; cat #111–095-003) at 1:500.

Study design

We first generated the serotonin sensors via molecular cloning, stably expressed them in budding yeast, and characterized them for luciferase activity. After we identified the iSero-Rluc_NT construct as a promising sensor for detecting cellular serotonin, we stably express this sensor in the BON neuroendocrine cancer cells [8]. Luciferase assays and western blot were used to confirmed stable sensor expression. We utilized the BON-iSero-Rluc cells to study effects of various growth states and nutrient stimulation/deprivation conditions on serotonin synthesis. We supported the BON-iSero-Rluc data with parallel immunocytochemistry and mass spectroscopy experiments. In addition, we performed western blotting for total and phosphorylated Tph1 to gain greater mechanistic insight into the effects of nutrient deprivation on serotonin synthesis. No Institutional Review Board (IRB) approval was required for this study.

Design and cloning of luminescent serotonin sensor

We designed two versions of the serotonin sensor containing the Rluc reporter (Figure 1A). Rluc was chosen because its enzymatic activity only requires O_2 and coelenterazine (CLZ) and displays a linear relationship with substrate availability [9]. The iSero-Rluc_NT construct contains the native Rluc with a two amino acid (a.a.) linker (SG) for enhanced flexibility. The iSero-Rluc_CP construct contains a circular permutation of Rluc sequence, cut site between a.a.110–111, linked with a flexible eight a.a. sequence (GGGGSGGG). In both constructs, the iSero sequence is divided into N-terminal (iSero-F1) and C-terminal (iSero-F2) sections, which flank Rluc. When bound to serotonin, the iSero and Rluc form

distinct domains (Figure 1B). Serotonin promotes the folding of the Rluc reporter and increase luminescent activity (Figure 1C). Without serotonin, Rluc enzymatic activity is low.

For the cloning of the sensor, all primers and DNA sequences were purchased from Integrated DNA Technologies. Rluc F1 and F2 gene fragments were amplified by PCR using primers listed on Table 1, *Taq* polymerase, and the following PCR conditions: 32 cycles consisting of 60 seconds at 95°C, 45 seconds at 55°C, and 60–120 seconds at 72°C. For Rluc_NT, F1 was amplified with primer pair Native-F1 and F2 with primers Native-F2. For Rluc_CP, F1 was amplified with primer pair Circ-F1 and F2 with primers Circ-F2. PCR products were purified, digested with *BamH1* restriction enzyme, and ligated with T4 DNA ligase to form complete Rluc_NT and Rluc_CP gene sequences. These sequences were then double digested with *Bspe1* and *Spe1* restriction enzymes and ligated into the linearized p413-Gal1-iSero vector. Ligation products were transformed into DH5α competent cells (ThermoFisher; cat #EC0112) using heat shock technique and plated on Luria Broth (LB) + ampicillin agar dishes. Colony PCR was used to select and screen colonies. Positive colonies were transferred to LB liquid culture and grown overnight at 37°C with shaking. Plasmids were purified from bacterial cultures using the GeneJet Plasmid Miniprep kit according to the manufacture's protocol (ThermoFisher; cat #K0701).

To prepare the sensor for mammalian expression, the iSero-Rluc_NT gene was amplified by PCR from the p413-Gal1-iSero-Rluc_NT plasmid using primer pair iSero-pLVX and subcloned into the linearized pLVX-TetOne-Puro vector using *EcoR1* and *Bgl11* restriction sites. This vector was transformed in high-efficiency competent E. coli (Takara; cat #636763) using heat-shock technique and positive colonies were determined by colony PCR following ampicillin selection. Plasmids were purified according to previously described protocol.

Recombinant yeast luciferase assays

BY4741 budding yeast strain was used for transforming the following plasmids: p413-Gal1 empty vector, p413-Gal1-iSero-Rluc_NT and p413-Gal1-iSero-Rluc_CP. Yeast were plated on synthetic complete medium lacking histidine (SC-His) agar plates and incubated at 30°C for 2–3 days until colonies formed. Well-defined colonies were inoculated and grown in liquid SC-His medium supplemented with 2% glucose for 48 hours at 30°C with shaking. Yeast were transferred to new SC-His medium containing 2% D-(+)-raffinose (RPI; cat #R20500) and incubated for another 24 hours to deplete glucose. To induce expression of the iSero-Rluc genes, medium was supplemented with 2% D-(+)-galactose (RPI; cat #33000) for 2 hours prior to performing the luciferase assay. Yeast cultures were thoroughly mixed, and aliquots of yeast suspension were plated in a 96-well flat bottom white plate. Water-soluble coelenterazine (CLZ-) (NanoLight; cat #3032) was dissolved in phosphate-buffered saline (PBS) and added to each well at a final concentration of 5 μM and the plate was incubated at 25°C in the dark for 2 minutes. Luminescent intensity (1 sec integration time) followed by optical density were measured.

Mammalian cell culture and generation of BON-iSero-Rluc cell line

All mammalian cell lines were maintained at 37°C under 5% CO_2. BON cells were obtained from Dr. M.B. Evers and Dr. C.M. Townsend. The BON cell line is derived from a lymph node metastasis of a serotonin-secreting pancreatic neuroendocrine tumor resected from a 28-year-old male patient [8]. BON cells were grown in DMEM/F12 medium supplemented with 10% Fetal Bovine Serum (FBS), 100 Units/mL penicillin + 100 μg/mL streptomycin, and 2 mM L-glutamine (Gibco, cat #26140079, #15140122, and #25030081). TSA201 cells were cultured in DMEM medium supplemented with 10% FBS and 1 mM pyruvate. The pLVX-TetOne-Puro-iSero-Rluc plasmid was transfected into TSA201 cells along with the pCMV_VSV-G and pCMV_psPAX2 vectors (addgene; cat #8454 and #12260) in a 10:1:9 ratio with the PolyFect Transfection Reagent (Qiagen; cat #301105). Following 16 hours incubation, medium was exchanged for DMEM/F12 + 30% FBS. After an additional 24-hour incubation, lentivirus-containing medium was collected, filtered through a 0.45 μm low protein binding syringe filter, and supplemented with 8 μg/mL polybrene (Sigma; cat #TR-1003). BON cells at 60% confluency were transduced with lentivirus twice consecutively for 24 hours each. Virus-containing medium was replaced with fresh medium and stables clones were selected using 2.5 μg/mL puromycin. Colonies with stable expression of iSero-Rluc were expanded and characterized.

Luciferase assays of BON-iSero-Rluc cells

BON-iSero-Rluc cells were seeded in 96-well plates at 5,000–10,000 cells/well. Plates were incubated 24 hours to facilitate cell attachment. Water-soluble CLZ (Biotium; cat #10126) dissolved in DPBS was added directly to the culture medium for a final concentration of 5 μM. Following a 2-minute incubation, luminescence (250–500 ms integration time) was recorded from each well every 30 seconds for 5 minutes while the plate was held at 37°C. Following luminescent reading, medium was removed, wells were washed with DPBS, cells were lysed with radioimmunoprecipitation buffer, and protein concentration of cell lysates was measured via bicinchoninic acid assay (ThermoFisher; cat #23225) to normalize luminescence to total protein. For determining sensor activity in each condition, raw relative luminescent unit (RLU) values were plotted against time. Then, the area under the curve (AUC) of this plot was calculated and used as a measure of overall Rluc activity. RLU and AUC values are unitless and therefore were normalized to the control condition for each experiment.

For adherent vs suspension assay, BON-iSero-Rluc cells were seeded in standard 96-well or ultra-low attachment plates at 10,000 cells/well and incubated for 24 hours. Immediately prior to luminescent assay, both adherent and suspension cells were dissociated into a single-cell suspension with trypsin to minimize differences from the two conditions unrelated to sensor activity from serotonin levels. In addition, resazurin metabolic reagent was used for normalization of cell number rather than total protein quantification.

Western blotting

Cells were lysed in LDS Sample Buffer (Invitrogen; cat #NP0008) supplemented with 50 mM DTT. Cell lysates were briefly boiled, separated on a 4–12% polyacrylamide gel (Invitrogen; cat #NP0323BOX), and transferred to PVDF membranes. Blots were

incubated overnight at 21°C with primary antibodies. Blots were then hybridized for 1 hour with corresponding HRP-conjugated secondary antibodies. Chemiluminescent detection was performed using ECL substrate (BioRad; cat #1705060).

Mass spectroscopy

BON cells grown as adherent or suspension in 6-well plates were washed with ice-cold DPBS, flash frozen with liquid nitrogen, and stored at −80°C. Gas chromatography coupled to mass spectroscopy (GC-MS) was performed by the University of Iowa Proteomics Facility.

Immunofluorescence staining

BON cells were washed with DPBS, fixed in 4% paraformaldehyde, and permeabilized with 0.25% Triton X-100. Cells were washed with 3% BSA in DPBS and incubated overnight at 21°C with anti-serotonin antibody. Cells were then incubated with FITC secondary antibody for 1 hour, washed, and mounted on glass slides with anti-fade mounting medium containing DAPI (Invitrogen; cat# S36967). Fluorescent images were acquired with a 200 ms exposure and 200X magnification.

Data analysis and statistics

ImageJ was used for calculating fluorescent intensity of immunofluorescence data. GraphPad v9.4.1 was to perform statistical analyses (GraphPad Software). Between group comparisons were made with two-tailed t-test.

RESULTS

Characterization of a functional luminescent serotonin biosensor

Saccharomyces cerevisiae (budding yeast) was selected as a model for proof-of-principle testing of the sensor constructs since stable protein expression can easily be achieved using plasmid systems. Plasmids containing no insert, the iSero-Rluc_NT, and the iSero-Rluc_CP genes under the Gal1inducible promoter were stably transformed in yeast. Following induction of sensor expression with exogenous galactose, luciferase assays in live yeast were performed in the presence of CLZ and serotonin (10 μM) or vehicle control. As shown in Figure 1D, the iSero-Rluc_NT sensor had a luminescence intensity ~2-fold higher in the presence of 10 μM serotonin compared to the control condition. For iSero-Rluc_CP, no significant difference in luminescent intensity between the 0 and 10 μM serotonin conditions was observed. Minimal luminescence was observed from the control yeast sample, attributable to auto-oxidation of CLZ in aqueous solution (Figure 1D). Further characterization of the iSero-Rluc_NT sensor with 0.1–500 μM exogenous serotonin revealed a dose-response relationship between serotonin concentration and Rluc reporter activity (Figure 1E).

The iSero-Rluc biosensor expressed in neuroendocrine cancer cell line identifies elevated serotonin synthesis in an anchorage-independent growth state

After identifying a promising serotonin sensor with the yeast model, we stably expressed this sensor in the neuroendocrine cell line BON [8]. We confirmed the BON-iSero-Rluc cells expressed the sensor via western blot and detected iSero-Rluc as a 66 kDa band (Figure 2A). Using water-soluble CLZ, robust luminescent signal was observed from the BON-iSero-Rluc cells, and minimal background luminescence was observed from wildtype BON cells, compared to the luminescence from auto-oxidation of CLZ (Figure 2B&C). Luminescence from parental BON cells is attributable to the oxidation of CLZ by cellular reactive oxygen species [10].

Recent findings suggest BON cells cultured in a suspension growth state secrete more serotonin than their adherent counterparts [11], however no direct measurement of intracellular serotonin has been made. We detected a greater Rluc activity in BON-iSero-Rluc cells cultured in suspension growth condition than cells in the adherent condition (Figure 3A). Area under the curve (AUC) analysis, described in the methods, revealed a statistically significant 2.10-fold (± 0.06) increase in Rluc activity in the suspension condition compared to adherent (Figure 3B). In addition, mass spectroscopy analysis confirmed a 1.87-fold (± 0.27) increase in serotonin levels in the suspension growth state (Figure 3C) and immunofluorescence (IF) staining revealed a 2.06-fold (± 0.53) increase in intracellular serotonin in suspension BON cells (Figure 3D&E). Considering the high level of agreement between these three experimental methods, we are confident that serotonin biosynthesis is elevated in BON cells in an anchorage-independent growth state.

Serum starvation induces serotonin biosynthesis in BON cells

Based on our finding that serotonin biosynthesis is elevated in an anchorage-independent growth state, we hypothesized that serotonin metabolism is important for tumor cell biology during the different stages of tumor metastasis. First, we investigated the effect of growth factor stimulation on serotonin synthesis with our BON-iSero-Rluc cell line. It's been reported that NETs proliferation *in vitro* is greatly dependent on growth factor (GF) signaling [12, 13] [14]. We stimulated the BON-iSero-Rluc cells for 1 or 24 hours with insulin-like growth factor I (IGF-1), hepatocyte growth factor (HGF), epidermal growth factor (EGF), or basic fibroblast growth factor (FGF-2) and measured sensor activity. Differences in Rluc activity were statistically significant, however the magnitude of change (2–10% variance from the control condition) was minor indicating little effect of GF stimulation on serotonin synthesis (Figure 4A&B). We also tested stimulation with each GF at a 10-fold higher concentration but did not observe any significant difference with the lower GF concentrations (data not shown). Interestingly, at the 1-hour timepoint, GF stimulation appeared to slightly reduce serotonin synthesis.

We revised our hypothesis to consider if lack of GFs and other cellular nutrients may induce serotonin synthesis. We assessed the effect of serum starvation in BON-iSero-Rluc cells by culturing them in medium with 10% fetal bovine serum (FBS) and 0% FBS for 24 hours. We found a significant increase in sensor activity in serum starved cells (Figure 4C). We performed western blot analysis of the Tph1 protein to investigate if this is due to an

increase in Tph1 expression level or due to Tph1 enzyme activation by phosphorylation at serine 58 (p-Tph1-S58) [15]. Our data showed no increased in total Tph1 level and a significant increase in p-Tph1-S58 when cells are grown in medium with 0% FBS (Figure 4D). We then supplemented with 0–8% FBS and compared sensor activity in cells cultured in control medium (10% FBS). Serum starvation significantly induced serotonin biosynthesis as sensor activity increased 2.33-fold (± 0.16) in the 0% FBS condition, with an evident dose-response relationship (Figure 4E). This was corroborated by the detection of increasing p-Tph1-S58 levels by western blot analysis (Figure 4F). We then tested serum starvation (1% FBS) at multiple timepoints to determine when serotonin synthesis begins to be upregulated (Figure 4G). Surprisingly, serotonin synthesis was substantially upregulated only 30 minutes following serum starvation, with maximum levels observed at 2 hours, again supporting post-translation modification of Tph1 via phosphorylation is the mechanism of serotonin upregulation (Figure 4H).

DISCUSSION

Serotonin metabolism is highly dynamic, and its physiological effects are exerted through diverse mechanisms including serotonin (5HT) receptor signaling, protein serotonylation, and free radical scavenging [1] [16] [17]. Understanding the role of serotonin metabolism during the development, progression, and therapeutic resistance of neuroendocrine cancers and other Tph1-positive cancers as well as carcinoid syndrome and carcinoid crisis is of great clinical importance. Progress on this research has been slow due to the challenging nature of studying serotonin metabolism in tumor cells [6]. By modifying an existing fluorescent serotonin sensor [7], we developed a novel genetically encoded serotonin biosensor termed iSero-Rluc that enables real-time luminescent detection of serotonin in live cells. iSero-Rluc gives strong luminescent signal with water-soluble native CLZ, providing simple and rapid experimental set-up, a high signal to noise ratio, and reproducible results.

The role of serotonin metabolism in the TME is not fully understood. Employing our BON-iSero-Rluc sensor in live cells, we determined that serotonin levels are elevated in an anchorage-independent growth state. Anchorage-independence is a prerequisite for cancer cells to metastasize to distant organs, therefore we hypothesize serotonin may confer an increased metastatic potential. Serotonin is known to promote fibroblast proliferation and upregulate profibrotic factor secretion [18]. In addition, serotonin induces hepatic IGF-1 secretion via $5HT_{2B}$, which in turn promotes NET cell proliferation [12, 13]. We evaluated the effect of IGF-1, HGF, EGF, and FGF, on serotonin synthesis using the iSero-Rluc sensor. We did not observe any substantial difference in serotonin synthesis at either timepoint or growth factor concentration. Interestingly, we observed that serum starvation upregulates serotonin biosynthesis. Our iSero-Rluc sensor assay detected an increase in serotonin levels when cells undergo serum deprivation. Considering that BON cells are highly dependent on serum supplementation and die after a few days in serum-free medium (unpublished observations), we believe this upregulation of serotonin synthesis is a stress-response mediated effect. In addition, our results suggest that the upregulation of serotonin synthesis occurs rapidly and is maintained over time, pointing to involvement of post-translational modifications. Phosphorylation of Tph1 at serine 58 has been reported to increase its enzymatic activity [15]. We found levels of phosphorylated Tph1 increased in serum

starved BON cells while total Tph1 remained constant. It's been previously reported that hypoxia induces serotonin synthesis in enterochromaffin cells through a similar mechanism [19]. Current theories of the solid TME posit that highly proliferating cells are on the exterior, necrotic cells make up the core, and quiescent cells fill the intervening space [20]. Regulation of serotonin metabolism may vary spatially across the tumor depending on local nutrient availability.

Serotonin is clinically significant in the surgical management of neuroendocrine tumors. Carcinoid crisis has been shown to be more likely to occur in patients with higher baseline serum serotonin levels, although the onset of carcinoid crisis is unpredictable solely based on serotonin levels [21]. While octreotide is being used for peri-operative management, its efficacy has been debated [22]. Given the potential effect of serotonin on vascular structures and its rapid dynamic process, studies on serotonin metabolism and serotonin targeting treatments can be an important avenue of investigation for preventing carcinoid crisis and reducing its incidence. *In vitro* drug screening with the BON-iSero-Rluc model and development of a BON-iSero-Rluc *in vivo* mouse model could be useful systems to pursue this line of research. Currently, we can detect signal from the iSero-Rluc sensor in a xenograft model when the substrate is delivered by tail vein injections and not by intraperitoneal injections. This restriction represents a limitation of the usage of this mouse model since tail vein injections are more difficult to perform. Often it is associated with high variability. Having a version of the substrate that could be injected into the peritoneal cavity and easily up taken systemically would resolve this issue.

The work described here provides a novel luminescent biosensor to investigate hypotheses on serotonin metabolism in Tph1-positive tumors. We have found serotonin synthesis to be upregulated under cellular stress *in vitro*, indicating the role of the TME in modulating serotonin metabolism. The improved study of serotonin metabolic pathways is essential for understanding the behavior of this oncometabolite in tumor biology and devising novel therapeutic strategies to target tumor serotonin metabolism.

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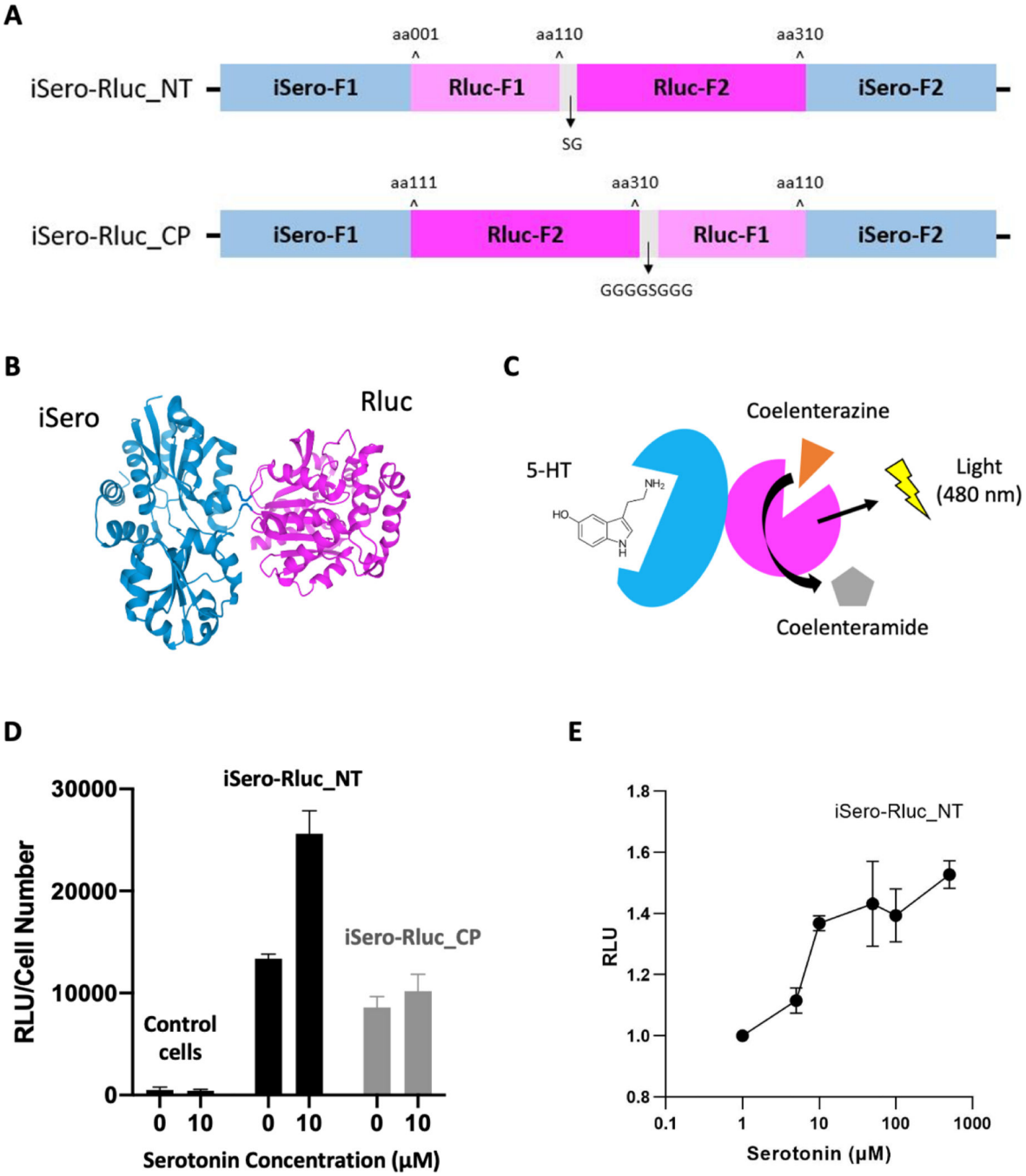

Figure 1.

Design and characterization of a luminescent serotonin sensor in budding yeast. **A)** Diagram of 2 versions of the serotonin sensor gene constructs based on the iSero protein and *Renilla* luciferase as a reporter (iSero-Rluc). iSero-Rluc_NT contains the native form of Rluc. iSero-Rluc_CP contains the circular permutation form of Rluc. **B)** Ribbon model of the iSero-Rluc_NT biosensor generated using structures of iSero sensor and Rluc (Protein Data Bank: 6PER and 2PSD). **C)** Schematic model of the iSero-Rluc biosensor for detecting serotonin in live cells. **D)** Rluc activity was measured in yeast expressing a control

plasmid or plasmids containing serotonin biosensor constructs in the presence of exogenous serotonin (10 μM) or vehicle control. **E)** Rluc activity was measured in yeast expressing the iSero-Rluc_NT biosensor in the presence of exogenous serotonin (1–500 μM). For (D) and (E), results from one representative experiment of three independent trials are presented as mean ± SD of 3 biological replicates.

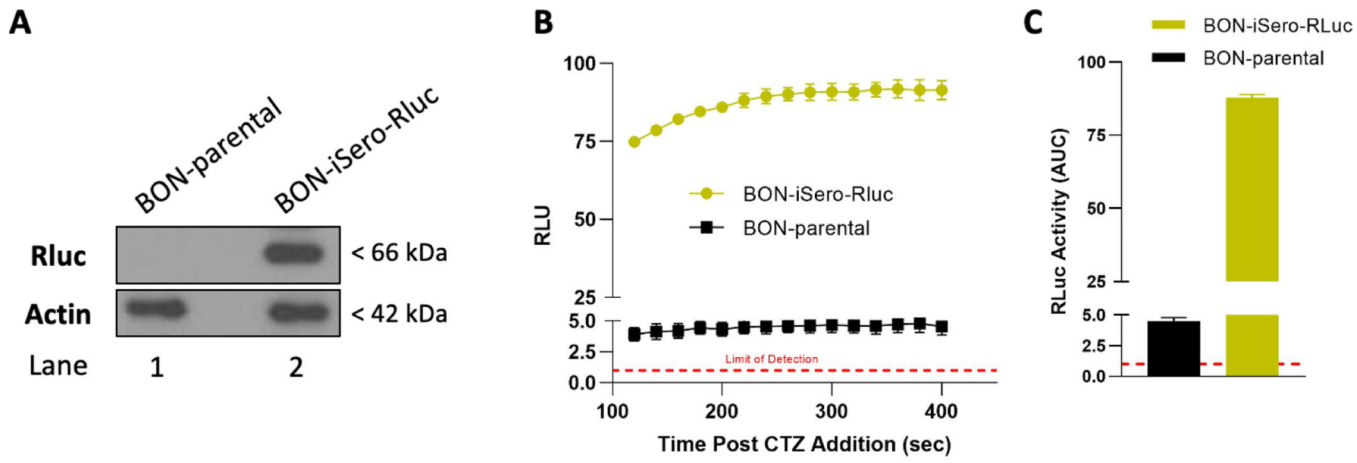

Figure 2.

Establishment of a neuroendocrine cancer cell line expressing the iSero-RLuc sensor. **A)** Western blot analysis of BON cells expressing the iSero-Rluc sensor compared to the parental BON cells using an anti-Rluc antibody. Anti-actin serves as loading control. **B)** Rluc activity was measured in BON-iSero-Rluc cells and BON-parental cells. RLU plotted against time. **C)** The area under the curve (AUC) of this plot is shown. Limit of detection was determined by measuring luminescence from the auto-oxidation of the coelenterazine (CLZ) in cell-free medium.

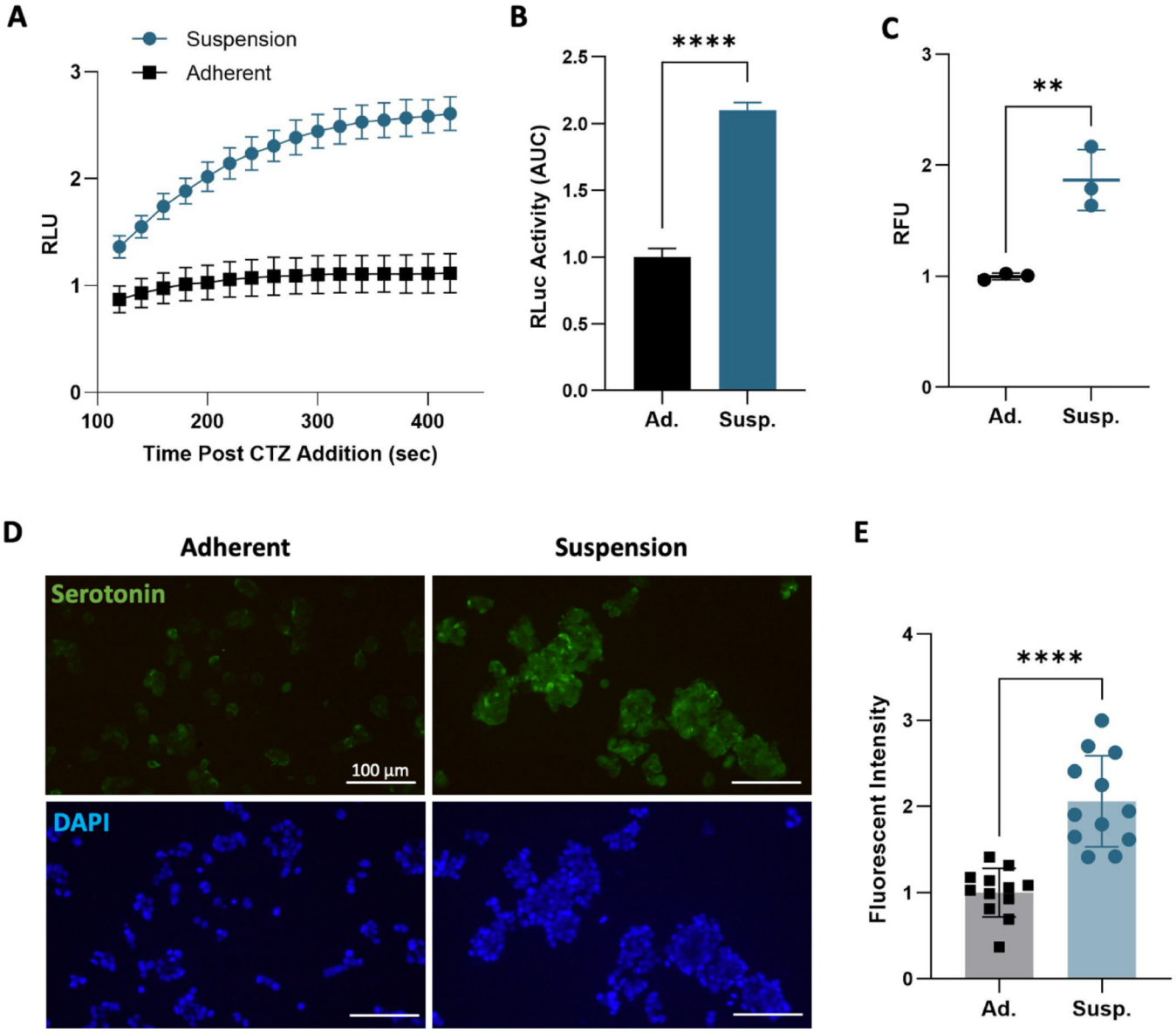

Figure 3.

iSero-Rluc biosensor identifies elevated serotonin in anchorage-independent growth **A)** Rluc activity was measured in BON-iSero-Rluc cells grown either in an anchorage-dependent (adherent) or anchorage-independent (suspension) culture state. RLU plotted against time is shown. **B)** The AUC of the previous plot is shown. **C)** Intracellular serotonin levels were measured by GC/MS in BON cells grown in either adherent or suspension culture (n = 3). **D)** Immunofluorescence (IF) staining of serotonin in BON cells grown in adherent or suspension culture. Representative images are shown in top panels along with corresponding 4′,6-diamidino-2-phenylindole (DAPI) staining in bottom panels. **E)** The mean fluorescent intensity ± SD of 4 fields of view per coverslip (n = 3) was calculated in ImageJ and normalized to the adherent condition.

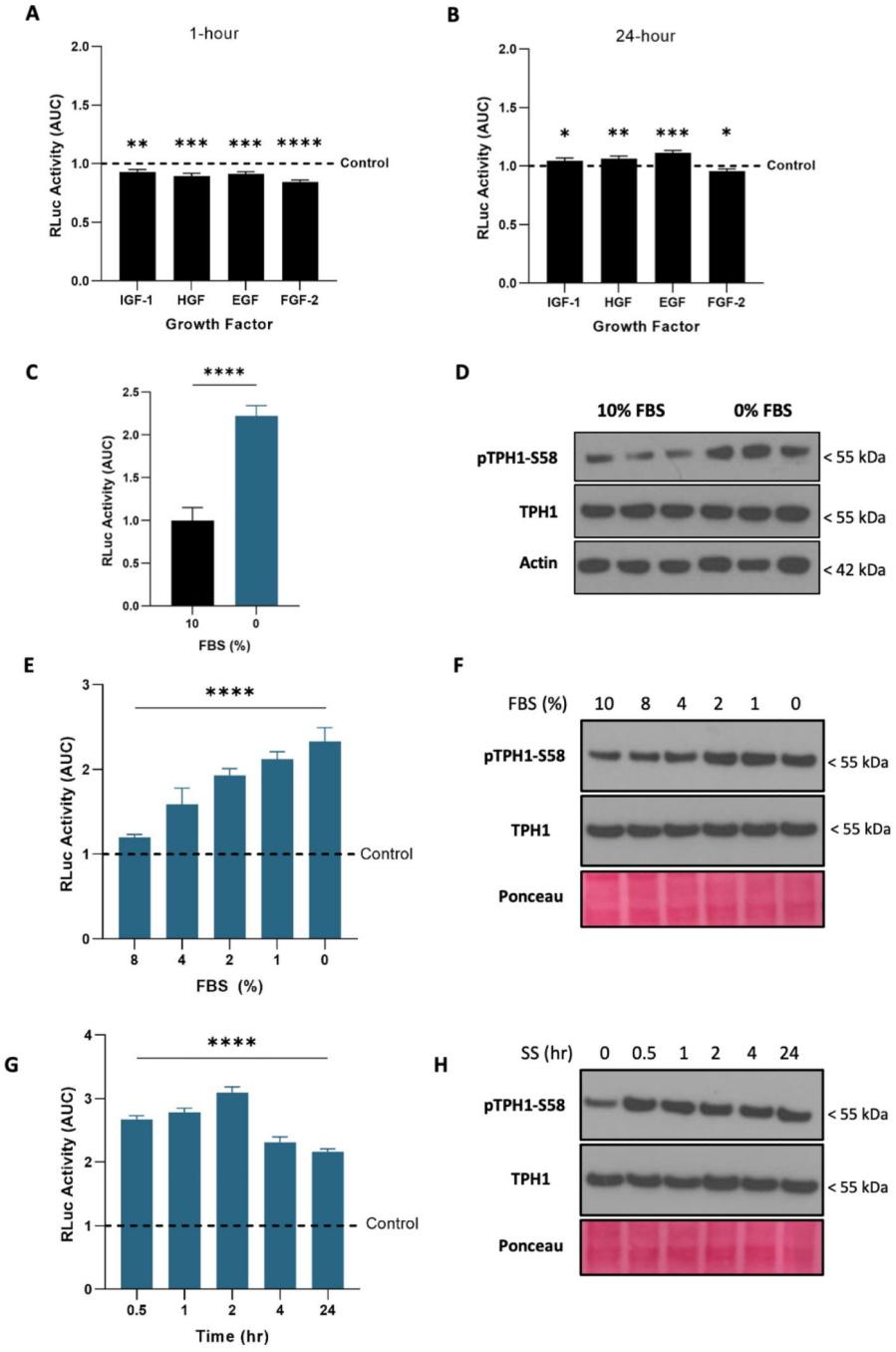

Figure 4.

Serum starvation induces serotonin biosynthesis in BON cells **A-B)** Rluc activity was measured in BON-iSero-Rluc cells treated for 1 hour (A) or 24 hours (B) with IGF-1 (2.5 ng/mL), HGF (10 ng/mL), EGF (5 ng/mL), FGF-2 (5 ng/mL), or vehicle control. RLU was plotted against time and used to calculate the AUC normalized to the control condition (represented as a dashed line). Results of one representative experiment of three independent trials are presented as mean ± SD of 4 biological replicates. **C)** Rluc activity was measured in BON-iSero-Rluc cells cultured in medium supplemented with 10% or

0% FBS. **D**) Detection of Tph1 and phosphorylated Tph1 at serine 58 (p-Tph1-S58) by western blot. **E**) iSero-Rluc activity in BON cells represented as AUC for the 0–8% FBS conditions normalized to the control condition (10% FBS). Three independent trials with 4–6 biological replicates were performed, and data is presented as mean ± SD of all three trials. **F**) Detection of Tph1 and p-Tph1-S58 by western blot from cells grown in different FBS concentration. **G)** Rluc activity was measured in BON-iSero-Rluc cells cultured in control medium (10% FBS) or serum reduced medium (1%) for varying length of time. AUC was calculated and the 1% FBS condition was normalized to control (10% FBS). Results from one representative experiment of three independent trials are presented as mean ± SD of 4 biological replicates. **H**) Detection of Tph1 and phos-Tph1-S58 by western blot for experiment described in panel **G**.

Table 1.

List of primers used for molecular cloning.

List of primers used for cloning iSero-Rluc sensor.

Name	Direction	Sequence (5’ – 3’)
Native-F1	Forward	CGGTTCCGGAGCTTCCAAGGTGTACGACCCCGAG
	Reverse	TGCCGGATCCAAGGTTCAGCAGCTCGAACCAAGC
Native-F2	Forward	AGGAGGATCCCCAAAGAAAATCATCTTTGTG
	Reverse	CGCCACTAGTCTGCTCGTTCTTCAGCACGCG
Circ-F1	Forward	CGGTTCCGGACCAAAGAAAATCATCTTTGTG
	Reverse	TGCCGGATCCTCCGCCACCCTGCTCGTTCTTCAGCACGCG
Circ-F2	Forward	AGGAGGATCCGGCGGTGGAGCTTCCAAGGTGTACGACCCCGAG
	Reverse	CGCCACTAGTAAGGTTCAGCAGCTCGAACCAAGC
iSero-pLVX	Forward	TATAGAATTCACCATGGCCAACGACACCGTGGTGGTG
	Reverse	CGTGAGATCTGCTAGATCAGGCCCTTCTCCTTCAG
pLVX-seq	Forward	CCAGGGCGCCTATAAAAGAG
	Reverse	GGCCCTCCTGTCTTAGGTTAG