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GENERATION OF TRANSGENIC MICE FOR *IN VIVO* DETECTION OF INSULIN-CONTAINING GRANULE EXOCYTOSIS AND QUANTIFICATION OF INSULIN SECRETION

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Insulin secretion is a complex and highly regulated process. Although much progress has been made in understanding the cellular mechanisms of insulin secretion and regulation, it remains unclear how conclusions from these studies apply to living animals. That few studies have been done to address these issues is largely due to the lack of suitable tools in detecting secretory events at high spatial and temporal resolution *in vivo*. When combined with genetically encoded biosensor, optical imaging is a powerful tool for visualization of molecular events *in vivo*. In this study, we generated a DNA construct encoding a secretory granule resident protein that is linked with two spectrally separate fluorescent proteins, a highly pH-sensitive green pHluorin on the intra-granular side and a red mCherry in the cytosol. Upon exocytosis of secretory granules, the dim pHluorin inside the acidic secretory granules became highly fluorescent outside the cells at neutral pH, while mCherry fluorescence remained constant in the process, thus allowing ratiometric quantification of insulin secretory events. Furthermore, mCherry fluorescence enabled tracking the movement of secretory granules in living cells. We validated this approach in insulin-secreting cells, and generated a transgenic mouse line expressing the optical sensor specifically in pancreatic β-cells. The transgenic mice will be a useful tool for future investigations of molecular mechanism of insulin secretion *in vitro* and *in vivo*.

Keywords: Insulin secretion; diabetes; optical imaging; secretory granule; exocytosis.

1. Introduction

Insulin, released from pancreatic β-cells, is the major anabolic hormone that regulates glucose homeostasis in the body. Insulin secretion is a complex and highly regulated process, which is under close control of blood glucose concentrations. Under physiological conditions, an elevation of blood glucose triggers rapid uptake of glucose

into pancreatic β-cells. The metabolism of glucose in β-cells results in increased ATP/ADP ratio and consequent closure of K_{ATP}-channels, membrane depolarization, opening of voltage-gated Ca^{2+} channels and rise in cytoplasmic Ca^{2+} concentration. The calcium influx and subsequent elevated intracellular Ca^{2+} concentrations trigger insulin granule exocytosis and the release of insulin into blood.[1–3] Defects in glucose-triggered insulin secretion, possibly exacerbated by a decrease in β-cell mass, are ultimately responsible for the development of type 2 diabetes.[4] In essence, diabetes is a disease of insulin deficiency, either due to lack of insulin production (type 1) because of autoimmunity triggered β-cell death and diminishing β-cell mass, or deficient insulin secretion (type 2) that fails to overcome peripheral insulin insensitivity.[5] Therefore, understanding insulin secretion and its regulation mechanisms over the disease progression will be instrumental for drug target identification and for evaluation of therapeutic strategies to treat diabetes, particularly type 2 diabetes.

Traditionally, insulin secretion is measured by enzyme-linked immuno-sorbent assay (ELISA) or radioimmunoassay (RIA).[6,7] These methods suffer from poor temporal resolution, complete lack of spatial resolution, and delayed results. The development of electrophysiological approaches made it possible to study insulin secretion on individual β-cells in real time, but without the spatial aspects of insulin release.[8] To overcome these limitations, optical imaging of genetically introduced exocytosis markers provides an alternative to detect insulin and other peptide/hormone secretion with high spatial and temporal resolutions, for example, green fluorescent protein (GFP) or its pH-sensitive variant pHluorin[9] was fused to insulin or Phogrin (phosphatase on the granule of insulinoma cells, a dense-core secretory granule-membrane glycoprotein) for detecting exocytosis of insulin granules in living cells by using confocal laser microscopy or total internal reflection fluorescence microscopy (TIRFM).[10,11] Besides the exocytosis of hormones, optical imaging has been widely used to detect other molecular events *in vivo* as well, such as enzyme activation,[12–15] calcium concentraction,[16] tumor growth and metastasis.[17,18]

In this study, we generated a DNA construct encoding a fusion protein of Phogrin and two spectrally separate fluorescent proteins: a highly pH-sensitive green pHluorin targeted to the secretory granule lumen and a red mCherry[19] localized to the cytosol. Upon exocytosis of secretory granules, the dim pHluorin inside the granule at pH 5.5–5.8[20] became brightly fluorescent outside the cells at pH $\sim$7.4, while mCherry fluorescence remained constant in the process, thus allowing ratiometric measurements of insulin release events. We validated this approach in insulin secreting INS-1 cells, and generated transgenic mouse lines carrying the optical reporter in pancreatic β-cells under the mouse insulin I promoter.

2. Materials and Methods

2.1. *Animal welfare*

All experiments involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of A*STAR (Agency for Science, Technology and Research).

2.2. *DNA plasmids*

pPhogrin-pHluorin-mCherry was constructed by first replacing the 0.7-kb AgeI-BsrGI fragment of Phogrin-EGFP-N1[21] (a generous gift of Dr. Takahashi Tsuboi) with the mCherry coding region (AgeI-BsrGI of mCherry-N1), then an MluI site was introduced immediately downstream of a KK cleavage site (amino acid 413–414 of Phogrin) by Quik-change mutagenesis (Stratagene), and finally pHluorin coding sequence was inserted in frame into the MluI site. To generate pLenti-Phogrin-pHluorin-mCherry for producing lentivirus expressing the fusion protein, we subcloned the EcoRI-HpaI fragment of pPhogrin-pHluorin-mCherry into the same sites of pLenti-Hiko vector.

2.3. *Cell culture and transfection*

INS-1 cells were cultured in advanced PRMI-1640 (Invitrogen) supplemented with 10% fetal bovine serum, 28.4 μM 2-mercaptoethanol, 10 mM HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin in an atmosphere of 5% CO_2 at 37°C. Transfection was performed by using Lipofectamin 2000 (Invitrogen) according to the manufacturer's protocol, and lentivirus infection was performed according to the Lentivector expression systems protocol (SBI).

2.4. *Generation of mIP-Phogrin-pHluorin-mCherry transgenic mice*

To generate the transgenic vector expressing Phogrin-pHluorin-mCherry specifically in pancreatic β-cells, we first modified the original transgenic vector pGEM-mIP-luc-hGH[22] (a generous gift from Dr. Graeme Bell) by introducing an SfiI site by Quik-change mutagenesis (Stratagene), and replacing the luciferase coding region with a short linker containing sites for restriction enzyme digest; we then cloned the 4.5-kb XhoI-HpaI fragment of pPhogrin-pHluorin-mCherry into the same sites of the modified pGEM-mIP-luc-hGH to generate the final transgenic vector pmIP-Phogrin-pHluorin-mCherry. The 14.9-kb mIP-Phogrin-pHluorin-mCherry fragment was released from the vector backbone by SfiI digestion and gel-purified (Qiagen). The purified transgene DNA was microinjected into the pronucleus derived from B6C3F1 mice. Tail DNA was used for screening the presence of the transgene by PCR. Three pairs of primers targeting different fragments of the transgene were used to ensure the integrity of the transgene: (1) 5′-TAC AAA GAG CTC AAG AAG GTA G-3′ and 5′-AGT GCT ATT GTG TAA GGA TAC C-3′ (amplifying the region between the promoter and Phogrin-pHluorin-mCherry coding sequence), (2) 5′-AAC TGA GGG GAC AGG ATG TC-3′ and 5′-ATT GTC CAT TGC AGT GAC GG-3′ (amplifying the Phogrin-pHluorin-mCherry coding region between Phogrin and mCherry), (3) 5′-TCC CTG CTC CAG GAG CTG TT-3′ and 5′-CGA GGA CTA CAC CAT CGTG-3′ (amplifying the region between the Phogrin-pHluorin-mCherry coding sequence and hGH cassette).

2.5. *Islet isolation*

Pancreas taken from euthanized mouse was incubated with a digesting solution containing 0.84 U/ml liberase blendzymes (Roche) in KRH buffer (130 mmol/l NaCl, 4.7 mmol/l KCl, 2.5 mmol/l $CaCl_2$, 1.2 mmol/l KH_2PO_4, 1.2 mmol/l $MgSO_4$, 20 mmol/l HEPES, pH 7.4, and 0.1% BSA) with shaking at 180 rpm for 20 minutes at 37°C. Then the mixture was washed several times with KRH buffer and individual islets were handpicked. Islets were plated on 35-mm coverslip bottom dishes for further staining and imaging.

2.6. *Immunohistochemistry*

Cryo-sections (20 μm) of pancreas tissue from transgenic mice were fixed with 4% paraformaldehyde for 20 minutes, and then blocked with 0.15% Triton X-100, 3% Goat Serum and 3% BSA in PBS for 30 minutes at room temperature. The sections were incubated with Guinea pig anti-swine insulin polyclonal antibodies (Dako cytomation) for 60 minutes, and then with Alexa Fluo 488 goat anti-guinea pig IgG (H+L) antibody (Invitrogen) for 60 minutes. Immunofluorescence staining was detected by confocal microscopy.

2.7. *Image acquisition*

Real-time detection of insulin granule exocytosis was performed on a dual-color Motorized laser TIRF system (Nikon), equipped with a high numerical aperture objective lens (CFI Apochromat, 100X oil, NA 1.49) and an environmental chamber (Stage top incubator, TOKAT HIT). Fluorescence signals from mCherry and pHluorin were imaged sequentially with excitation of 561 nm and 488 nm laser lines, respectively. The exposure time of mCherry and pHluorin channels was set to 300 ms and 700 ms, respectively. Images were acquired for five minutes with a five-second interval between images. Exocytotic events were analyzed by NIS-Elements viewer software.

The images of cryo-sections and islets were acquired on a Nikon A1R confocal imaging system equipped with a 40-mW argon laser and a 100×1.4 NA oil immersion objective. The images of pHluorin and Alexa Fluo 488 were obtained through photomultiplier 1 (PMT1, bandpass filter 500–530 nm) with 488 nm excitation, while the images of mCherry were obtained through PMT2 (bandpass filter 570–620 nm) with 561 nm excitation.

3. Results

3.1. *Expression of Phogrin-pHluorin-mCherry in INS-1 cells*

Phogrin-pHluorin fusion protein was shown previously to localize to insulin secretory granules and used to detect insulin granule exocytosis.[10] However, because pHluorin is highly pH sensitive, and is almost non-fluorescent inside the acidic environment of the secretory granules (pH 5.5–5.8) prior to exocytosis, Phogrin-pHluorin could not be used to

visualize or to track the movement of insulin granules under resting conditions. Furthermore, quantification of insulin release could only be assessed by the fluorescence change of a single chromophore, making it highly variable from one experimental condition to the next. To circumvent these caveats in imaging pancreatic β-cell secretory granules and quantifying insulin release, we generated a DNA construct encoding Phogrin that was linked with two spectrally separate fluorescent proteins: a red mCherry at the C-terminus and green pHluorin upstream of the transmembrane region [Fig. 1(a)]. At resting conditions, fluorescence from pHluorin inside the acidic secretory granules was very low, while mCherry signal was clearly visible. Upon stimulation and exocytosis of secretory granules, pHluorin changed from acidic intra-granular environment to neutral extracellular environment, leading to a significant increase in pHluorin fluorescence [Fig. 1(b)]. mCherry, on the other hand, remained in the cytosol before and after secretory granule exocytosis, and its fluorescence remained unchanged in the process, thus allowing ratiometric measurements of insulin secretory events [Fig. 1(b)].

To test whether the optical sensor was localized to acidic secretory granules, we expressed the fusion protein in insulin-secreting INS-1 cells by lentiviral infection. Only the mCherry fluorescence, but not the pHluorin signal, was clearly visible as distinct puncta in INS-1 cells [Fig. 1(c)]. Application of 50 mM NH_4Cl, which collapsed proton gradient across secretory granule membrane, led to a rapid increase in pHluorin fluorescence. Moreover, fluorescence signal from pHluorin was co-localized with that from mCherry [Fig. 1(c)]. These results indicate that the optical sensor was properly targeted and oriented to the membrane of acidic secretory granules.

3.2. *Real-time imaging of insulin-containing secretory granule exocytosis*

GFP-fused neuropeptides or hormones, as well as Phogrin-GFP have been used previously for monitoring the dynamic behavior of secretory granules and for quantifying peptide/hormone secretion.[10,11,23–26] To determine whether the optical sensor could be used to monitor granule exocytosis, we expressed the Phogrin-pHluorin-mCherry fusion protein in INS-1 cells by lentiviral infection, and stimulated the cells with 22 mM glucose.

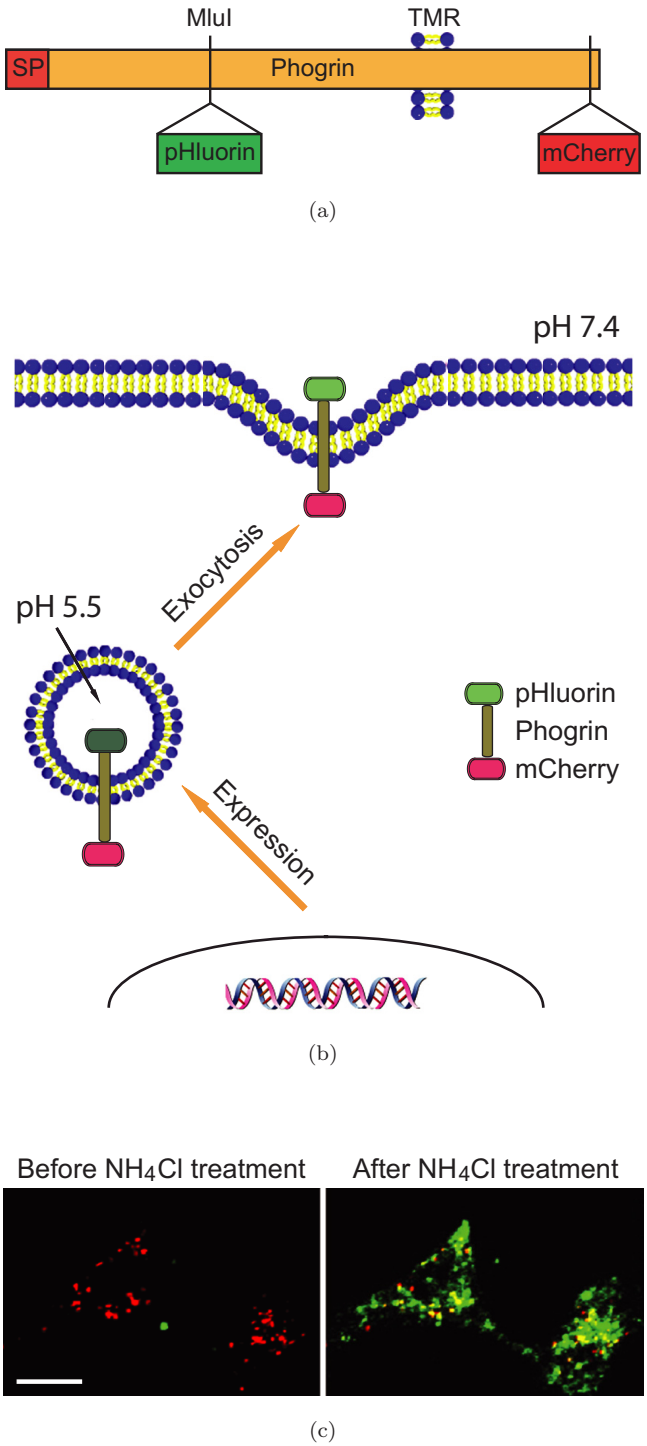


Fig. 1. Optical sensor for visualization of secretory granule exocytosis. (a) The optical biosensor consists of a dense-core secretory granule-membrane protein Phogrin, a highly pH-sensitive green pHluorin and a red mCherry. TMR: transmembrane region; SP: signal peptide. (b) pHluorin inside the acidic secretory granule was dim prior to exocytosis. Upon exocytosis, pHluorin became significantly brighter when facing neutral pH. (c) Increase of pHluorin fluorescence following exposure to NH_4Cl. INS-1 cells expressing Phogrin-pHluorin-mCherry were exposed to 50 mM NH_4Cl to neutralize intragranular pH. Scale bar = 10 μm.

We recorded the experiments in real time using a total internal reflection fluorescence microscope (TIRFM). Because pHluorin only becomes fluorescent at or near neutral pH, such as when facing extracellular bath, the appearance of green fluorescent spots after secretagogue stimulation indicates exocytosis of secretory granules. Figure 2(a) shows a collection of representative time-lapse images of exocytosis of insulin secretory granules under 22 mM glucose stimulation. At 220 seconds after glucose stimulation, a green fluorescent spot appeared, and its brightness increased over time until it reached plateau at around 260 seconds. The changes of pHluorin and mCherry fluorescence in the region of interest (ROI) in Fig. 2(a) were shown in Fig. 2(b). The intensity of mCherry fluorescence was stable for ∼100 seconds after glucose stimulation, and increased gradually over the next 100 seconds to reach a higher fluorescent, but more dynamic state. In contrast, pHluorin signal increased sharply only at ∼200 seconds after glucose stimulation, and reached its peak within ∼60 seconds. The exocytotic event was also shown as the ratio of signals from pHluorin and mCherry [Fig. 2(c)]. These results demonstrated that secretory granule exocytosis could be detected by ratiometric imaging of pHluorin and mCherry fluorescence of the optical sensor.

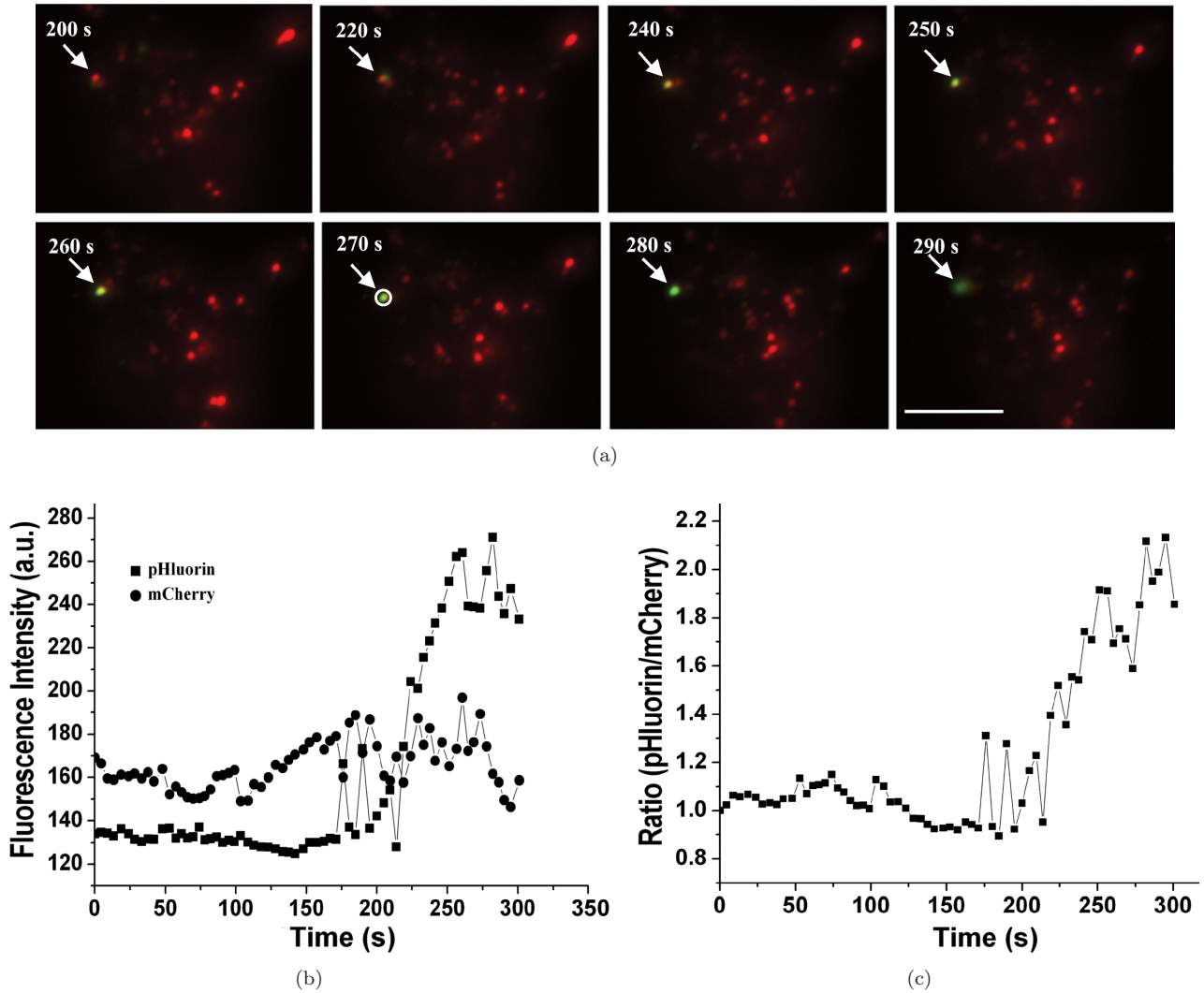


Fig. 2. Real-time detection and quantification of glucose-stimulated insulin secretory granule exocytosis in an INS-1 cell. (a) Time-lapse fluorescence images of INS-1 cells under 22 mM glucose stimulation. INS-1 cells were infected with lentivirus expressing Phogrin-pHluorin-mCherry. Images were obtained every five seconds for five minutes. Note the appearance of a green punctuate (arrows) at 220 seconds, which became brighter over time. Red: mCherry; Green: pHluorin. Scale bar = 10 μm. (b) Time course of fluorescence intensities of mCherry and pHluorin in the region of interest (ROI), shown as a white circle in (a) a.u.: arbitrary unit. (c) Time course of fluorescence ratio (pHluorin/mCherry).

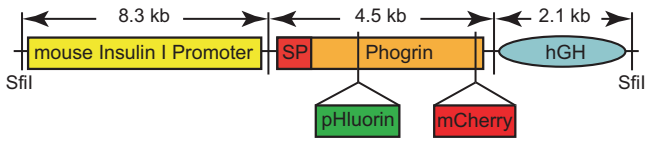


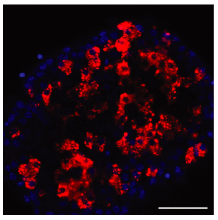
Fig. 3. Transgenic vector for pancreatic β-cell specific expression of Phogrin-pHluorin-mCherry. The 4.5-kb Phogrin-pHluorin-mCherry coding sequence was placed downstream of the 8.3-kb fragment of the mouse insulin I promoter, followed by a 2.1-kb fragment of the human growth hormone cassette gene (hGH). Transgene could be released from the cloning vector by SfiI digestion.

3.3. *Generation of mIP-Phogrin-pHluorin-mCherry transgenic mice*

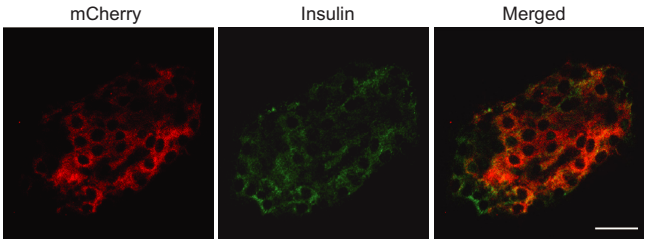
We then constructed the transgenic vector to express the optical sensor, Phogrin-pHluorin-mCherry, under the control of mouse insulin I promoter. The 14.9-kb mIP-Phogrin-pHluorin-mCherry-hGH fragment (Fig. 3), containing the 8.3-kb fragment of the mouse insulin I promoter, 4.5-kb Phogrin-pHluorin-mCherry coding region, and 2.1-kb fragment of human growth hormone cassette gene (hGH), was released by SfiI restriction enzyme digestion. hGH cassette was added to enhance protein expression levels. We obtained seven founders that were tested positive to contain the transgene by all three genotyping strategies (please refer to methods for details). The transgene was stably transmitted in all seven lines, and the fusion protein was expressed in islets of all lines, although at varied levels. Two lines with the highest levels of expression were kept for further analysis. The transgene was maintained on the B6C3F1 background, and mice were housed under specific pathogen-free (SPF) conditions with free access to food and water.

3.4. *Expression of Phogrin-pHluorin-mCherry in pancreatic β-cells*

Rodent pancreatic islets consist of several endocrine cells: glucagon-producing α-cells (15–20%), insulin-producing β-cells (65–80%), somatostatin-producing δ-cells (3–10%), pancreatic polypeptide-producing PP cells (3–5%), and ghrelin-producing ε-cells (<1%).[27] Histological sections from mouse and rat islets demonstrate a characteristic architecture in which a predominant proportion of



(a)



(b)

Fig. 4. Phogrin-pHluorin-mCherry expression in mouse pancreatic β-cells. (a) Pancreatic islets were isolated from Phogrin-pHluorin-mCherry transgenic mice and stained with DAPI nuclear staining solution. Blue: DAPI; Red: mCherry. Scale bar = 50 μm. (b) 20-μm pancreatic sections of transgenic mice were labeled with insulin (green). Red: mCherry. Scale bar = 20 μm.

insulin-producing β cells are located in the core of the cluster and surrounded by α, δ and PP cells in the periphery.[28,29] The islets of the mIP-Phogrin-pHluorin-mCherry transgenic mice were isolated and imaged by confocal microscopy. We found that the optical sensor was expressed in the core of the islets, and most of the cells at the islet periphery were not fluorescent [Fig. 4(a)], consistent with expected specific expression in pancreatic β-cells. To further test whether the fusion protein Phogrin-pHluorin-mCherry was expressed in pancreatic β-cells, we labeled transgenic mouse pancreas sections with insulin antibody. mCherry signals completely overlapped with fluorescence from the insulin signal [Fig. 4(b)], indicating that the optical sensor was expressed only in insulin-containing cells. We also examined the expressions of Phogrin-pHluorin-mCherry in other tissues, such as brain, fat, muscle, liver, and spleen, and found no evidence of expression in these non-pancreatic tissues (data not shown). These analyses revealed that the fluorescent reporter was specifically expressed in pancreatic β-cells, but not in extra-pancreatic tissues, consistent with the previous report on the tight regulation of the 8.3-kb mouse insulin I promoter.[30]

4. Discussion

Since the discovery of insulin almost a century ago, past research efforts have been focused on understanding the insulin secretion mechanisms, and more recently, on the molecular regulation of insulin granule exocytosis. The established insulin secretion mechanism is mostly based on *in vitro* studies. Although it is reasonable to assume that the fundamental mechanisms derived from *in vitro* studies apply to *in vivo* situations, many details have not been tested in *in vivo* settings. This is largely due to the lack of appropriate tools to measure and quantify insulin secretion at high spatial and temporal resolutions. In this study, we generated a transgenic mouse line expressing an optical sensor of insulin granule exocytosis in pancreatic β-cells. We envisage that the mouse line will be a useful tool for future studies on the molecular mechanism of insulin secretion *in vitro* and *in vivo* that require high spatial and temporal resolutions.

Under the control of mouse insulin I promoter, the optical sensor was exclusively expressed in pancreatic β-cells (Fig. 4). The β-cells with the fluorescence signal can be easily recognized from the other cells in the islet. Previously, subpopulations of dispersed β-cells were identified from non-β-cells by NAD(P)H autofluorescence, cell size or electrophysiological signatures.[31,32] More recently, GFP and luciferase reporter genes were introduced into β-cells under the control of mouse insulin I promoter.[22,30] These transgenic mouse lines allow easier identification and separation of β-cells from other endocrine cell types than previous methods. However, it is still necessary to isolate the β-cells for further insulin secretion measurements by biochemical or electrophysiological methods. In our transgenic mice, pancreatic β-cells expressing the optical sensor with dual colors can be easily identified by mCherry fluorescence despite dim pHluorin signal, and secretory granule dynamics and exocytosis can be studied by ratiometric quantification of mCherry and pHluorin levels in intact islets in real time. As with most of the GFP-fused secretory granule cargo or membrane proteins, the labeling is often only on a subset of the secretory granules. This makes it particularly challenging to catch the exocytotic events in insulin secretion from clonal β-cells or islets, because only $\sim$1–5% of the insulin granules undergo exocytosis during glucose-stimulated insulin secretion. Currently, we are testing various DNA constructs to identify a fusion protein that gives the highest percentage of insulin-granule labeling.

Phogrin, a dense-core secretory granule-membrane glycoprotein, was shown previously to be an excellent insulin granule maker.[10,11] Bright *et al.* generated a transgenic mouse line expressing Phogrin-EGFP in pancreatic β-cells under the control of rat insulin II promoter. The Phogrin-EGFP mice appeared to be healthy, with normal weight and glucose tolerance.[33] Similarly, our transgenic mice were healthy, fertile, and did not exhibit obvious neurological and behavioral phenotypes. Detailed testing on metabolic responses of these mice is under way.

In summary, we have generated a mouse line with specific expression of an optical sensor for secretory granules in pancreatic β-cells. The transgenic mice may be used in future studies of the molecular mechanism of insulin secretion *in vitro* and *in vivo*.

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