Video Article

Coculture Analysis of Extracellular Protein Interactions Affecting Insulin Secretion by Pancreatic Beta Cells

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Abstract

Interactions between cell-surface proteins help coordinate the function of neighboring cells. Pancreatic beta cells are clustered together within pancreatic islets and act in a coordinated fashion to maintain glucose homeostasis. It is becoming increasingly clear that interactions between transmembrane proteins on the surfaces of adjacent beta cells are important determinants of beta-cell function.

Elucidation of the roles of particular transcellular interactions by knockdown, knockout or overexpression studies in cultured beta cells or *in vivo* necessitates direct perturbation of mRNA and protein expression, potentially affecting beta-cell health and/or function in ways that could confound analyses of the effects of specific interactions. These approaches also alter levels of the intracellular domains of the targeted proteins and may prevent effects due to interactions between proteins within the same cell membrane to be distinguished from the effects of transcellular interactions.

Here a method for determining the effect of specific transcellular interactions on the insulin secreting capacity and responsiveness of beta cells is presented. This method is applicable to beta-cell lines, such as INS-1 cells, and to dissociated primary beta cells. It is based on coculture models developed by neurobiologists, who found that exposure of cultured neurons to specific neuronal proteins expressed on HEK293 (or COS) cell layers identified proteins important for driving synapse formation. Given the parallels between the secretory machinery of neuronal synapses and of beta cells, we reasoned that beta-cell functional maturation might be driven by similar transcellular interactions. We developed a system where beta cells are cultured on a layer of HEK293 cells expressing a protein of interest. In this model, the beta-cell cytoplasm is untouched while extracellular protein-protein interactions are manipulated. Although we focus here primarily on studies of glucose-stimulated insulin secretion, other processes can be analyzed; for example, changes in gene expression as determined by immunoblotting or qPCR.

Video Link

The video component of this article can be found at https://www.jove.com/video/50365/

Introduction

We describe here a method to facilitate investigations of how the extracellular domains of specific transmembrane proteins affect insulin secretion. The method probes the effects of interactions of the protein of interest with proteins (or possibly other molecules) on the pancreatic beta-cell surface. The method allows investigations of how cell-surface proteins expressed by beta cells or by other neighboring cells (*e.g.* endothelial cells, neurons, pancreatic alpha cells) affect beta-cell function through transcellular interactions (*i.e.* through interactions with interaction partners on the surface of adjacent beta cells).

The cellular plasma membrane contains a complex array of structural and functional proteins serving as bridges to the extracellular environment. By formation of transcellular connections or by initiation of plastic signaling events, interactions between cell-surface proteins can help coordinate the function of neighboring cells. Pancreatic beta cells are clustered together within the pancreatic islets and act in a coordinated fashion to maintain glucose homeostasis[1]. As revealed, for example, by the importance of extracellular EphA-ephrinA and neuroligin-2 interactions in the regulation of glucose-stimulated insulin secretion, it is becoming ever more clear that increased knowledge of the extracellular interactions occurring between proteins on the surfaces of adjacent beta cells will be of great importance for gaining a full understanding of insulin secretion, beta cell functional maturation and the maintenance of glucose homeostasis[1-3]. The goal of the method described here is to enable investigations of the effects on beta cell function of transcellular interactions involving specific transmembrane or otherwise-cell-surface-associated proteins. By co-culturing beta cells with HEK293 cells transfected with different expression constructs, the effects on beta cell function of different cell-surface proteins or mutated variants thereof can be efficiently probed. This is accomplished without having to transfect the beta cells themselves.

Elucidation of the roles of particular transcellular interactions by knockdown, knockout or overexpression studies in cultured beta cells or *in vivo* necessitates direct perturbation of beta-cell mRNA and protein expression, potentially affecting beta cell health and/or function in ways that could confound analyses of the effects of specific extracellular interactions. These approaches also alter levels of the intracellular domains of the targeted proteins and, further, do not allow effects due to interactions between proteins on or in the same cell to be distinguished from the effects of transcellular interactions. Here, a method for determining the effect of specific transcellular interactions on the insulin secreting capacity and responsiveness of beta cells is described. This method is applicable to insulin-secreting beta-cell lines, such as INS-1 cells[4], and to dissociated primary rodent or human beta cells. It is based on coculture models developed by neurobiologists, who found that exposure of cultured neurons to specific neuronal proteins expressed on HEK293 (or COS) cell layers could identify proteins that drive synapse formation[5,6]. Given the parallels between the secretory machinery of neuronal synapses and of beta cells, we reasoned that beta-cell function and functional maturation might be driven by similar transcellular interactions[7-9]. In order to probe these interactions, we developed the system described herein in which beta cells are cocultured on a layer of HEK293 cells expressing a protein of interest[10]. This system allows the beta-cell cytoplasm to remain untouched while extracellular protein-protein interactions are manipulated.

Protocol

1. Transfection of HEK293 Layer

1. Prepare HEK293 cell medium by adding to 500 ml bottles of DMEM (with 4.5 g/ml glucose and phenol red and without glutamine): 50 ml FBS, 5 ml 100X penicillin/streptomycin solution, 5 ml 100x L-glutamine solution and 500 µl amphotericin B.
2. Plate out HEK293 cells in a 24-well plate using 0.5 ml of HEK293 media per well. Ensure that the cells are spread evenly across the bottom of the plate.
3. When HEK293 cells reach 100% confluency, transfect with 0.8 µg of plasmid coding the protein of interest (inserted in a mammalian expression vector) and 2 µl of Lipofectamine 2000 according to the Lipofectamine protocol. We have opted to use the pcDNA 3.3 backbone designed for expression in mammalian cells.
4. Optionally, after 6 hr of incubation, exchange the transfection media described in the Lipofectamine protocol with normal HEK293 media.
5. To evaluate expression of the transfected protein, a subset of the transfected tissue culture wells can be used for immunofluorescent detection of the expressed protein or for western blot analysis of protein expression using standard techniques.

2. Optional Fixation of HEK293 Cells Expressing Transfected Protein

Transfected HEK293 cells can be gently fixed in order to facilitate coculture in media that might be harmful to living HEK293 cells (*e.g.* step 3.9 below) or to allow the efficient preparation in advance of plates for several experiments all at once (see also Discussion).

1. Conduct pilot studies to determine the time course of the expression of the protein in the HEK293 cells.
2. After transfecting the HEK293 cell layer, aspirate the media from the cells at the post-transfection time point associated with highest level of expression. If this occurs within the first 24 hr, do not aspirate until 24 hr after transfection.
3. Wash HEK293 cells gently with D-PBS then add 500 µl 4% paraformaldehyde (PFA) and incubate at RT for 30 min. (To make 4% PFA solution, start with 16% solution in 1X PBS and dilute 1:4 with 1X PBS.)
4. Using sterile PBS, gently wash the cells 3 times and incubate in PBS O/N at 4 °C.
5. Wash cells 3 times with sterile PBS, then add PBS and leave at 4 °C until needed (maximum storage time may vary depending on protein expressed. We store HEK293 cells transfected to express neuroligin isoforms for up to 2 weeks without significant changes in observed effects on insulin secretion).

3. Co-culturing of INS-1 Beta Cells with HEK293 Cells

1. Prepare INS-1 media by adding to 500 ml RPMI 1640 (with glucose and phenol red and without glutamine): 50 ml FBS, 5 ml 100X penicillin/streptomycin, 5 ml 100X L-glutamine solution, 5 ml sodium pyruvate, 500 µl amphotericin B, 500 µl beta-mercaptoethanol (this is nearly identical to the medium typically used for primary islet culture except for the addition of beta-mercaptoethanol).
2. Using Cell-stripper (or another non-enzymatic cell remover), harvest INS-1 cells at approximately 70-80% confluency off the bottom of a T75 culture flask. Each flask will provide roughly enough cells for 48 wells.
3. After spinning down cells in the centrifuge for 3 min at 1,200 rpm, resuspend in a 50/50 mix of INS-1 and HEK media. For a 70-80% confluent flask of INS-1 cells, resuspend in approximately 25 ml of mixed media.
4. If using HEK293 cells that have been fixed in paraformaldehyde rather than live HEK293 cells, resuspend the INS-1 cells in 100% INS-1 media rather than mixed media.
5. Using a 10 ml pipette, dislodge the cells from the pellet to make a homogenous cell suspension.
6. Aspirate to remove the media on the HEK293 cells.
7. Using a p1000 pipette, gently add 500 µl of INS-1 cell suspension onto the HEK cell layer along the sides of the well. After every 6 wells, use a 10 ml pipette to resuspend the INS-1 cells again to ensure a homogenous cell suspension. The overall scheme of the coculture set-up is depicted in **Figure 1.**
8. Incubate the cells for 24-48 hr depending on the experimental protocol.
9. If more than 48 hr coculture period is required, use optional steps in section 2 above to fix the HEK293 cells.
10. If using primary dissociated rodent or human islets [*e.g.* see prior protocols in JoVE[11-15]], ensure that an appropriate media such as RPMI with 5 ml pen/strep, 5 ml L-Glut, 0.5 ml amphotericin-B is used instead of INS-1 media. Although one can use dissociated islet cells in a 24 well plate, in practice because of the large number of islets potentially needed, it is recommended that consideration be given to scaling down to a 48 well plate (necessitating fewer islet cells per well). On a 48 well plate, add approximately 100 islets worth of dissociated cells per plate to start. Depending on the efficacy of dissociation and the method used, fewer islets may be required.

4. Glucose Stimulated Insulin Secretion

1. Preincubate cells in 250 µl of 2.5 mM glucose in KRB (Krebs-Ringer bicarbonate buffer) for a minimum of 1 hr. To preincubate, remove coculture medium and immediately replace with KRB. This exchange should be done one well at a time to minimize exposure of INS-1 cells to environmental oxygen. Aspirate with one hand while pipetting the low (2.5 mM) glucose KRB with the other.
2. Exchange preincubation KRB to 250 µl of either 2.5 mM or 20 mM glucose in KRB buffer one well at a time.
3. If appropriate for the specific experiment, additional secretagogues such as 100 µM IBMX may be added to produce a more robust stimulated insulin secretory response. Dissociated primary islet beta cells in particular are poorly responsive to increased glucose concentrations alone, so here especially addition of IBMX or other secretagogues should be considered[16,17] .
4. After 1 hr of incubation, transfer media to microfuge tubes and spin 1,500 x g for 5 min.
5. Remove 200 µl of the supernatant and use this for analysis by insulin RIA or ELISA.
6. To prepare cell lysate, add 100 µl of RIPA cell lysis buffer (150 mM NaCl, 1.0% IGEPALCA-630 or Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) with protease inhibitors to the plate immediately after removing media and incubate at 4 °C for 20 min.
7. Spin down lysate for 15 min at 10,000 x g and remove 50 µl of supernatant for analysis by insulin RIA or ELISA.

Representative Results

Using the method described here, we have tested the effect of different variants of the protein neuroligin on insulin secretion. This complements our published work investigating the effect of neuroligin-2 on beta-cell function[10]. **Figure 2**, for example, depicts results obtained from coculturing INS-1 beta cells with HEK293 cells transfected to express a neuroligin isoform referred to here as NL-X. This experiment was designed to test the hypothesis that NL-X engages in transcellular interactions that increase insulin secretion. In **Figure 2A**, it can be seen that expression of NL-X increased basal and stimulated insulin secretion by cocultured INS-1 cells. The approach of transfecting HEK293 cells so that they express NL-X allows us to know that it is the extracellular domain-which is the region displayed on the HEK293 cell surface and thus able to come into contact with cocultured INS-1 cells-that brings about the observed increase in secretion. The NL-X extracellular domain must be interacting with another protein (or perhaps some other molecule) on the INS-1 cell surface to cause the INS-1 cells to secrete more insulin. If desired, secreted insulin could also be expressed as a percent of cellular insulin content, which can be measured after lysing the cells.

In **Figures 2B** and **2C,** results from other types of analyses using the coculture system are depicted: specifically, analyses of the effect of transcellular interactions involving neuroligin variant NL-Y on cellular insulin content and on PDX-1 and insulin mRNA levels. NL-Y increased the insulin content of the cocultured INS-1 cells (**Figure 2B**). Further testing would be necessary to determine whether this was due to an increase in the amount of insulin per cell or due to increased INS-1 cell proliferation. In **Figure 2C**, RNA was harvested from the cell layer and analyzed by RT-qPCR. Since the HEK293 cells are of human origin, for transcripts that might be present in both HEK293 and INS-1 cells, use of rat-specific PCR primers (INS-1 cells are of rat origin) ensures that only mRNA of INS-1 origin is measured. Here it can be seen that coculture with NL-Y increased insulin and PDX-1 mRNA levels (normalization was to 18S RNA). The primers and methodology used for this RT-qPCR analysis were previously described[10], online supplement.

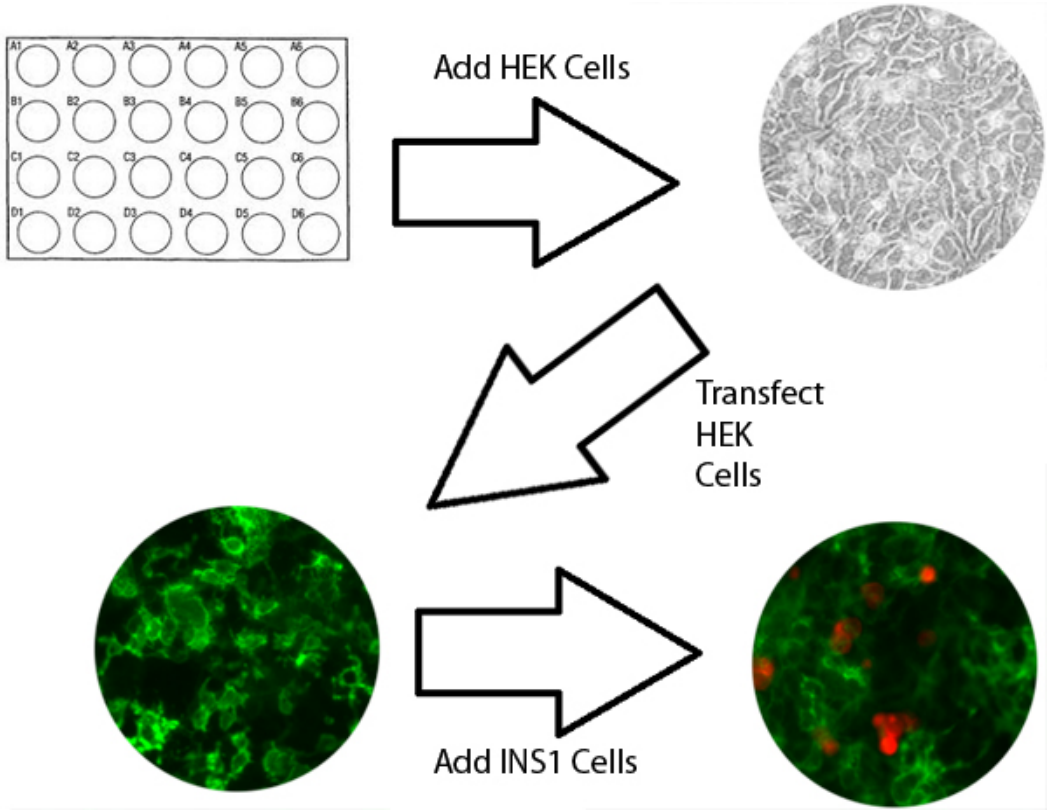

Figure 1. Coculture set-up. HEK293 (HEK) cells are added to 24-well tissue culture plates and then transfected. A bright field image of a representative HEK293 cell layer is shown on the top, right. The HEK cells are transfected to express an epitope-tagged transmembrane protein. Here, immunofluorescent staining is used to reveal the transfected cells (green). Next, INS-1 cells are added. Immunofluorescent staining for insulin was performed to allow visualization of the cocultured INS-1 cells (red).

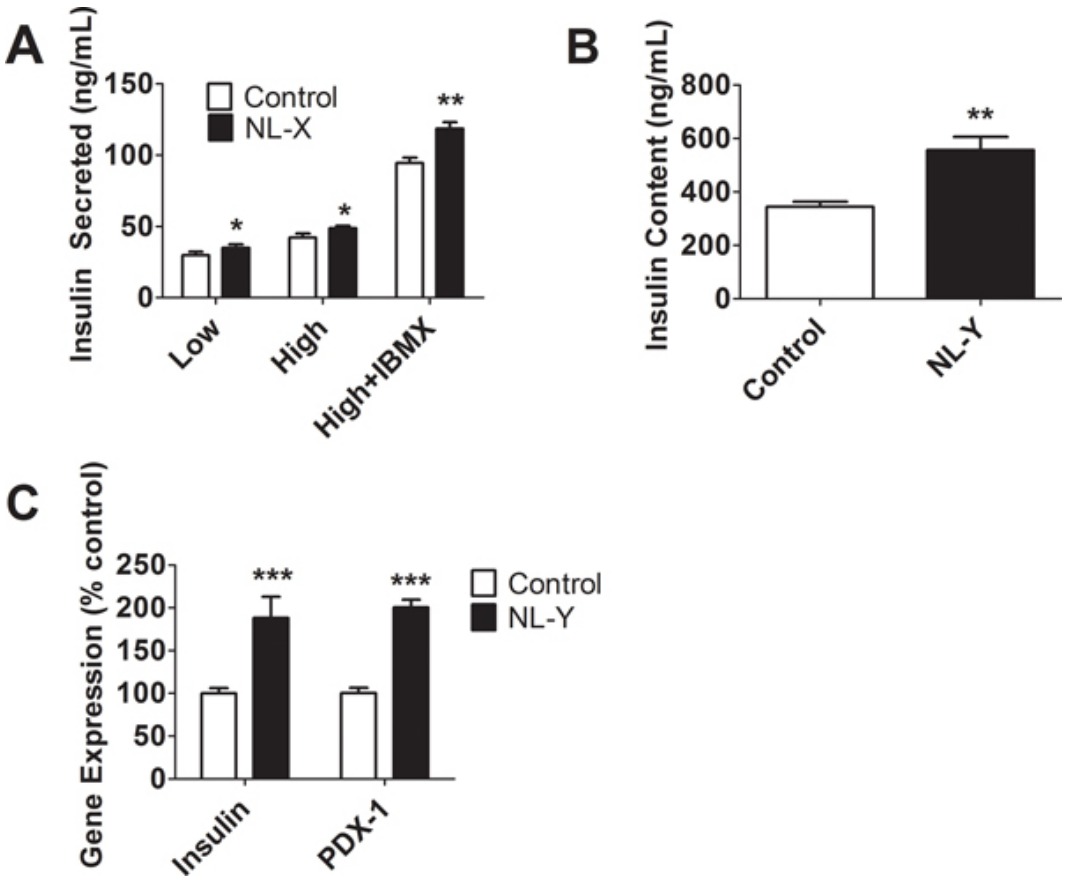

Figure 2. Results obtained from coculturing INS-1 beta cells with HEK293 cells transfected to express the neuroligin protein variants NL-X or NL-Y (filled-in, black columns). Control HEK293 cells were transfected with the same plasmid constructs altered so as not to express protein (white columns). **A**, insulin secretion by cocultured INS-1 cells under low (2.5 nM) and high (20 mM) glucose conditions and also after stimulation by high glucose together with IBMX. **B,** INS-1 cell insulin content after coculture with HEK293 cells expressing NL-Y. **C**, analysis of insulin and PDX-1 transcript levels by RT-qPCR using RNA isolated from INS-1 cells cocultured with either HEK293 cells transfected with NL-Y or with control-transfected HEK293 cells. (*, P<0.05; **, P<0.01; ***, P<0.001).

Discussion

The coculture method described here provides an effective way to determine the physiological importance of specific beta-cell-surface, transmembrane proteins, and specifically of their extracellular domains. By culturing beta cells or insulinoma cells (such as the INS-1 cells employed here) in contact with HEK293 cells displaying a protein of interest on the cell surface, experiments can be designed to determine the effects of extracellular protein-protein interactions without directly disturbing the intracellular milieu of the cells. Since the proteins of interest are expressed by HEK293 cells, any effects on β-cell function that could be mediated by the intracellular domains of the transfected proteins would be absent and therefore would not complicate analysis of the effects of extracellular interactions.

Effects on insulin secretion can be readily determined by comparing secretion from beta cells cocultured with HEK293 cells expressing a protein of interest to secretion from beta cells cocultured in contact with control HEK293 cells. The control HEK293 cells can be mock-transfected or, alternatively, transfected with a variant of the experimental plasmid construct mutated to produce a non-functional variant of the protein of interest. In our hands, both types of controls have yielded identical results. Since the HEK293 cell surface inherently contains a great variety of proteins, either type of control allows the function of beta cells in contact with many different cell-surface molecules, including the protein of interest, to be compared to the function of beta cells in contact with the same set of cell surface molecules with the exception of the protein of interest (or including a mutated version of the protein of interest).

INS-1 cells (or other beta cells) are added to the HEK293 layers at low density so that there is minimal or no contact of INS-1 cells with other INS-1 cells. This allows the effects of transcellular interactions between the protein of interest expressed on the HEK293 cell surface and proteins on the INS-1 cell surface to be observed in the absence of potential background noise caused by transcellular interactions involving the same protein expressed endogenously on the INS-1 cell surface.

After transfection, the HEK293 cell layer can be gently fixed. This optional fixation can be utilized to allow multiple coculture plates to be prepared at once for subsequent addition of beta cells at a later date. Fixing the HEK293 cells would also be advantageous if there were concerns that HEK293 metabolism or secretory function would somehow interfere with analysis of beta cell function. Lastly, the fixation protocol can be employed to facilitate prolonged coculture experiments. Here, the chief advantage of using fixed HEK293 cells is that the optimal culture medium for the beta cells can be employed rather than using a mixed media (*e.g.* see step 3.3) necessary for maintaining cocultured HEK293

and beta cells simultaneously. In prolonged coculture experiments, use of the mixed media-which is not optimal for either cell type-can impair beta cell viability.

HEK293 cells are employed here because they are highly amenable to transfection: they can be transfected with high efficiency using a variety of different transfection reagents[18,19]. HEK293 is one of two cell types-along with COS cells--that are routinely employed in similar neuronal coculture systems[5]. Lipofectamine 2000 is a proven transfection reagent very commonly used with HEK293 cells and routinely achieving transfection efficiencies (percent of cells transfected) of greater than 95% in published studies[18-20]. Other reagents can achieve similar efficiencies of transfection[19,20]. With our neuroligin-2 plasmid construct, transfection efficiency is generally ~80%. Although we focus here on studies of glucose-stimulated insulin secretion, other modes of analysis are possible, for example by FACS, immunohistochemistry, western blotting and/or, as seen in **Figure 2C**, qPCR.

Despite being a very useful tool in helping uncover the function of particular extracellular protein interactions, the method described here is not without its limits. Because the beta cells need to be in direct contact with the HEK293 cell layer, insulinoma cell lines such as INS-1 cells are particularly well-suited for this coculture system[4]. Translating the results to primary islet cells requires the dispersion of isolated islets. Since the islet structure itself is integral to the proper signaling and response to glucose, the stimulated insulin response is blunted in dispersed primary beta cells relative to intact islets[1,3,17]. Furthermore, because transient transfection does not result in uniform expression of the protein across the HEK lawn, when performing imaging-based (immunohistochemical) experiments, a method to normalize to protein expression levels may be required to interpret results. An experimental approach that takes advantage of this non-uniform expression to analyze the effect of the transfected protein on the secretory machinery of cocultured INS-1 cells is depicted in Figure 3 in Suckow *et al*., 2012[10].

It should also be noted that the extent of contact between any given beta cell and neighboring cells expressing the protein of interest is likely to be less in this system than in intact islets *in vivo*, in which beta cells might be surrounded on all sides by cells expressing the protein. This relatively reduced cell-to-cell contact could reduce the magnitude of changes in insulin secretion to below what would be observed if the relevant transcellular interactions were somehow eliminated from native islets.

Use of a clonal population of stably-transfected HEK293 cells rather than transient transfection would result in more uniform expression and possibly simplify experimental set-up, but would require substantially more up-front time and effort in order to generate the stably-expressing cells. Future studies will determine whether, by transfecting two or more transmembrane proteins into the HEK293 cell layer, possible synergistic effects could be observed.

Disclosures

We have nothing to disclose.

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