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REVIEW

Connexins and microRNAs: Interlinked players in regulating islet function?

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ABSTRACT

Pancreatic β-cells are connected to neighboring endocrine cells through the adherin proteins and gap junctions. Connexin 36 (Cx36) is one of the most well-studied and abundantly expressed gap-junction proteins within rodent islets, which is important in coordinated insulin secretion. The expression of connexins is regulated at various levels and by several mechanisms; one of which is via microRNAs. In past 2 decades, microRNAs (miRNAs) have emerged as key molecules in developmental, physiologic and pathological processes. However, very few studies have demonstrated miRNA-mediated regulation of connexins. Even though there are no reports yet on miRNAs and Cx36; we envisage that considering the important role of connexins and microRNAs in insulin secretion, there would be common pathways interlinking these biomolecules. Here, we discuss the current literature on connexins and miRNAs specifically with reference to islet function.

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Increasing complexity in multi-cellular organisms requires a higher order of cell-cell coupling for optimal tissue function. In the islets of Langerhans, the secretion of insulin and other hormones needs to be carefully coordinated through membrane depolarization and calcium influx that drive the release of insulin in response to increasing concentrations of glucose. Gap junctions are cross-border gateways that connect adjoining cells, which readily transfer ions, metabolites and other small molecules between cells. Intercellular cross-talk is an essential component of normal cellular physiologic processes that integrate a range of environmental cues from their interactions with soluble factors, signaling molecules, extracellular matrix components as well as from their neighboring cells. A defect in signaling across such gap junctional proteins is implicated in various diseases, including diabetes. The goal of this article is to discuss the existing literature on connexin expression and islet cell function, then to introduce the literature on regulation of non-islet connexin expression by microRNAs and finally to emphasize the need for future research in regulation of islet-enriched connexins by microRNAs. Even though connexins in the pancreas have not been shown to be regulated by pancreas-specific/-enriched microRNAs, there is plenty of evidence suggesting the role of each of them (connexins and microRNAs) in insulin secretion, an important function of pancreatic islet cells. Bioinformatically, one can predict different microRNAs targeting pancreatic connexins and therefore it is very likely that connexins and microRNAs could be interlinked with each other in regulating pancreatic insulin secretion. We hope that future studies, which investigate microRNA-mediated-regulation of connexins in islet cells and their role in β-cell dysfunction and diabetes would lead to developing novel therapies for improving islet cell function, and survival, through regulation of gap junctional coupling.

Gap junctions and connexins

A pancreatic β-cell as well as other specialized endocrine cells, represent a highly active entity where several molecules are synthesized and degraded continuously. Most of the cellular physiologic processes are very well regulated and this regulation occurs at multiple levels. In a multicellular organism, where several cell types give rise to a single tissue that then serves a particular function for

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survival of that organism, it is extremely important that all cells efficiently communicate with each other. This is also true for maximizing the functional capacity of mini organs such as the islets of Langerhans. Most cells communicate with each other via secretion of effector molecules such as proteins, peptides, nucleic acids, hormones and ligands, and by responding to them. This communication can be either contact-independent (paracrine, endocrine and neuronal) or contact-dependent. In a contact-dependent communication, two of the cells need to be in a close vicinity to one another and should be connected to each other via a ligand-receptor complex or via gap junctions or via other cell adhesion molecules.

Gap junctions are important for the cell to cell communication, intercellular signaling and coordinated cellular functions.[1,3] In eukaryotes, metabolites, nutrients, signaling molecules; eg. small RNAs,[4] ions as well as electrical signals are exchanged via gap junctions, thus maintaining cellular homeostasis.[5] Apart from their role in cellular function, gap junctions are also important for normal development, proliferation, and differentiation of stem/progenitor cells. In vertebrates, connexins are building blocks of gap junctions, while innexins are proteins that form gap junctions in invertebrates.[6] There are 21 connexin proteins in humans and 20 connexins in mice.[7] Another family of connexin-like proteins; the pannexins, is known to be essential in mammals and consists of 3 members.[8]

A single connexin protein is a non-glycosylated, transmembrane molecule that spans the plasma membrane 4 times and has both C- and N-terminals in the cytoplasm. Six connexin protein units bunch together in a cylindrical fashion and form a single connexon/hemichannel. Two hemichannels, one contributed by each of the adjoining cells, connect with each other and create a hydrophilic gap junction that allows transfer of small molecules (up to 900 daltons). Accordingly, the gap junctions are called as homotypic gap junction and heterotypic gap junction (Fig. 1). In homotypic gap junction, both hemichannels/connexons are identical, while heterotypic gap junction consists of 2 different connexons.[9] There are 2 types of connexons; homomeric and heteromeric connexon (Fig. 1). Homomeric connexons have identical connexins while heteromeric connexons are composed of different types of connexins.[9] It is also observed in some cases that a hemichannel does not connect to another cell but could open in the extracellular environment, facilitating the transports of ions, ATP and glutamate.[1,10]

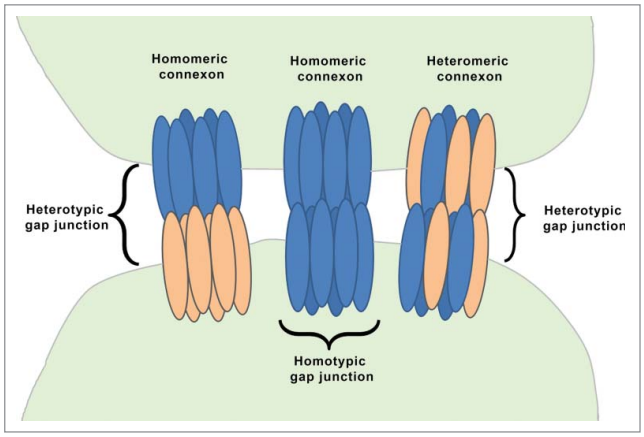

Figure 1. Organization of gap junction proteins. This is a schematic presentation of different types of connexons. A homomeric connexon refers to a connexon formed when 6 identical connexin proteins form the pore for a gap junction. A heteromeric connexon refers to a connexon formed between different connexin subunits. Two identical connexons form a homotypic gap junction, whereas a heterotypic gap junction consists of 2 different hemichannels.

Cell to cell communication via gap junctions is seen in almost all cells excluding erythrocytes, circulating lymphocytes and skeletal muscle cells.[2] Any changes or inhibition of gap junction-mediated cellular crosstalk leads to abnormal biology potentially contributing to the development of diseases including cancer and diabetes. Connexins have been well known as tumor supressors in cancer biology, as reviewed elsewhere.[11] With reference to the islet-enriched connexin; i.e. Cx36, it is well known that the gene encoding Cx36 is located on the 14q region of chromosome 15, which is a susceptibility locus for Type 2 diabetes, indicating a possible role in reduced β-cell function associated with progression to Type 2 diabetes.[12,13] Thus, there is significant evidence to understand the role of connexin dysfunction in disease. Although causality remains to be conclusively identified, the current evidence recognizes the need for normal connexin expression in optimal tissue function. Connexin proteins have a very short half-life of around 3 hours and are internalized or degraded by lysosomes or proteasomes.[1,2] Connexin expression and gap junctional intercellular communication are regulated at many levels including electrochemical gradient across the chanel, change in pH, change in the concentration of calcium ion, phosphorylation of connexin molecules, rearrangement of the structure as well as voltage and chemical gating.[2,3]

Gap junctions in the pancreas

The pancreas is a unique organ wherein its endocrine portion; the islets of Langerhans, is embedded within the exocrine counterpart (acini and ducts). Islets consist of 5 different endocrine cell types; α, β, δ, *pp and* ε cells. The most abundant islet cell type is the β-cell, which produce and secrete insulin – the islet hormone that works toward reducing circulating glucose concentrations. Although there are significant differences observed between human and rodent islet architecture,[14-16] β-cells are always in contact with other β-cells or other endocrine pancreatic (non-β) cells and "hard-wired" or connected via specific connexins. Synchronized and pulsatile secretion of insulin requires co-ordination and electrical coupling with adjacent cells for transfer of the depolarizing signal from the adjacent endocrine cells in the islet to achieve normal insulin secretion; a process that is known to be coordinated via gap junction signaling.[17] Connexin 43 (Cx43) was demonstrated to be important in rat pancreas function;[18] however later on it was demonstrated that Cx36 is the most abundant and exclusive connexin expressed in pancreatic insulin-producing cells[19-21] while Cx43 and Cx45 are expressed by vascular endothelial cells that are abundant in islets.[22,23] Recently, connexin 30.2 has been demonstrated to be present in mouse β-cells along with Cx36,[24] however, its functional role in insulin secretion is not yet confirmed. Connexins 26 and 32 have been reported in pancreatic exocrine/acinar cells during mouse pancreas development.[19] Cx36 is shown to be β-cell specific and is seen between adjacent β-cells,[21] while a probability of heterotypic gap junction consisting of Cx36 and Cx43 is suggested that may mediate β-cell and intra-islet endothelial cell interactions.[25]

Cx36 knockout mouse islets fail to demonstrate intracellular calcium oscillations as well as the synchronous and pulsatile release of insulin.[26-28] Similar observations were reported in an *in vitro* manipulated (MIN6) cell line.[29] Cx36 knockout mice have normal fasting glucose, but display abnormal glucose clearance following an intraperitoneal glucose tolerance test, suggesting glucose intolerance.[17] There are also contradictory reports on the increase in basal insulin release in Cx36 deficient islets.[27,30] Studies in Cx36 knockout animals have indicated that these gap junctions in β-cells not only regulate insulin secretion but also regulate intra-islet blood flow.[31] High fat fed mice, which show insulin resistance, obesity and pre-diabetes have a significant reduction in islet Cx36 protein and around 30% less β-cell to β-cell coupling.[32] These data suggest that Cx36 gap junctions are affected and may contribute to islet β-cell dysfunction during the progression from impaired glucose tolerance to Type 2 diabetes. Although the role of connexins in β-cell dysfunction is well established, their role in β-cell survival is not demonstrated as yet. It is well understood that β-cell dysfunction precedes their death in progression to Type 2 diabetes.[33] The role of connexins in β-cell survival would need long-term assessment of β-cells from connexin-specific knockout mice during different stages of progression to Type 2 diabetes and insulin-requiring Type 2 diabetes. Overall, there is significant evidence to confirm the role of Cx36-dependent intercellular communication in glucose-stimulated insulin secretion (GSIS) and in β-cell dysfunction, leading to the development of Type 2 diabetes.

As mentioned above, connexin expression is regulated at multiple levels. However, there is not much information on regulation of Cx36 in islets. In the retinal AII amacrine cells, Cx36 is phosphorylated by protein kinase A (PKA) and it results in reduced coupling, decreased permeability across the Cx36 gap junction in these cells mediating visual adaptation.[34] Recently, another protein kinase PKCδ is shown to alter Cx36 coupling in islet cells and is believed to be a mechanism of islet dysfunction during cytokine exposure leading to diabetes.[35] Another study where islets were co-cultured with endothelial progenitor cells *in vitro*, reported decreased expression of Cx36 with increase in basal insulin secretion.[36] Even though the exact molecular mechanism was not investigated, a possibility of crosstalk between β-cells and endothelial cells is evident. All these reports point to the intricate regulatory processes within islet cells including those controlling connexin expression and coupling in pancreatic islets. Recently, microRNAs have been shown to regulate connexin expression and function in multiple non-islet tissues and in several species;[37-40] however, no reports on microRNA regulation of islet-specific/-enriched connexins are available till date. Considering the existence of such a mechanism in non-islet cell systems, this article presents a review of the known microRNAs that target connexins and attempts to initiate discussion in this area of biology that we think would emerge to be an important component of islet cell organization and function.

microRNAs

microRNAs are small, non-(protein) coding RNA molecules that are about 22 nucleotides in length and act as negative regulators of gene expression.[41] They are synthesized as a long transcript (primary miRNA) in the nucleus, which undergoes 2 rounds of processing by enzyme complexes (Drosha and Dicer in nucleus and cytoplasm respectively) to generate mature miRNAs in the cytoplasm.[42,43] These mature miRNAs are single-stranded molecules, which act at post-transcriptional level via incorporation into RISC (RNA-induced silencing complex) and regulate the target mRNA expression largely through 2 processes; i) degradation of target mRNA or ii) translational inhibition. Most miRNAs target 3′UTR of the mRNAs and in some cases its 5′UTR or the coding region, functioning as key regulators of developmental, physiologic and pathological conditions.[45,46]

It is now known that microRNAs themselves are regulated by various other mechanisms including RNA-binding proteins, SNPs, methylation, miRNA-editing and circadian rhythms as reviewed elsewhere.[47] It has been demonstrated that a RNA-binding protein Deadend-1 (Dnd1) can physically block the access to a miRNA target site, thereby sterically hindering the normal function of RISC.[48] Although not much is known about the structural aspects of miR-RISC target recognition and Dnd1 binding, such molecules may offer another layer of regulation. It is also speculated that Dnd1 may change the subcellular localization of mRNA molecules, taking it out of the reach of its targeting miRNAs. Indeed, Dnd1 has been shown to localize to discrete perinuclear granules in primordial germ cells.[49]

miRNAs are required for normal pancreas development, regeneration and function.[41,50-52] miR-7 and -375 are some of the most abundant miRs within the pancreas[53] and are shown to regulate α and β-cell mass,[54,55] insulin expression,[56] insulin secretion,[57,58] as well as β-cell secretome.[59] Apart from these, several other miRNAs are implicated in regulating pancreas development by targeting important transcription factors.[41,50] Several microRNAs have been shown to be altered during β-cell dysfunction and/or apoptosis; especially miR-34a, miR-21, miR-29, and miR-146 have been reported to date;[51,60-63] as well as in diabetes progression/complications.[64-66] Insulin secretion is also regulated by several different miRNAs, apart from the miR-7 and miR-375 mentioned above. These include miR-30d that targets MAP4K4,[67] miR-124a targeting sirt1, NeuroD1, FoxA2 and Rab27a,[68] miR-96 that targets Noc2 and granuphilin,[69] miR-33a/b targeting ABCA1[70] and multiple others. Although these studies underscore the importance of miRNAs in pancreas development, function, and disease (diabetes) progression, there are no reports yet on microRNA regulation of connexins in the pancreatic β-cells.

microRNAs and connexins

Till date, there are few studies demonstrating microRNA-mediated regulation of connexins. Most of these demonstrate regulation of Cx43, one of the most commonly expressed connexins in the body.[71,72] Bioinformatics analysis of microRNA and connexin interactions also predict Cx43 to be extensively regulated by miRNAs compared with other gap junction proteins.[73]

In one of the reports, miR-206 and miR-1 microRNAs are shown to negatively regulate Cx43 during *in vitro* myoblast fusion[37] and a similar mechanism is implicated during muscle development *in vivo*. Elevated expression of miR-1, a muscle-specific miRNA, is observed during coronary artery disease and arrhythmia.[74,75] The proposed mechanism involves post-transcriptional repression of Kir2.1 and Cx43, thereby lowering conduction potential and depolarization of cardiac muscles. Three miRs (miR-1, -206 and -133) are also seen to be upregulated during *in vitro* myoblast differentiation with a concomitant reduction in Cx43 mRNA, suggesting that Cx43 is a potential target of these microRNAs.[76] MiR-206 is observed to be involved in osteoblast differentiation, where its abundance decreases during normal differentiation.[77] Overexpression and knockdown studies of this miRNA indicated that Cx43 is a target of miR-206 during bone formation. MiR-1 regulates Cx43 in cardiac hypertrophy[78] and the interaction between these 2 molcules is also important in bladder cells, thereby controlling bladder development and sensitivity.[79] Cx43 is also reported to be targeted by miR-218 in cancer cell lines,[80] miR-145 in corneal epithelial progenitor cells,[81] miR-221/222 cluster in glioblastoma cells,[38] miR-20a in prostate cancer cell lines[82] and miR-19a/b in murine cardiac cells.[83] Among other connexins, Cx40 is shown to be regulated by miR-208a, which is also necessary for normal cardiac conduction and function.[84,85]

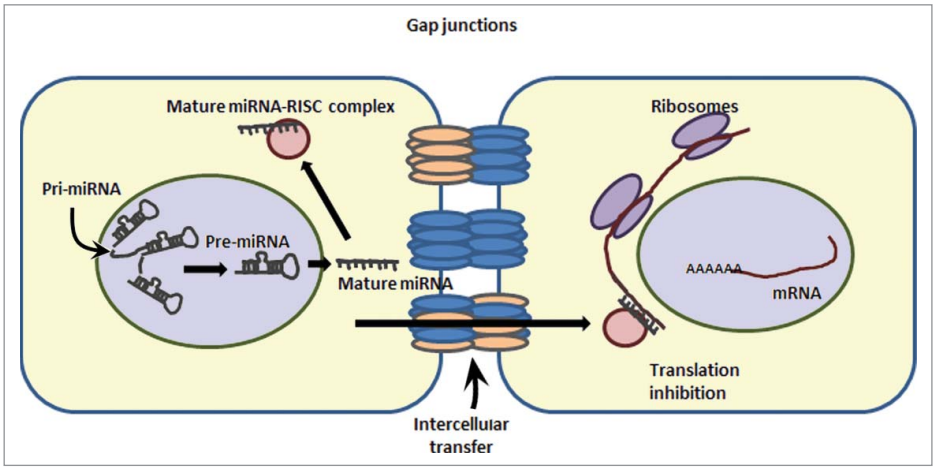

Figure 2. Intercellular transcript regulation via miRNA transfer. miRNAs can enter into neighboring cells via gap junction-mediated transfer. This cartoon demonstrates the ability of a cell (on the left) to transcribe and process the pre-miRNA to mature (single stranded) microRNA. A mature microRNA gets incorporated into RISC (RNA-induced silencing complex) and can target the expression of mRNAs in the same cell via translation inhibition/ transcript degradation or may enter the adjoining cell(s) to inhibit the expression of a specific set of genes in the neighboring cells.

miRNAs transfer via connexins/gap junction

Apart from miRNA-mediated regulation of connexins, another interesting interaction exhibited by connexins/gap junctions and miRNAs involves intercellular communication via gap junction-mediated transfer of miRNAs (Fig. 2). With this novel mechanism, miRNAs cannot only regulate various gene transcripts within a cell of their origin but also have the ability to do so in their neighboring cells. It is yet uncertain as to what signals drive the shuttling of miRNAs between the cells via gap junctions and also whether this transfer is an active mechanism or a passive flow. Different *in vitro* co-culture studies have demonstrated the transfer of miRNAs between donor and recipient cells, especially in cancer cells.[86-89] There are no *in vivo* studies reported as yet, however, this specific and efficient mode of intercellular miRNA transfer would have clinical applications that include cell-specific delivery of small RNAs as a cancer therapy or for regenerative medicine. Whether such transfer exists between adjacent β-cells for regulation of insulin secretion and calcium signaling is yet unclear.

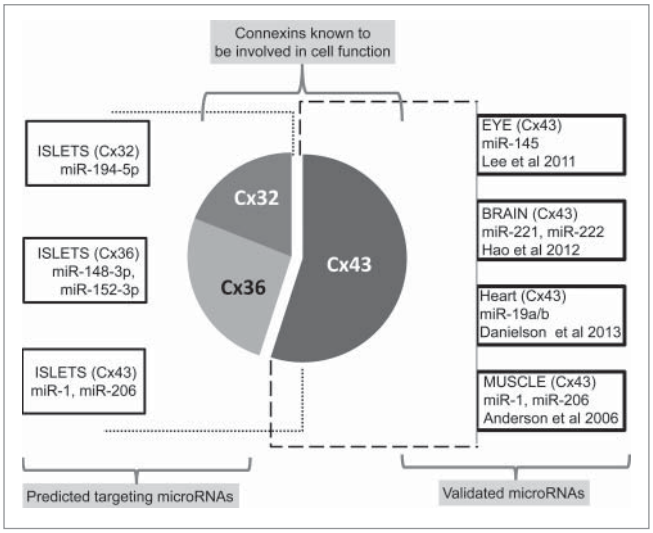

Figure 3. Regulatory microRNAs targeting connexin gene transcripts. Schematic illustrating different microRNAs targeting the expression of specific connexin molecules demonstrated herein. The most widely studied Cx43 is experimentally shown to be targeted by several different miRNAs in various tissues (Right side panel). Left side panel represents different connexins in islets and bioinformatically identified, highly conserved (using the publically available site http://www.targetscan.org) microRNAs predicted to bind to a specific connexin.

Conclusion

MicroRNAs have emerged as important regulators of several physiologic and pathological processes. Though most of the current literature demonstrates the role of miRNAs in Cx43 regulation (Fig. 3); it is most likely that such regulatory effect on other connexins will be discovered in near future. Gap junctions in pancreatic β-cells are made of Cx36; which is shown to be important for normal islet function including coordinated insulin release. The decrease in expression of Cx36 is now linked to β-cell dysfunction and prediabetes. Whether such progressive loss of Cx36 is causal to β-cell failure, leading to apoptosis, is not yet

understood. Cx43 is also present in pancreatic islets but mainly in islet vasculature, which has a regulatory role in β-cell function. Given the importance of connexins in insulin secretion and the known regulation of miRs on insulin secretion (miR-375 via myotrophin and PDK1), it would be interesting to see if any of the "pancreatic" miRs regulate connexin expression and thereby insulin secretion. The potential regulation of cell-to-cell communication channels via microRNAs adds a first level of regulatory control. Interestingly, the demonstration of the regulatory molecule (*dead end 1*/dnd1), which can antagonize the action of these microRNAs[48,90] present a potentially intricate mechanism wherein such molecules could modulate connexin expression through regulation of targeting microRNAs. Further research is needed to understand if connexins, which are known to be altered in disease state, can be rescued using molecules that can either inhibit the miRNA binding or localize them to sub-cellular compartments that offer protection from miRNA-mediated degradation. If such RNA-binding proteins proffer the proposed regulatory mechanisms, then these could be used as modulators of miRNAs with potential for translational research. Studies focused on understanding the role of miRNAs in the regulation of islet-associated connexins could lead to the identification of strategies for enhancing insulin secretion in differentiating islet progenitor/stem cells.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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