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Connexins and steroidogenesis in mouse Leydig cells

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Abstract

Connexin43 has been recognized as forming gap junctions in Leydig cells. However, previous work had shown that mouse Leydig cells lacking this connexin do not suffer any limitation of their ability to produce testosterone when stimulated with luteinizing hormone. The objective of the present study was to identify additional connexins in mouse Leydig cells that could be required for steroidogenesis. An RT-PCR screen involving isolated adult Leydig cells identified connexin36 and connexin45 as expressed along with connexin43. Treatment of dissociated testes with carbenoxolone, a non-specific blocker of gap junctional coupling, significantly reduced testosterone output as did treatment with quinine, which disrupts coupling provided by connexin36 and connexin45 gap junctions but not those composed of connexin43, indicating that either or both of connexins 36 and 45 could be involved in supporting Leydig cell steroidogenesis. Immunolabeling of adult mouse testis sections confirmed the localization of connexin36 along with connexin43 in Leydig cell gap junctions but not connexin45, which is distributed throughout the cells. It was concluded that connexin36, connexin43, and connexin45 are co-expressed in Leydig cells with connexins 36 and 43 contributing to gap junctions. The role of connexin45 remains to be elucidated.

Introduction

The connexins are a family of proteins that form the intercellular membrane channels of gap junctions. There are 20 or more connexins encoded in mammalian genomes, with each forming membrane channels exhibiting distinct properties and with a characteristic distribution among different organs and cell types. However, these expression patterns are not unique to individual connexins; rather, co-expression of different connexins in individual cell types is common. This situation complicates attempts to understand the physiological roles of specific connexins in different cell types.

The Leydig cells of the testis are a case in point. Leydig cells are the main constituent cell type in the interstitial compartment of the testis where they are responsible for synthesizing and releasing androgens in response to luteinizing hormone (LH) released from the pituitary gland. Several studies have identified connexin43 (Cx43, also known as GJA1) in Leydig cells and, based on a variety of observations, it has been surmised that Cx43 is the only connexin expressed in those cells (reviewed by Pointis et al., 2010). However, Kahiri et al.

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(2006) detected residual intercellular fluorescent dye transfer (“dye coupling”, an indication of the presence of gap junctions) between Leydig cells isolated from mouse testes which lacked Cx43 due to targeted deletion of the *Gjal* gene. In the same study it was demonstrated that LH-stimulated androgen production by Cx43-deficient Leydig cells was not impaired: both the amounts and types of androgens produced were unaltered. This was somewhat surprising given that gap junctional intercellular communication (GJIC) was suspected of being involved in regulating the stimulated release of hormones in other endocrine organs, a role that has now been confirmed in the pancreas (Head et al., 2012) and adrenal medulla (Colomer et al., 2012). In those organs, GJIC is proposed to coordinate the responses of individual cells to the external stimulus, optimizing stimulus-secretion coupling. We therefore hypothesized that Cx43 is not essential for Leydig cell steroid production because another connexin is present to coordinate stimulus-secretion coupling, either alone or in parallel with Cx43. The present experiments were designed to test this hypothesis.

Materials and methods

Leydig cell isolation

Adult CD1 mice were purchased from Charles River Laboratories (Saint-Constant, PQ). Care and euthanasia of the mice conformed to a protocol that was approved by the Animal Use Subcommittee of the University Council on Animal Care, the University of Western Ontario. Each Leydig cell isolation was performed using the testes from 6 mice. The testes were decapsulated and minced with scissors. Dispersion of the tissue was carried out in a 50 mL tube containing prewarmed dissociation medium (5–10 mL/testis) and shaken at 60 cycles/min at 37°C for 10 min. The dissociation medium consisted of medium 199 with Hank’s salts containing 12 µg/mL DNase I (both from Sigma-Aldrich, Oakville, ON). The suspension was repeatedly dissociated with a fire-polished Pasteur pipette to break up large clumps, and then with a 16G needle. The suspension was then filtered through a nylon mesh (70 µm) into two 50 mL centrifuge tubes and each was topped up with medium 199 to the 50 mL mark. The tubes were then centrifuged for 10 minutes at 300 x g at 4°C after which the supernatant was discarded. The pellets were resuspended and combined in a total volume of 4 mL.

For preparation of an enriched Leydig cell fraction, 0.5 mL of cell suspension was layered on each 45% Percoll (GE Healthcare, Baie d’Urfe, PQ) gradient previously prepared in medium 199 by centrifugation 1 hr at 20,000 x g. Centrifugation of the cell suspension was carried out for 20 minutes at 800 x g to produce a band of Leydig cells close to the bottom of the tube. A hypodermic needle was used to remove 2 mL from the bottom of the gradient, then the next 2 mL fraction that included the Leydig cell band was removed. The Leydig cell fractions from several gradients were combined in a single 50 mL tube which was then filled with medium 199 to the 50 mL mark. This tube was spun three times for 10 min each at 1,000 rpm, each time resuspending the pellet in a small volume of medium 199 before refilling the tube to the 50 mL mark. The final pellet was resuspended in 1–2 mL of medium. To identify the Leydig cells for counting, they were cytochemically stained for 3β-hydroxysteroid dehydrogenase (3β-HSD) activity using 5β-androstan-3β-ol-17-one (Sigma - Aldrich) as substrate, nicotinamide adenine dinucleotide (NAD⁺, Sigma - Aldrich) as coenzyme, and nitro blue tetrazolium (NBT, Sigma -Aldrich) as final hydrogen acceptor. The stained Leydig cells were counted using a haemocytometer.

RT-PCR screen for connexin transcripts

RNA was extracted from the enriched Leydig cell fraction using a QIAshredder kit combined with the RNeasy kit (both from Qiagen, Toronto, ON) according to the

manufacturer's instructions. The reverse transcription reaction was carried out using 11 μ l of mRNA solution and 1 μ l of oligo(dT) primer (50 pmol). The reactions were incubated at 65°C for 15 min then cooled on ice for 5 min. This was followed by addition of 4.3 μ l of 5X first strand buffer, 1.6 μ l of dithiothreitol (DTT) solution (1 M), 1.1 μ l of 10 mM deoxyribonucleotide triphosphate (dNTP) mix, and 1 μ l (200 U) reverse transcriptase enzyme (Superscript II R) (all components from Life Technologies, Burlington, ON). The reaction mix was incubated at 37°C for 1 h followed by treatment at 95°C for 5 min before storage of the cDNA preparations at -20°.

PCR was performed using a Perkin Elmer 2400 thermal cycler. Each reaction contained 10.7 μ l of ddH₂O, 1.25 μ l of 10X PCR buffer, 0.75 μ l of MgCl₂ solution [50 mM], 1.5 μ l of 2 mM dNTP mix, 2.5U of Taq polymerase (all reagents from Life Technologies), 0.3 μ l of cDNA solution, and 0.75 μ l each of the 3' and 5' primers [10 μ M] (Table 1). The cDNA preparations were first tested using primers designed to amplify an intron-spanning segment of the *Actb* gene in order to rule out the presence of contaminating genomic DNA. Once the cDNA was confirmed to be uncontaminated, amplification reactions were carried out for various numbers of cycles using connexin specific primers. Primers for *Gapdh* were used as a positive control and a sample without cDNA was used as a negative control. Before the cycles began there was a 5 min denaturation period at 94°C. Each subsequent cycle then consisted of denaturation at 94°C for 30 sec, annealing at a temperature specific to the primers for 30 sec, and an elongation step at 72°C for 30 sec. Following the last cycle there was a 7 min extension period at 72°C. The PCR products were separated on 3% agarose gels with images obtained using the ChemiDoc XRS imaging system and Quantity One software from Bio-Rad (Mississauga, ON).

LH-stimulated testosterone production

The testes were removed from four 6-wk old CD1 mice and those from each mouse were divided between control (no drug treatment) and experimental groups [200 μ M carbenoxolone (CBX) or 300 μ M quinine, in separate experiments; both Sigma -Aldrich]. The testes were manually decapsulated and placed into 1 ml of M199 with Earle's salts and 1% bovine serum albumen (BSA). After adding CBX or quinine to the experimental groups, the testes were incubated at 37°C for a total of 8 h. After the first 4 h, a 50 μ l sample of the medium was removed for radioimmunoassay (RIA) of testosterone, and LH (NIH oLH26) was added to both the control and experimental groups to a concentration of 400 pg/ml. After another 4 h, a second 50 μ l sample was taken for RIA. A two-way ANOVA was used to analyze the data. RIA was performed by the Neuroendocrinology Assay Lab, Department Psychology, at the University of Western Ontario.

Immunolocalization

Testes were fixed in 4% paraformaldehyde (PFA) and embedded in paraffin for sectioning at 5 μ m thickness. Sections were processed for immunolabeling as described in Roscoe et al., 2001. Immunostaining for connexin36 (Cx36, also known as GJD2) utilized a mouse monoclonal antibody (Invitrogen cat. no. 37-4600, from Life Technologies, Burlington, ON) raised against a synthetic peptide from the C-terminal region of the rat/mouse protein, at 1/100 dilution. We used three different antibodies to visualize the distribution of connexin45 (Cx45, also known as GJA7) in Leydig cells: a rabbit polyclonal antibody raised against a synthetic peptide corresponding to residues 97-127 of the N-terminal portion of human Cx45 (cat. no. AP1547a from Abgent, San Diego, CA) at 1/100 dilution; a mouse monoclonal antibody raised against a synthetic peptide corresponding to residues 354-367 of the C-terminal portion of human Cx45 (cat. no. ab78408 from Abcam, Cambridge, MA) at 1/1000 dilution; and an undiluted mouse monoclonal hybridoma supernatant (antibody P3C9) raised against a synthetic peptide corresponding to residues 285-298 of the C-

terminal portion of human/rat/mouse Cx45. To localize Cx43 we used a rabbit polyclonal antibody raised against a synthetic peptide corresponding to residues 363-382 of the C-terminal portion of rat/human Cx43 (cat. no. C6219 from Sigma-Aldrich, St. Louis, MO) at 1/1000 dilution. β -HSD was immunolocalized using a polyclonal antibody raised against the human placental protein in the laboratory of Dr. Ian Mason at the University of Edinburgh, UK; it was used at a dilution of 1/500. Primary antibodies were detected using Alexa Fluor goat anti-mouse IgG 488 or Alexa Fluor goat anti-rabbit IgG 594 (1/1000 dilution; cat. nos. A11001 and A11005, respectively, from Life Technologies). Immunohistochemical detection of Cx45 and β -HSD was performed using the ABC Staining System from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

To provide a positive control for Cx45 localization in gap junctions, mouse hearts were frozen in OCT (VWR, West Chester, PA) and 5 μ m sections were cut on a cryostat microtome. The sections were fixed in ice-cold acetone and methanol (1:1), washed twice in PBS and then incubated with 5% normal serum and 1% BSA in PBS with 0.25% Tween-20 for 1 hour at room temperature. Slices were then incubated with the P3C9 antibody in blocking solution for 1.5 hour. After four washes in PBS with 0.05% Tween-20, the sections were incubated with Alexa-Fluor-546-conjugated goat anti-mouse secondary antibody (Invitrogen) for 1 hour. After washing, the slices were mounted in ProGold anti-fade medium (Invitrogen). Images were acquired using a Deltavision deconvolution microscope with a 60 $\times$ oil objective.

Results

Identification of connexin transcripts in Leydig cells

To screen for transcripts that could encode gap junction proteins in adult mouse Leydig cells, RT-PCR was performed on RNA isolated from the enriched Leydig cell fraction using the connexin primer sets listed in Table 1 (we did not screen for transcripts encoding connexin23 since this connexin is structurally aberrant and does not form functional intercellular channels; Sonntag et al., 2009). Strong amplicon bands representing connexins 36, 43, and 45 were consistently detected on gels with other bands being less prominent and more variable (Figure 1). Failure to detect transcripts encoding Cx33, which is restricted to the tubular compartment of rodent testes (Fiorini et al. 2004, 2006), provided assurance that contamination of the enriched Leydig cell fraction from the tubular compartment was minimal. Based on these results, we focussed our attention on Cx36 and Cx45 as possibly being co-expressed with Cx43 in mouse Leydig cells.

Involvement of connexins in testosterone production

To test whether Cx36 or Cx45 plays an essential role in Leydig cell function, we tested the effects of gap junction channel blockers on testosterone production in vitro. Adult mouse testes were first decapsulated to facilitate access of the channel blockers, carbenoxolone and quinine, to the interstitial compartment and to allow release of testosterone into the culture medium. While not specific for connexin-based membrane channels, both of these agents are potent blockers of gap junction channel conductance with carbenoxolone being effective regardless of which connexin contributes to the channel (reviewed by Juszczak and Swiergiel, 2009). In contrast, quinine has been shown to discriminate between channels composed of Cx36 or Cx45 (both of which are sensitive to the drug) and Cx43 (insensitive up to 300 μ M; Srinivas et al., 2001). We compared the effects of these two drugs on LH-induced testosterone production as outlined in Figure 2A. As shown in Figure 2B, carbenoxolone significantly reduced the level of testosterone measured in the culture medium of decapsulated testes after 4 h of LH stimulation, consistent with one or more types of gap junction channel being involved in Leydig cell steroidogenesis. Similar results

were obtained with quinine (Figure 2C). We concluded that either Cx36 or Cx45 (or both) could contribute to gap junctions and be required for testosterone production by mouse Leydig cells.

Localization of connexins in Leydig cells

To confirm that Cx36 and Cx45 are present in Leydig cell gap junctions, we used antibodies raised against these connexins, as well as an antibody against Cx43, for immunolabeling testis sections. Immunofluorescence analysis revealed the presence of Cx36 in gap junction-like plaques in the Leydig cells (Figure 3). In some images, co-localization of Cx36 and Cx43 could be detected, indicating that these two connexins are expressed in the same cells and are located together in some gap junctions. In contrast, Cx45 showed no localization in structures resembling gap junctions but was distributed throughout the Leydig cell cytoplasm (Figure 4). Whether by immunofluorescence or immunohistochemistry, there was no suggestion that Cx45 assembled into prototypical gap junction arrays in 3β -HSD expressing Leydig cells, either alone or colocalizing with Cx43. This unusual subcellular distribution was revealed by immunolabeling with three different Cx45 antibodies, independently derived (Figures 4 and 5), and contrasted starkly with the localization of Cx45 in mouse heart gap junctions (Figure 5B). We conclude that Cx45 is expressed in Leydig cells along with Cx43 and Cx36 but it is unlikely that Cx45 assembles into gap junctions in those cells.

Discussion

This study was initiated in response to the finding (Kahiri et al., 2006) that Leydig cells lacking Cx43, previously thought to be the only connexin contributing to gap junctions in those cells, retain the capacity for gap junctional communication and remain capable of responding to LH by producing androgens. One interpretation of those findings is that at least one other member of the connexin family is expressed in Leydig cells and it, rather than Cx43, is required for their steroidogenic function. In the present study, our RT-PCR screen for connexin transcripts in Leydig cell-enriched fractions from adult testes consistently identified transcripts encoding both Cx36 and Cx45 along with Cx43. Furthermore, the ability of quinine to inhibit testosterone production when used at a concentration known to be effective in disrupting gap junction channels composed of Cx36 or Cx45 but not Cx43 implicates either or both of those two connexins as playing a role in Leydig cell steroidogenesis. It remains to be determined what role Cx43 plays in Leydig cells but the evidence indicates it is not essential for steroidogenesis (Roscoe et al., 2001; Kahiri et al., 2006).

There is evidence for the involvement of connexins in hormone production or secretion in other organs. In the ovary, Cx43 is essential for granulosa cell proliferation (Ackert et al., 2001) and thus also for the rising estradiol levels associated with follicular growth, but there is no evidence for direct regulation of estradiol production/secretion by gap junctional communication (GJIC) in those cells. Likewise, Cx43 expression level is correlated with progesterone output in luteal cells but again, a direct cause-and-effect relationship has not been established (Borowczyk et al., 2007). On the other hand, there is ample evidence for a connexin or GJIC regulating stimulus-secretion coupling. Cx36, for example, is expressed in pancreatic islets where it forms gap junctions that govern the dynamics of insulin secretion by coordinating the responses of individual β cells to glucose (Benninger et al., 2011; Head et al., 2012). A similar coordinating function has been proposed for Cx36 and/or Cx43 gap junctions in adrenal chromaffin cells releasing catecholamines in response to cholinergic synaptic transmission (reviewed by Colomer et al., 2009). Cx45 has also been cited as being involved in stimulus-secretion coupling, both in the juxtaglomerular apparatus of the kidney

(for renin secretion; Hanner et al., 2008) and in the bone marrow (along with Cx43, for chemokine secretion; Schajnovitz et al., 2011).

Either or both Cx36 and Cx45 could play an essential role in the LH-stimulated production of testosterone by Leydig cells but, given the intracellular distribution of Cx45 and its apparent absence from gap junctions, their roles must be quite different. Our evidence is consistent with Cx36 contributing to gap junctions in Leydig cells as it does in other cell types including neurons (Al-Ubaidi et al., 2000) in addition to pancreatic β -cells (Serre-Beinier et al., 2000; Theis et al., 2004). If, in fact, both Cx36 and Cx43 contribute to Leydig cell gap junctions, then the properties of the population of channels linking those cells must be more complex than previously realized. The channels formed by these two connexins have distinct biophysical properties: Cx36 channels are cation-selective with unitary conductance of only ~15 pS whereas Cx43 channels are relatively non-selective with unitary conductance of ~100 pS (Teubner et al., 2000; Harris, 2001; Srinivas et al., 2001; Bukauskas, 2012). An electrophysiological analysis of the properties of mouse Leydig cell gap junction channels using double whole cell patch clamp (Varanda et al., 1994) confirmed the predominance of Cx43 channels but also provided evidence of a subpopulation of channels with much smaller unitary conductance (~21 pS in that study) that could possibly incorporate a different connexin, although a subsequent analysis (Pérez-Armendariz et al., 1994) failed to confirm this. Given that Cx36 co-assembles with Cx43 in gap junction plaques in mouse Leydig cells as shown in Figure 3, a subpopulation of homomeric/homotypic Cx36 gap junctions may be too small to identify electrophysiologically. A mixed population of gap junction channel types provided by co-oligomerization and/or co-assembly of two different connexins into gap junctions would provide Leydig cells with a greater repertoire of intercellular channels and hemichannels with different permeabilities (Harris 2001). It should be noted, however, that male knockout mice lacking Cx36 are capable of impregnating females and are thus considered “fertile” (Deans et al., 2001; Hormuzdi et al., 2001). These mice will need to be examined in greater detail to confirm that there are no deficiencies in sperm number or quality that would render the males subfertile as was demonstrated for males carrying the dominant loss-of-function Cx43^{G60S} mutant (Gregory et al., 2011).

In contrast to Cx43 and Cx36, the likely function of Cx45 in Leydig cells is elusive: we could find no indication of its presence in gap junctions using any of the three independently-derived antibodies we employed, including one which we demonstrated to stain prototypical gap junction-like structures in heart tissue. This is despite the well-established contribution of Cx45 to intercellular communication in other cell types, including co-localization with Cx36 in neuronal gap junctions (Li et al., 2008). Furthermore, the possibility that Cx45 and Cx36 can provide equivalent functions has been confirmed in knockin mice in which Cx36 replaced Cx45 in retinal neurons (Frank et al., 2010). Thus a strictly non-junctional distribution of Cx45, as revealed in the present study in Leydig cells, is highly unusual. One possible role for Cx45 in Leydig cells that might be consistent with its unusual distribution is that of a scaffolding protein. Several connexins including Cx45 (Kausalya et al., 2001; Laing et al., 2005), have been shown to be able to link with the actin cytoskeleton via binding to the tight junction proteins, ZO1 or ZO3. Whereas Leydig cells are not epithelial and are not known to have tight junctions, there is evidence of ZO1 immunoreactivity in cells of the testicular interstitial region (Hejmej et al., 2012). The presence of ZO1 in Leydig cells and its linkage of Cx45 with the actin cytoskeleton would provide one possible explanation for its intracellular distribution, if not its function. Unfortunately, knockout mice lacking Cx45 die in utero due to cardiac and vascular defects (Krüger et al., 2000; Kumai et al., 2000), hence their Leydig cell function and fertility could not be tested. Further research—for example, employing mice with Cx45 deleted specifically in Leydig cells—will be needed to elucidate the role, if any, of Cx45 in those cells.

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Figure 1. RT-PCR screen for connexin transcripts in the Leydig cell fraction of mouse testis. L, DNA ladder (base pairs); +, positive control (GAPDH); -, negative control (no template). All PCR reactions were carried out for 30 cycles with the amplicons being separated on three parallel gels.

Figure 2.

Pharmacological evidence of a role for Cx36 and/or Cx45 in Leydig cell function. A, time line of the experiments. B, effect of treatment with carbenoxolone (CBX, 200 μ M). C, effect of treatment with quinine (300 μ M). In both experiments, testosterone levels between control and treated testes were significantly different at the 8 h time point ($p < 0.001$).

Figure 3.

Localization of Cx36 and Cx43 in mouse Leydig cells by immunofluorescence. A, bright field (left images) and fluorescence (right images) of testis sections immunostained for Cx36 (green, arrows) and Cx43 (red, small arrowheads). Points of overlap of the two fluorescence signals (orange) are indicated by large arrowheads. Nuclei were stained with Hoechst (blue). B, immunostain for Cx36 in connexin-negative HeLa cells transfected with *Gjd2* cDNA, confirming antibody specificity. Nuclei were stained with Hoechst (blue). Scale bars: A, 50 μ m; B, 25 μ m.

Figure 4.

Localization of Cx45 in mouse Leydig cells by immunofluorescence (A–G) and immunohistochemistry (H–K). A, brightfield image of an interstitial region from a testis section. B, Cx45 immunostain using the Abcam antibody. C, 3 β -HSD immunostain. D, Cx45 and 3 β -HSD immunostain merged (the very bright spots are autofluorescence from erythrocytes). E, co-immunostain for Cx45 (green, using the Abcam antibody) and Cx43 (red) with nuclei labeled with Hoechst (blue); arrows indicate Cx43-containing gap junctions. F and G, negative control (brightfield and darkfield, respectively) of a testis section treated with secondary antibody only. H, Cx45 immunostain using the Abcam antibody. I, negative control. J, 3 β -HSD immunostain. K, negative control. Interstitial regions are labeled i. Scale bars = A, 25 μ m; E, 25 μ m; F and H–K, 100 μ m.

Figure 5.

Localization of Cx45 and Cx43 in mouse Leydig cells by immunofluorescence. A, conventional fluorescence micrograph showing co-immunostaining for Cx45 (green, using the P3C9 antibody) and Cx43 (red). B, visualization of Cx45-containing gap junctions (red, using the P3C9 antibody) and nuclei (blue) in mouse heart. C, confocal fluorescence micrograph showing co-immunostaining for Cx45 (red, using the Abgent antibody) and Cx43 (green). Scale bars = 10 μ m.

Table 1

Connexin PCR primers.

Connexin	Upstream/Downstream Sequences	Annealing Temp. (°C)	Amplicon Length (bp)
Cx26	5'-CGGAAGTTCATGAAGGGAGAGAT-3' 5'-GGTCTTTTGGACTTTCCTGAGCA-3'	60	365
Cx29	5'-GCTCATGGGATTCCGTCTC-3' 5'-GGTTGTGCTGCCAATACAAGG-3'	55	460
Cx30	5'-AATGTGGCCGAGTTGTGTTA-3' 5'-CCAAGGCCAGTTGTCAC-3'	53	399
Cx30.2	5'-TCATGCTGATCTTCCGCATC-3' 5'-GGCCTGGTGTCATGGAGTAGAT-3'	55	218
Cx30.3	5'-TCAAACATGGGCCCAATG-3' 5'-GGGAGTCACAGAGCAAGC-3'	60	182
Cx31	5'-AGAAGCACGGGGAGCAAT-3' 5'-TACTATGCTGGCGCACTG-3'	60	182
Cx31.1	5'-ATATACCCTCCCTTCTATGGT-3' 5'-TCACAGAATGGTTTCTTCA-3'	55	357
Cx32	5'-CTGCTCTACCCGGCTATGC-3' 5'-CAGGCTGAGCATCGGTGCGTCTT-3'	60	386
Cx33	5'-GAGGCAGATTGCTGCTAAC-3' 5'-AGGGCATGGTAGCTCATCAC-3'	56	323
Cx36	5'-GGGGTGCTGCAGAACACAGAGA-3' 5'-ACCACACAAATGCCGCTCACA-3'	60	344
Cx37	5'-GGCTGGACCATGGAGCCGGT-3' 5'-TTTCGGCCACCCTGGGGAGC-3'	60	422
Cx39	5'-TTGCTCGCATGTACCCTG-3' 5'-CACCTGACCTTGGCTAAGATA-3'	55	265
Cx40	5'-CTGTCCCCACCCAGTCAACT-3' 5'-CGGTTGTCACTATGGTAGC-3'	57	460
Cx43	5'-TACCACGCCACCACCGGCCA-3' 5'-GGCATTTTGGCTGTCGTCAGGGAA-3'	60	294
Cx45	5'-TTCCAAGTCCACCATTTTAT-3' 5'-ATCGTTCCTGAGCCATTCTGA-3'	55	444
Cx46	5'-GGAAAGGCCACAGGGTTTCCTGG-3' 5'-GGGTCCAGGAGGACCAACGG-3'	60	332
Cx47	5'-ATGGGATTGCTTCGTGTCGC-3' 5'-ACGCACCACCAGGCTGTAGTCTG-3'	60	256
Cx50	5'-CTTGTATCCCGGCCTACTGA-3' 5'-CTTCTCTCCCACTTCCGGTTCCA-3'	60	466
Cx57	5'-CCACATGACCGCAGTG-3' 5'-GGTGATGGGCTGTTTCT-3'	60	392