Expression, Characterization, Cloning and Functional Impact of Slc12a5 in the Endocrine Pancreas

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Expression, characterization, cloning and functional impact of Slc12a5 
in the endocrine pancreas

A thesis submitted in partial fulfillment

Of the requirements for the degree of

Master of Science

By

SHAMS KURSAN

B.S., Damascus University 2010

2014

Wright State University
I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY SHAMS KURSAN ENTITLED EXPRESSION, CHARACTERIZATION, CLONING AND FUNCTIONAL IMPACT OF Slc12a5 IN THE ENDOCRINE PANCREAS BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Kursan, Shams. M.S. Department of Pharmacology and Toxicology, Wright State University, Boonshoft School of Medicine, 2014: Expression, characterization, cloning and functional impact of Slc12a5 in the endocrine pancreas.

The Slc12a5 gene encodes the neuron-specific electroneutral K⁺Cl⁻ cotransporter-2 (KCC2), a constitutively active Cl⁻ extruder. In mature neurons, KCC2 functionally predominates over other members of the Slc12a family, particularly the Na⁺K⁺2Cl⁻ cotransporter-1 (NKCC1), a ubiquitous Cl⁻ loader. Therefore, KCC2 keeps and maintains the intracellular chloride concentration ([Cl⁻]) below values predicted by thermodynamic equilibrium and in doing so, KCC2 plays a key role in neuron physiology: it makes possible the inhibitory action of neurotransmitters such as γ-aminobutyric acid (GABA) and glycine. Neurons and insulin-secreting pancreatic β-cells share many genes and functions. Indeed, neuron-specific genes have been also found in β-cells whereas both neurons and β-cells are electrically excitable. Our experiments demonstrate that, like in neurons, KCC2 is expressed in β-cells and suggest that the functional balance NKCC1/KCC2 modulates insulin secretion in response to glucose.
# Table of contents:

**Introduction** ......................................................................................................................................................... 1

1. The *SLC12A* family of genes: an overview ........................................................................................................ 1

2. The *SLC12A* family of Cl⁻ loaders ...................................................................................................................... 4

3. The *SLC12A* family of Cl⁻ extruders .................................................................................................................. 5

4. Functional regulation of KCCs ............................................................................................................................... 9

5. KCC2 in neuronal signaling and function ............................................................................................................. 11

6. Molecular characterization of KCC2 and variants ............................................................................................... 14

7. Molecular determinants of KCC2 function ........................................................................................................... 19

8. The concept of neuron-specific expression of KCC2 .......................................................................................... 21

9. Transcription factors involved in neuronal KCC2 expression ........................................................................... 23

10. Expression of KCC2 in non-neuronal cells ......................................................................................................... 25

11. Similarities between neurons and insulin-secreting β-cells ............................................................................. 28

12. Insulin secretion depends on [Cl⁻] of β-cells ...................................................................................................... 29

**Hypothesis and Specific aims** ............................................................................................................................. 32

**Methods** ............................................................................................................................................................... 33

1. Reagents, kits and immunochemicals .................................................................................................................... 33

2. Cell culture ............................................................................................................................................................ 34

3. Islets extraction and purification .......................................................................................................................... 36

4. Reverse transcription-polymerase chain reaction (RT-PCR) ............................................................................. 37

5. Long-range PCR ....................................................................................................................................................... 38

6. Pfx/Pfu-generated amplicon purification, 3'-A overhanging and pGEM-T cloning ........................................... 40

7. Molecular cloning of long-range PCR products ................................................................................................... 41

8. Transformation of competent *E. coli* bacteria ...................................................................................................... 41

9. Plasmid purification, screening, characterization and sequencing ...................................................................... 42

10. Site directed mutagenesis ................................................................................................................................... 43
11. Subcloning and tagging ................................................................. 44
12. DNA Ligation .............................................................................. 44
13. Western Blotting ......................................................................... 45
14. Immunofluorescence microscopy .................................................... 46
15. Cell transfection .......................................................................... 48
16. Potentiometric determination of total cellular Cl⁻ content .................. 48
17. Insulin Secretion .......................................................................... 51
18. Statistics ..................................................................................... 52
Results ............................................................................................... 54
1. Expression of the Slc12a family of Cl⁻ extruders in β-cells .................. 54
2. Expression and localization of KCC2 in insulin-secreting β-cells .......... 58
3. Cloning and characterization of KCC2a and KCC2b ......................... 62
4. Expression pattern of KCC2a, KCC2b and KCC2a-S25 in mouse tissues .... 69
5. Functional evidence of KCC2 in MIN6 β-cells and impact on insulin secretion .... 72
Discussion .......................................................................................... 76
Conclusions ....................................................................................... 86
Acknowledgments .............................................................................. 87
References .......................................................................................... 88
List of figures:

Figure 1: Cladogram of the *Slc12a* family members and predicted protein topologies…2

Figure 2: Regulation of \([Cl^-]\), in cells.................................................................13

Figure 3: Partial representation of KCC2a and KCC2b mRNAs.................................16

Figure 4: Representation of aligned human KCC2 transcripts posted in *GenBank*......18

Figure 5: Calibration curves for the ion-selective electrode........................................50

Figure 6: Insulin standard curves.............................................................................53

Figure 7: KCC transcripts and their primer sets used in PCRs.................................55

Figure 8: Expression of KCC mRNAs in MIN6 β-cells.............................................56

Figure 9: Expression of KCC2 mRNAs in rat β-cells and mouse or human islets........57

Figure 10: KCC2 protein expression analysis using monoclonal antibodies.............59

Figure 11: KCC2 protein expression analysis using polyclonal antibodies...............61

Figure 12: KCC2 protein expression in pancreas and islets using polyclonal

Antibodies.............................................................................................................63

Figure 13: KCC2 protein localization in islet β-cells of the mouse and human

pancreas using polyclonal antibodies..............................................................64

Figure 14: Cloning and characterization of KCC2a mRNAs expressed in

MIN6 β-cells........................................................................................................66

Figure 15: Predicted primary structure of cloned mKCC2s......................................68

Figure 16: Transient expression of HA-mKCC2a and myc-mKCC2a-S25

in MIN6 and COS7 cells....................................................................................70

Figure 17: Expression pattern of KCC2 mRNAs and protein in mouse tissues.........71

Figure 18: KCC2a/b-S25 expression in mouse endocrine tissues............................73
Figure 19: Impact of KCC2 function on Cl⁻ uptake and insulin secretion
in MIN6 β-cells.................................................................75
List of tables:

**Table1**: Slc12a5 homologs, variants, RefSeqs and sources ............................................17

**Table 2**: Primary and secondary antibodies .................................................................35

**Table 3**: Sense and antisense primers ........................................................................39
Introduction

1. The SLC12A family of genes: an overview

The database of the Human Genome Organization (HUGO) Nomenclature Committee identifies 43 gene families nucleating 298 genes, the majority of them encoding proteins with transport ability and overall named as solute carriers (SLCs). These SLC proteins include cotransporters, exchangers, passive transporters, mitochondrial and vesicular transporters. Each representative member of these families has different pharmacological, physiological and pathological properties (Hediger, et al. 2004). The genes grouped in the SLC family 12, group A (SLC12A) encode at least 7 secondary active cation-chloride (Cl\(^–\)) cotransporters (CCCs) identified with numbers from 1 to 7 (SLC12A1-7) plus two additional members of undefined transport capabilities i.e., SLC12A8-9 (Hartmann, et al. 2014) (Figure 1). Many of the protein products encoded by SLC12A1-7 genes have been extensively characterized at the molecular, pharmacological and functional levels. In general, CCCs are considered key regulators of the cellular volume and of the intracellular chloride concentration ([Cl\(^–\)]) (Gamba 2005).

SLC12A1-3 upload Cl\(^–\) into cells by using the thermodynamic energy stored in the inwardly directed Na\(^+\) gradient, whereas SLC12A4-7 use the driving force of the outwardly directed K\(^+\) gradient to extrude Cl\(^–\) from cells. Both, the Na\(^+\) and K\(^+\) gradients are generated and maintained in all cells by the primary active transporter Na\(^+\)/K\(^+\)-ATPase (Russell 2000). The active transport of Cl\(^–\) into cells increase its concentration above levels predicted by Nernstian equilibrium thus generating an outwardly directed
Figure 1. Cladogram of Slc12a family members and predicted protein topologies. *In silico* phylogenetic neighbor-joining analysis using the genetic distance model of Jukes & Cantor (Evolution of Protein Molecules. New York: Academic Press. pp. 21-132, 1969). Shown are the three branches of the Slc12a family as well as their respective predicted protein topologies.
gradient with the potential to generate electrogenic work. It is the non-equilibrium distribution of Cl$^-$ the one responsible for the depolarizing current carried by Cl$^-$ upon opening of Cl$^-$ channels of any nature. Conversely, active Cl$^-$ extrusion from cells, a task mainly performed by SLC12A4-7 results in [Cl$^-$] below thermodynamic equilibrium thus promoting the hyperpolarizing and classically inhibitory entry of the anion upon opening of Cl$^-$ channels (Alvarez-Leefmans 2012).

The main products of the SLC12A1-7 genes share several properties, most notably the electroneutral transport stoichiometry (Gamba 2005) and therefore, it is assumed that this kind of ion transport neither contributes to the steady state membrane potential (E$_m$) nor is affected by it (Jacob, et al. 1984). The molar ratio of ions transported by SLC12A1-2, SLC12A3 or SLC12A4-7 is: 1Na$^+$, 1K$^+$ and 2Cl$^-$, 1Na$^+$, 1Cl$^-$ or 1K$^+$, 1Cl$^-$, respectively, and therefore the functional activity of these transporters results in no transfer of net charge across plasma membrane i.e., there is no change in E$_m$. Another shared feature among SLC12A members is their predicted in silico topology. This is based on the amino acid sequences of SLC12A1-7 proteins, which are highly similar (>50%) but not identical (Gamba 2005). However, the actual structure of these transporters has not been elucidated yet and several in silico models have been proposed based on different algorithms (Di Fulvio and Alvarez-Leefmans 2009). The simplest proposed topology based on the Kyte-Doolittle hydropathy algorithm (Kyte and Doolittle 1982) includes two large intracellular N- and C-termini separated by at least twelve trans-membrane (TM) domains. The predicted extracellular loop connecting TM7 and TM8 in SLC12A1-3 or TM5 and TM6 in SLC12A4-7 contains conserved consensus sites for N-glycosylation and therefore, these loops are considered to face the extracellular side of the plasma membrane (see Figure 1). These N-glycosylation sites
are usually occupied by N-glycans of diverse nature and molecular weights (MWs). Indeed, \textit{SLC12A1-7} are found expressed in cells as proteins with MWs ranging from ~125 kDa (core/high-mannose N-glycans) to ~130-170 kDa (hybrid/complex N-glycans).

2. The \textit{SLC12A} family of Cl\textsuperscript{−} loaders

Although \textit{SLC12A1}, \textit{SLC12A2} and \textit{SLC12A3} belong to the same family of genes based on phylogenetic analysis, sequence identity, predicted topologies, potential N-glycan states, ability to upload Cl\textsuperscript{−} into cells and maintain [Cl\textsuperscript{−}] above electrochemical equilibrium, these three genes produce protein transporters that can be functionally distinguished. For instance, \textit{SLC12A2} also known as the Na\textsuperscript{+}K\textsuperscript{+}2Cl\textsuperscript{−} cotransporter-1 (NKCC1) transports ~590 molecules of water per 1Na\textsuperscript{+}, 1K\textsuperscript{+}, 2Cl\textsuperscript{−} ions uploaded (Hamann, et al. 2010) whereas \textit{SLC12A1} i.e., NKCC2 is a "dry" cotransporter (Zeuthen and Macaulay 2012). Whether \textit{SLC12A3} (the Na\textsuperscript{+}Cl\textsuperscript{−} cotransporter, NCC) transports water remain untested, but this co-transporter can be distinguished from NKCCs by the fact that NCCs do not translocate K\textsuperscript{+} into the cell (Gamba, et al. 1994). In addition to these transport properties, NKCCs and NCCs exhibit different sensitivities to widely prescribed diuretics. NKCCs are the targets of the loop-diuretics bumetanide (BTD) or furosemide, whereas NCCs are inhibited by thiazides (Gamba 2005). Further, NKCCs and NCCs differ in their expression and localization patterns among tissues and cell types. Indeed, NKCC1 is considered the ubiquitous secretory isoform of NKCCs because it is found in the basolateral side of epithelial cells where it plays a role in fluid secretion whereas NKCC2 is regarded as the absorptive kidney-specific NKCC because its location in the apical side of the epithelial cells of the thick ascending limb of Henle's loop where is involved in NaCl absorption (Ares, et al. 2011; Arroyo, et al. 2013; Gamba 2005). NCC is abundantly, although not exclusively expressed in epithelial cells of the
distal convoluted tubule of the kidney where it participates in salt reabsorption (Arroyo et al. 2013; Reilly and Ellison 2000).

Nevertheless, it is important to mention that the epithets commonly used in the literature and textbooks to identify NKCC1 (e.g., "ubiquitous", "basolateral", "secretory") or NKCC2 (e.g., "kidney-specific", "absorptive") do not make justice to the fact that NKCC1 is expressed in the apical side of the choroid plexus cells where it may not play a role in secretion (Brown, et al. 2009; Plotkin, et al. 1997) and it has not been found in many cell types (Kaplan, et al. 1996; Majid, et al. 2001; Shillingford, et al. 2002), whereas NKCC2 is found outside of the kidney (Akiyama, et al. 2010; Akiyama, et al. 2007; Alshahrani, et al. 2012; Alshahrani and Di Fulvio 2012; Nickell, et al. 2007; Xue, et al. 2009; Xue, et al. 2014; Zhu, et al. 2011) a fact that has been recognized in a recent review (Castrop and Schiessl 2014).

3. The SLC12A family of Cl⁻ extruders

The K⁺Cl⁻ cotransporters (KCCs) are involved in Cl⁻ extrusion and cell volume regulation (Adragna, et al. 2004). As many other transport systems, KCCs are secondary active cotransporters with the ability to function in both directions depending on the sum of the chemical potentials of Cl⁻ and K⁺, a characteristic that enables KCCs to play a role as a dynamic K⁺ buffering i.e., capturing high extracellular K⁺ upon cellular activity (Payne 1997). KCCs were originally described in red blood cells as a 1K⁺, 1Cl⁻ coupled cotransport mechanism with the remarkable ability to respond to N-ethylmaleimide (NEM) and cell swelling upon acute hyposmotic shock (Dunham and Ellory 1981; Lauf and Theg 1980). After being well defined in volume regulation of red cells, the role of KCCs in epithelial salt transport was reported (Reuss 1983). In
mammals, there are four known KCC isoforms i.e., *SLC12A4* (KCC1), *SLC12A5* (KCC2), *SLC12A6* (KCC3) and *SLC12A7* (KCC4). Phylogenetic distance analysis of KCC main proteins revealed that KCC1 has higher identity to KCC3 whereas KCC2 shares abundant similarity with KCC4 (Hartmann et al. 2014). KCCs are represented by many splice variants. Most of them differ at their N- or C-termini as the result of the election of alternative promoters, first exons, alternative splicing, alternative election of first codons or internal splicing sites as well as alternative ends in last exons (Gagnon and Di Fulvio 2013). Relative to KCC1 or KCC3, which have several cloned alternatively spliced variants (Crable, et al. 2005; Mercado, et al. 2005; Pellegrino, et al. 1998), KCC2 and KCC4 genes appear to have fewer transcripts, some of them of uncertain function (Lauf, et al. 2012; Nickell et al. 2007; Tao, et al. 2012). Within the group of KCCs, KCC3 has the greatest number of alternatively spliced variants, particularly within in the 5'-UTR and sequences encoding the N-terminus (Gagnon and Di Fulvio 2013). For instance, KCC3a and KCC3b, the two major KCC3 variants described, differ in their first coding exons, which in turn appear to be subjected to additional splicing events (Mercado et al. 2005; Pearson, et al. 2001). Although the physiological difference between all KCC3 variants is not known, KCC3a and KCC3b exhibit different potential protein kinase C (PKC) phosphosites suggesting that different regulatory mechanisms may impact KCC3a or KCC3b function (Mercado, et al. 2004). Unlike KCC3, only one variant of KCC4 has been characterized at the molecular and functional levels *in vitro* and *in vivo* (Boettger, et al. 2002; Fujii, et al. 2011; Hartmann and Nothwang 2011; Hartmann, et al. 2010; Karadsheh, et al. 2004; Melo, et al. 2013a; Mount, et al. 1999; Weng, et al. 2013). However, KCC4 may have additional C-terminal variants, as it has been suggested (Nickell et al. 2007).
At the pharmacological level, all studied KCCs are inhibited by furosemide, BTD and several other compounds, either directly, indirectly or at high concentrations (Gamba 2005; Lauf, et al. 1984). Interestingly, the di-hydro-indenyloxyalkanoic acid (DIOA) is considered a specific inhibitor for KCCs (Chee, et al. 2006; Olivieri, et al. 1991). However, DIOA also inhibits anion exchangers (Garay, et al. 1988) and Ca\(^{2+}\)-activated K\(^{+}\) channels (Lauf, et al. 2008). Recently, more potent and selective inhibitors and agonists of KCC2 have been reported. For instance, the compound VU0240551 (VU) was optimized as a selective KCC2 antagonist showing ~100 times more preference for KCC2 over NKCC1 and higher potency relative to other analogs (IC\(_{50}\) = 61nM) (Delpire, et al. 2012; Delpire, et al. 2009). Recently, CLP257 was characterized as a specific enhancer of KCC2 transport function and plasma membrane location (Gagnon, et al. 2013), opening the interesting possibility that this KCC2 agonist could be used to alleviate neuronal disease that results from loss of KCC2 activity such as neuropathic pain (Lavertu, et al. 2014). Additional modulators of KCC2 function with potential in vivo relevance have also been described (Lebon, et al. 2012; Pegurier, et al. 2010).

In vivo, KCC2, KCC3 and KCC4 isoforms appear to have very different physiological roles, at least when studied in mice genetically engineered to lack one of them. Disruption of the \textit{Slc12a4} gene in mice lead to undetectable levels of KCC1 in the tissues tested. However, and in spite of its ubiquitous tissue distribution (Gillen, et al. 1996) and proposed role in cell volume regulation (Adragna et al. 2004), the absence of KCC1 in vivo did not result in overt phenotypic defects (Rust, et al. 2007). This surprising finding may suggest that the function of the housekeeping KCC1 is compensated by another mechanism such as KCC3 (Rust et al. 2007), or that KCC1 function is
redundant or silent under most physiological conditions. Notably, mice lacking KCC3 suffer from deafness, reduced threshold to seizures and neuronal degeneration in the peripheral and central nervous systems (Boettger, et al. 2003), a phenotype resembling Anderman's syndrome (peripheral nephropathy with agenesis in corpus callosum), which in humans is the result of mutations in the SLC12A6 gene (Howard, et al. 2002). However, KCC3 is not a transporter exclusive of neurons, as it may be the case for KCC2 (Blaesse, et al. 2009; Chamma, et al. 2012; Kaila, et al. 2014; Medina, et al. 2014), indicating that KCC3 along with KCC2 may have a role in the regulation of neuronal Cl\(^{-}\) homeostasis (Blaesse et al. 2009). This hypothesis is illustrated by the increased [Cl\(^{-}\)], measured in neurons of mice lacking KCC3 and their reduced seizure threshold (Boettger et al. 2003). In the case of KCC4, mice lacking this isoform showed distinctive tubular acidosis and deafness (Boettger et al. 2002), a finding that cannot be explained by the relative wide tissue distribution of this isoform (Becker, et al. 2003; Di Fulvio, et al. 2001; Karadsheh et al. 2004; Mount et al. 1999; Velazquez and Silva 2003). Contrary to KCC1, KCC3 or KCC4 knockout mice, elimination of all isoforms of KCC2 result in lethality at birth (Hubner, et al. 2001; Woo, et al. 2002) and therefore, KCC2 function cannot be replaced or compensated by other KCCs or any other Cl\(^{-}\) extruder.

In the case of KCC2, two main splice variants have been functionally identified in rodents and humans: KCC2a and KCC2b. However, more than two KCC2 variants are known, particularly those produced by the human SLC12A5 gene (see Section 6). Both, KCC2a and KCC2b are distinguished from other members of the KCC family most notably by their function and tissue distribution. For instance, KCC2a and KCC2b are constitutively active Cl\(^{-}\) extruders under most physiological conditions (Payne 1997; Uvarov, et al. 2007) whereas the studied splice variants of KCC1, KCC3 and KCC4
require hyposmotic challenge to extrude Cl\textsuperscript{−} i.e., they are activated by cell swelling (Melo, et al. 2013b; Mercado, et al. 2006; Mercado, et al. 2001; Mercado, et al. 2000; Mercado et al. 2005). At the tissue level, all KCCs, except KCC2 are expressed in a widespread fashion whereas the latter is considered "neuron-specific" in virtue of its exceptionally high expression levels in the brain and spinal cord relative to other tissues (Gagnon and Di Fulvio 2013; Kaila et al. 2014; Medina et al. 2014; Payne, et al. 1996). It is in the neuron where KCC2 is proposed to play a fundamental role in the maintenance of [Cl\textsuperscript{−}] below thermodynamic equilibrium and synaptic transmission (Kaila et al. 2014). In virtue of this non-equilibrium distribution of Cl\textsuperscript{−}, KCC2 determines the inhibitory potential of activated γ-aminobutyric acid (GABA) receptor type A (GABA\textsubscript{A}) and glycine receptors, two Cl\textsuperscript{−} channels activated by their respective neurotransmitters (Kaila et al. 2014; Medina et al. 2014; Rivera, et al. 1999) (see Section 5).

4. Functional regulation of KCCs

The main splice variants of each KCC isoforms have been extensively characterized at the functional level [reviewed in (Adragna et al. 2004; Payne 2009)]. As mentioned, all studied KCCs are activated by N-ethylmaleimide (NEM) (Lauf and Theg 1980) and are proposed as the ones involved in the restoration of cell volume upon cell swelling (Dunham and Ellory 1981), a phenomenon known as regulatory volume decrease (RVD). However, we need to keep in mind that KCC2 is slightly activated by cell swelling whereas KCC4 activity increases dramatically under hypotonic conditions when over-expressed in oocytes (Mercado et al. 2000). In general terms and in spite of several exceptions, the function of plasma membrane located KCCs are stimulated by staurosporine, a general protein kinase inhibitor (Adragna et al. 2004). Interestingly, KCCs and NKCCs appear to be reciprocally regulated by phosphorylation and
dephosphorylation events. For instance, phosphorylation mediated by WNKS (with no lysine kinases) and downstream kinases i.e., SPAKs (Ste20-related proline alanine-rich) or ORS1 (oxidative stress response-1) deactivate KCCs while activating NKCCs (Delpire and Gagnon 2006). These discoveries gave the basis for the original observations that KCCs are deactivated by phosphatase inhibitors such as calyculin A or by okadiac acid (Adragna et al. 2004; Bize, et al. 1999; Kaji and Tsukitani 1991). The diuretic BTD also inhibit KCCs, but at concentration at least an order of magnitude higher than the doses required to inhibit NKCCs whereas KCCs are more sensitive to furosemide-mediated inhibition than NKCCs (Alvarez-Leefmans 2012).

The functional properties of KCC2 have been defined in over-expression experiments using several cell lines including human embryonic kidney cells (HEK293), Chinese hamster ovary (CHO) cells, the pheochromocytoma cells line PC12, neuronal cell line C17, *Xenopus laevis* oocytes and primary neurons (Gamba 2005). When over-expressed in those cell models, KCC2 is found mostly as a hybrid/complex N-glycosylated protein of ~150-160 kDa that can be clearly seen towards the edges of the cells as well as in intracellular compartments (Payne 1997). At the plasma membrane, KCC2 exhibits constitutive K⁺ transport, which is dependent on Cl⁻ (Payne 1997; Song, et al. 2002; Strange, et al. 2000) and is further activated by cell swelling in some cells but not in all of them. For instance, hypotonic cell swelling does not activate KCC2 over-expressed in HEK293 (Payne 1997) or CHO cells (Strange et al. 2000) due to unknown mechanisms (Payne 1997; Payne, et al. 2003). At the pharmacological level, KCC2 appears to be the only KCC with relatively specific inhibitors and agonists (Delpire et al. 2012; Delpire et al. 2009; Gagnon et al. 2013; Lebon et al. 2012; Pegurier et al. 2010), (see Section 3).
5. **KCC2 in neuronal signaling and function**

The fact that most mature neurons studied keep and maintain $[\text{Cl}^-]_i$ at levels below electrochemical equilibrium is attributed mainly to the predominant functional presence of KCC2 over Cl$^-$ loaders such as NKCC1 (Dubreil, et al. 1995; Stein, et al. 2004; Thompson, et al. 1988). Most notably, the role for KCC2 in the regulation of the inhibitory post-synaptic potential via GABA$_A$ receptor-mediated plasma membrane hyperpolarization has been identified in invertebrates and most vertebrates studied (Aickin, et al. 1982; Dubreil et al. 1995; Thompson et al. 1988). In addition, KCC2 co-localizes with GABA$_A$ receptors in neurons (Payne 1997; Payne et al. 1996; Williams, et al. 1999), a finding that supports the concept that KCC2 makes possible the hyperpolarizing inhibitory effect of GABA. Indeed, KCC2 is proposed to actively extrude Cl$^-$ from mature neurons shifting the equilibrium potential for Cl$^-$ ($E_{Cl}$) \textit{i.e.}, the membrane potential at which the net flux of Cl$^-$ ions is zero, to values lower than the resting $E_m$ (Alvarez-Leefmans 2012; Alvarez-Leefmans 2009). Therefore, the opening of Cl$^-$ channels such as those activated by GABA, result in hyperpolarization and neuronal inhibition (Akerman and Cline 2006; Canciedda, et al. 2007; Chudotvorova, et al. 2005; Lee, et al. 2005; Rivera et al. 1999). Hence, in mature neurons GABA activation of its Cl$^-$-channel associated receptor GABA$_A$ hyperpolarizes the cell due to massive Cl$^-$ influx. In immature neurons, however, GABA$_A$ activation has clear depolarizing and excitatory actions (Blaesse et al. 2009; Chamma et al. 2012; Lu, et al. 1999; Medina et al. 2014; Rivera et al. 1999; Stein et al. 2004). The unique neuronal localization and function of KCC2 and its increased expression pattern during neuronal maturation formed the basis for the proposal that KCC2 is the responsible for the classical developmental GABAergic switch of neurotransmission \textit{i.e.}, from its depolarizing stimulatory action in immature
neurons to its hyperpolarizing and inhibitory role in adult neurons (see Figure 2). In fact, the discovery of the splice variant KCC2a and the observation that KCC2a levels are kept relatively constant during neuronal development led to the refinement of that hypothesis to introduce KCC2b, the first discovered KCC2 spliced variant, as the one involved in the developmental GABAergic switch (Ludwig, et al. 2011a; Ludwig, et al. 2011b; Uvarov et al. 2007). The involvement of KCC2 on this switch has been documented in many settings (Akerman and Cline 2006; Cancedda et al. 2007; Chudotvorova et al. 2005; Ganguly, et al. 2001; Ge, et al. 2006; Kelsch, et al. 2001) and is now considered a universal biological phenomenon characteristic of most central neurons (Medina et al. 2014). Indeed, in the worm *Caenorhabditis elegans* or the fruit fly *Drosophila melanogaster* mutations in the *Slc12a5* gene increase the excitatory GABA effect decreasing the threshold for seizures (Hekmat-Scafe, et al. 2006; Tanis, et al. 2009). Further, the importance of KCC2 in neuronal physiology has been dramatically demonstrated in mice genetically engineered to lack functional expression of all known KCC2 variants. Most notably, elimination of KCC2 expression by targeted disruption of exon 4 in the *Slc12a5* gene produced KCC2-KO mice that died immediately after birth due to severe motor defects and respiratory failure (Hubner et al. 2001). Mice deficient in KCC2b, however, survive ~3 weeks after birth while suffering from spasticity and generalized seizures, highlighting the importance of KCC2a in postnatal development (Woo et al. 2002). In humans, KCC2 has been linked to bipolar disorders (Freedman, et al. 2001) and schizophrenia (Freedman et al. 2001; Hyde, et al. 2011; Kalkman 2011; Tao et al. 2012), and point mutations in the human *SLC12A5* gene, which result in KCC2s with reduced surface expression/function (KCC2-R952H and -R1049C) have been recently implicated in febrile seizures and idiopathic generalized seizures in human patients (Kahle, et al. 2014; Puskarjov, et al. 2014)
Cl\textsuperscript{−} enters (hyperpolarization)\[Cl\textsuperscript{−}\] extruders: KCCs...

Figure 2. Regulation of [Cl\textsuperscript{−}] \textsubscript{i} in cells. The predominant functional presence of Cl\textsuperscript{−} loaders relative to extruders (left side) sets and maintains [Cl\textsuperscript{−}] \textsubscript{i} above values predicted by the Nernst equation and therefore, Cl\textsuperscript{−} ions tend to exit the cell upon opening of Cl\textsuperscript{−} channels. This exiting following the electrochemical gradient is naturally electrogenic and depolarizing. The predominant functional presence of Cl\textsuperscript{−} extruders relative to loaders (right side) sets and maintains [Cl\textsuperscript{−}] \textsubscript{i} below values predicted at thermodynamic equilibrium and therefore, Cl\textsuperscript{−} ions tend to enter the cell upon opening of Cl\textsuperscript{−} channels in an electrogenic and hyperpolarizing fashion.
Although the paradigm that KCC2 makes possible the inhibitory actions of GABA, a phenomenon that has been corroborated in most species and in many different mature neurons (Medina et al. 2014), not all neurons or cells can be included in this general rule. For instance, nociceptors of the dorsal root ganglion exhibit $[\text{Cl}^-]$ above thermodynamic equilibrium due to the predominant functional presence of NKCC1 over KCC2, which is almost undetectable in these neurons (Alvarez-Leefmans 2009; Mao, et al. 2012). Therefore, GABA$_A$ activation in nociceptors or any neuron or cell expressing GABA$_A$ and exhibiting $[\text{Cl}^-]$ above thermodynamic equilibrium results in the electrogenic efflux of Cl$^-$ and plasma membrane depolarization (Alvarez-Leefmans 2009). Examples of non-neuronal cells with the ability to depolarize in a GABA$_A$-dependent manner are the mammalian insulin-secreting $\beta$-cell (Braun, et al. 2010; Braun, et al. 2004; Pizarro-Delgado, et al. 2010; Rorsman, et al. 1989; Sehlin 1978; Taneera, et al. 2012; Wendt, et al. 2004) (see Section 12), several hormone-secreting cells [reviewed in (Stojilkovic, et al. 2010)] and GnRH-neurons [reviewed in (Watanabe, et al. 2014)]

6. Molecular characterization of KCC2 and variants

KCC2 was first identified as a neuron-specific KCC isoform in 1996 (Payne et al. 1996), a finding that sparked a series of experiments aimed at its functional characterization (Payne 1997). KCC2 was originally cloned from a rat brain cDNA library by screening with primers designed against human expressed sequence tags (hESTs) harboring ~35% identity to human NKCC1. After aligning the nucleotide sequences of many positive clones, two different cDNAs were identified and named rKCC1 and rKCC2 (identified in GenBank with the accession numbers U55815 and U55816, respectively), both sharing 67% identity among themselves but 25% amino acid identity against other Slc12a members known at that time (Gillen et al. 1996; Payne et al. 1996). The primary
structure for rKCC2 revealed the expected in silico topology proposed for other Slc12a family members i.e., 12 hydrophobic TM segments flanked by two large intracellular N- and C-termini and consensus N-glycan sites between TM7 and TM8 (see Figure 1). The N-terminus of rKCC2 (and KCCs in general) was the most divergent region within Slc12a members while TMs were the most conserved ones. Eleven years later, Uvarov and collaborators discovered a new neuron-specific, furosemide-sensitive variant of KCC2 in the mouse brain named KCC2a (Uvarov et al. 2007). KCC2a differs from KCC2b by the inclusion of an alternative first coding exon located 7.5 kb upstream the second exon of the Slc12a5 gene i.e., the one included in KCC2b transcripts. This finding implies that the production of KCC2a may require an alternative promoter and a splicing event involving the elimination of exon 2 (1b) to join exons 1 (1a) and 3. Therefore KCC2a and KCC2b transcripts are characterized by unique 5’-ends, which translate into distinctive N-terminal regions (Figure 3). In spite of these predicted molecular differences, mouse KCC2a and rat KCC2b could not be functionally distinguished when over-expressed in HEK293 cells (Uvarov et al. 2007). Nevertheless, disruption of the mouse Slc12a5 gene aimed at elimination of KCC2b produced mice with a life span of few weeks (Woo et al. 2002), whereas elimination of KCC2a and KCC2b, as mentioned, resulted in lethality shortly after birth (Hubner et al. 2001; Tornberg, et al. 2005) indicating that these two KCC2 variants may have non-redundant functions, a phenomenon recently proposed to be linked to differential expression pattern of these isoforms in some regions of the mature mouse brain (Markkanen, et al. 2013).

Soon after the initial cloning of rKCC2 cDNAs, KCC2s for other species were also identified (see Table 1). Most notably, the different full-length KCC2b cDNA clones obtained from human (Figure 4) or mouse brains indicates that KCC2b mRNAs are...
Figure 3. Partial representation of KCC2a and KCC2b mRNAs. The election of an alternative promoter located upstream exon 1 (1a) coupled to an alternative splicing event involving exon 2 (1b) in the Slc12a5 gene results in the generation of KCC2a transcripts, whereas gene transcription from a promoter located 5' of exon 2 (1b) directs expression of KCC2b mRNAs. Both, KCC2a and KCC2b transcripts differ in their 5'-ends and in the first coding nucleotides. This results in mRNAs with unique regulatory, 5'-UTRs and in proteins with unique N-termini, as depicted in colored residues. The 5'-UTR of KCC2b mRNAs presents unique TF-responsive elements.
Table 1. *Slc12a5* homologs, variants, *RefSeqs* and sources

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*Obtained in experiments performed as part of the present Thesis*
Figure 4. Representation of aligned human KCC2 transcripts posted in GenBank. Human KCC2a and KCC2b transcripts posted in GenBank database under the indicated accession numbers were aligned in silico to highlight splicing of exons (blue arrowed boxes) or retention of introns (grey arrow boxes). These splicing events predict the generation of proteins (red lines) of different lengths and unknown functional properties. Note the enrichment of KCC2b clones relative to KCC2a.
subjected to splicing events beyond those involving first exons. The first one to be
described corresponds to a shorter, truncated variant of KCC2b recently detected in
human brain (Lauf et al. 2012) and subsequently cloned, tagged and over-expressed in
human neuroblastoma SH-SY5Y cells (Tao et al. 2012). This KCC2b-v1 (GenBank
accession AK165769) ends in exon 8 and retains sequences of intron 8 therefore
generating a shorter protein of unknown function (Tao et al. 2012). Additional short
KCC2b transcripts have also been described (see Table 1 and Figure 4), their tissue
distribution or function, however, remain unknown.

7. Molecular determinants of KCC2 function

In silico alignment of KCCs protein sequences originally identified revealed a
unique feature in KCC2: a conserved region of 42 C-terminal amino acids (residues 932-
1043 in rKCC2) encoded by the last nucleotides of exon 24 in the Slc12a5 gene
(Mercado et al. 2006). Therefore, antibodies against this particular region of KCC2 were
developed and tested in Western blotting (WB) and immunohistochemistry experiments
(Williams et al. 1999). This antibody proved to be an invaluable tool for the detection and
characterization of the expression pattern of KCC2 (KCC2a and/or KCC2b) in the brain
and spinal cord and it has become one of the most reliable KCC2 antibodies so far made
(Chamma et al. 2012). Western blot using this KCC2 antibody in rat brain revealed
abundant signal centered at ~150 kDa and corresponding to N-glycosylated forms of
KCC2 and minor bands with MWs ranging from ~125 to ~135 kDa corresponding to core
and intermediate high-mannose/hybrid-type N-glycans. The KCC2 band of higher MW is
typically shifted to ~125 kDa after treatment of protein samples with N-glycosidase F, a
widely used criterion for the identification of N-glycosylated proteins (Williams et al.
1999).
As mentioned, KCC2 is active under most physiological conditions (Mercado et al. 2001). It is now apparent that the intracellular C-terminus of KCC2 is involved in its functional regulation while subjected to positive and negative phosphorylation signals [reviewed in (Chamma et al. 2012)]. Indeed, the C-terminus of KCC2 carries several putative phosphosites including: Y\textsuperscript{926}, T\textsuperscript{929}, S\textsuperscript{963}, T\textsuperscript{1029}, S\textsuperscript{1056} or Y\textsuperscript{1109} (residues are numbered according to mKCC2a) all involved in the regulation of KCC2 function, and the "ISO domain" consisting of residues \textsuperscript{1043}PSPVSSEGIKDFSMKP\textsuperscript{1059}, which endows KCC2 with its unique constitutive activity under isotonic conditions (Chamma et al. 2012; Kahle, et al. 2013; Medina et al. 2014; Mercado et al. 2006). Notably, inhibition of protein phosphatase-1 with calyculin did not affect basal KCC2 isotonic activity but abolished that activated by cell swelling indicating that KCC2 isotonic and swelling-induced activities are regulated by two different mechanisms, at least in oocytes (Mercado et al. 2006). Interestingly, the conserved T\textsuperscript{929} and T\textsuperscript{1029} in KCC2 and the homologue T residues in other KCCs or NKCCs have been identified as sites for WNK-mediated phosphorylation (Kahle et al. 2013; Medina et al. 2014). Further, phosphorylation of S\textsuperscript{963} by PKC increased cell surface expression and stability of KCC2b by reducing the rate of endocytosis (Lee, et al. 2007). Therefore, phosphorylation of key residues in the C-terminus of KCC2 may regulate its function directly or indirectly through regulation of its trafficking to the plasma membrane. In addition, mutagenesis of Y\textsuperscript{1087} in rKCC2b (equivalent to Y\textsuperscript{1109} in mKCC2a), a consensus site for tyrosine kinase-mediated phosphorylation, reduced KCC2 activity under basal isotonic or hypotonic conditions when over-expressed in oocytes without affecting its trafficking (Strange et al. 2000). Further, mutagenesis of Y\textsuperscript{1056} in rKCC1 (equivalent to Y\textsuperscript{1109} in mKCC2a) yielded similar results to those obtained with Y\textsuperscript{1087} highlighting the role of this C-terminally conserved Y residue in regulating KCC1 and KCC2 activities. However, it is important to keep in mind
that mutations in the human *SLC12A5* gene (R952H and R1049C, for instance) lying outside consensus phosphosites also impact phosphorylation of neighbor S residues (Kahle et al. 2014) raising the possibility that mutagenesis in a single Y or S residue may impact KCC function indirectly.

**8. The concept of neuron-specific expression of KCC2**

KCC2b is considered a neuron-specific cotransporter (Kaila et al. 2014; Medina et al. 2014) based on the exceptional abundance of this KCC2 variant in the mammalian brain as judged by Northern blot analysis of eleven major rat tissues probed against KCC2 or by RT-PCR of total RNA from astrocytes, C6 glioma and PC12 pheochromocytoma cell lines and whole brain (Payne et al. 1996). The brain distribution of KCC2 was further confirmed by *in situ* hybridization of brain tissue slides, where KCC2 was found in all layers of the cortex, hippocampus and granular layer of the cerebellum but not in the white matter of corpus callosum (Payne et al. 1996). However, KCC2 expression outside neurons was rarely tested and therefore not validated. Following the development of specific antibodies against KCC2, the transporter was clearly detected in neuronal somata and dendrites of retinal amacrine cells but not in axons, glia (Williams et al. 1999) and neurons of the dorsal root ganglion (Mao et al. 2012; Rivera et al. 1999). The finding the KCC2 co-localizes with GABA<sub>A</sub> in cerebellum together with its particular functional properties led to the proposal that KCC2 is indeed neuron-specific and responsible for regulating [Cl<sup>−</sup>]<sub>i</sub> in the mature central nervous system causing the well-known postsynaptic inhibition mediated by GABA and glycine (Kaila et al. 2014; Medina et al. 2014; Payne 1997; Payne et al. 2003; Payne et al. 1996; Williams et al. 1999).
The KCC2a variant is also considered neuron-specific (Uvarov et al. 2007). This conclusion has its roots in the fact that KCC2a is prominently expressed in whole brain, hippocampus and cerebellum but not in liver, kidney or lung, while clearly detectable in testis (Uvarov et al. 2007). Furthermore, it was found that KCC2a and KCC2b exhibit similar expression levels in brain stem and spinal cord in postnatal stage; however, KCC2b is the one that undergoes developmental up-regulation in the cortex and hippocampus (Markkanen et al. 2013; Uvarov et al. 2007). Indeed, immunohistochemistry of mouse brain using isoform specific antibodies revealed that KCC2a and KCC2b have a similar pattern of expression in embryonic and neonatal mouse brainstem. In adult mouse brain KCC2b expression increases in the cortex and hippocampus regions while KCC2a is more restricted to basal forebrain, hypothalamus and brainstem. At the cellular level, KCC2b localizes in the soma and membranes of neurons while KCC2a signal was more prominent than that of KCC2b with some co-localization between the two variants in dendrites. This different pattern of expression between the two KCC2 spliced variants was interpreted as indicative of distinctive regulatory mechanism and different or overlapping functions (Markkanen et al. 2013). However, the extra-neuronal expression pattern of KCC2 using KCC2a- or KCC2b-specific antibodies was not studied.

The idea that KCC2 is neuron-specific is also supported by the studies performed in mice lacking one or two copies of KCC2 due to the fact that disruption of the Slc12a5 gene in mice results in obvious neurological abnormalities leading to death soon after birth or within few weeks of life (Hubner et al. 2001; Woo et al. 2002) whereas hypomorphic mice lacking 50% or more of total KCC2 activity resulted in increased anxiety-like behavior and susceptibility to pentylenetetrazole-induced seizures while
exhibiting decreased sensitivity to tactile or thermal stimuli (Tornberg et al. 2005). Interestingly, these mice are leaner than WT indicating that reduction of KCC2 function may also impact overall metabolism. However, the non-neurological phenotypic characteristics of mice deficient in KCC2 have never been studied.

9. Transcription factors involved in neuronal KCC2 expression

Since the study of KCC2 was (still is) concentrated in neuronal tissues, it was not only widely and immediately accepted that KCC2 is exclusive of neurons but also fueled a series of experiments aimed at elucidating the mechanisms involved in the neuron-specific pattern of KCC2 expression. The first to be proposed involved the neuron-restricted silencing element (NRSE), a 21 bp sequence located within intron 2 i.e., non-coding sequences 3’ of exon 2 (exon 1b) and 5’ of exon 3 in mouse and human KCC2 genes (Karadsheh and Delpire 2001; Song et al. 2002). This transcriptional element is involved in the repression of several genes in non-neuronal tissues. However, NRSE participates in silencing or repression of some but not all NRSE-containing genes in tissues expressing the neuron-restricted silencing factor (NRSF), which binds with high affinity to NRSEs (Schoenherr and Anderson 1995). Further, mice lacking NRSF does not exhibit the phenotype observed in mice lacking KCC2 (Chen, et al. 1998) indicating that NRSF may be dispensable for KCC2 expression. In line, mice genetically engineered to harbor \textit{Slc12a5} genes deficient in identified NRSEs show a similar expression pattern of KCC2 in neurons compared to WT mice, including intact developmental up-regulation (Uvarov, et al. 2005). In addition, luciferase reporter experiments using an \textit{Slc12a5} genomic fragment containing 1.4 kb of putative promoter region upstream exon 2 (1b), intron 2 lacking NRSE and exon 3 was highly active when nuclear extracts from cerebral cortex were tested whereas such activity was 30 to 50
times lower in peripheral sensory ganglia and almost undetectable when using nuclear extracts from muscle, kidney, liver, lung, pituitary or adrenal glands demonstrating that NRSE is not necessary for KCC2 expression (Uvarov et al. 2005). These results imply that other factors may be involved in neuron-specific expression of the Slc12a5 gene. At the same time, these experiments cannot rule out the possibility that the high expression levels of KCC2 observed in neurons may not be necessarily and exclusively related to gene transcription. Nevertheless, Uvarov and collaborators began a meticulous search for clues in support of the concept that neuron-specific expression of KCC2 is transcriptionally driven (Uvarov, et al. 2006). A genomic region containing the putative promoter of the mouse, human, rat and chimpanzee Slc12a5 gene plus contiguous sequences encompassing exon 2 (1b) were analyzed in silico to find potential consensus binding sites for transcription factors (TFs). Notably, TATA and CAAT boxes were not found in the genomic sequences analyzed, but several potential binding sites for general, basal or inducible TFs were, including: SP1, AP2 and AP1 as well as binding sites for TFs with a more restricted expression pattern such as MEF2 [highly expressed in the frontal and entorhinal cortices, amygdala and dentate gyrus and other regions of the adult brain (Flavell, et al. 2006; Leifer, et al. 1994; Lyons, et al. 1995)], E-BOX [expressed in B lymphocytes (Ephrussi, et al. 1985), pancreatic β-cells (Whelan, et al. 1990), neurons and muscle (Massari and Murre 2000)], or EGRs1-4 [expressed in neurons, muscle spindle (O’Donovan, et al. 1999), insulin secreting β-cells (Muller, et al. 2012; Thiel, et al. 2014)]. Of these TFs, the role of EGR4 and its binding sites [located in exon 2 (1b), position −345 to −357 relative to A˚TG and part of the 5 ′-UTR of mKCC2b, see Figure 3)] was chosen for further studies based on the fact that EGR4 is abundantly expressed and up-regulated in the mouse hippocampus and cerebellum (Uvarov et al. 2006). Over-expression or silencing of ERG4 in cultured neurons increased or
decreased, respectively, luciferase reporters driven by \textit{Slc12a5} promoters harboring ERG1-4 elements demonstrating for the first time the involvement of EGR4 in the regulation of \textit{Slc12a5} gene expression (Uvarov et al. 2006). However, \textit{in vivo} elimination of functional EGR4 genes generates mice with impaired male fertility suggesting a role for EGR4 in spermatogenesis (Hogarth, et al. 2010) and indicating that EGR4 is not necessary for \textit{Slc12a5} neuron-specific expression. Interestingly, low expression levels of KCC2 could be detected in mouse testes (Uvarov et al. 2007) raising the intriguing possibility that KCC2 may also be implicated in fertility. In addition, EGR4 was not detected in the rat cerebellum (Crosby, et al. 1992) where KCC2 is abundantly expressed (Mikawa, et al. 2002; Seja, et al. 2012). Therefore, the molecular basis responsible for the neuron-specific expression of KCC2 still remains unknown.

\textbf{10. Expression of KCC2 in non-neuronal cells}

As delineated above, the concept that KCC2 is neuron-specific is strongly supported by its abundant expression in neurons, its functional properties \textit{in vitro}, physiological impact \textit{in vivo}. This is intriguing in the light of the fact that not all neurons express KCC2 and that the transporter was found in non-neuronal tissues even in parallel experiments performed during the development of the notion that KCC2 is neuron-specific. Indeed, since its initial characterization until very recently [see (Antrobus, et al. 2012; Lauf et al. 2012; Wei, et al. 2011; Yu, et al. 2014)], KCC2 function was exclusively studied within the context of neuron physiology (Kaila et al. 2014; Medina et al. 2014). In addition, several reports have shown variable levels of KCC2 mRNA expression in many non-neuronal tissues and primary cells in culture. The presence or absence of KCC2 in neuronal or non-neuronal cells could be related not only to cell type or species but also to methodological issues, cut-off expression criteria.
(Kang, et al. 2011) or the extent to which we arbitrarily define a gene as "expressed" vs. "not expressed". Regardless, low levels of KCC2 transcripts have been detected in most tissues by micro-array analysis, sometimes at higher levels than other KCC transcripts considered as "expressed" (Gagnon and Di Fulvio 2013). Interestingly, experiments performed in cell lines of non-neuronal origin such as C6 glioma cells, rat primary astrocytes, neuronal SH-SY5Y, GT1-7, N1E-115, NG-108 or PC12 cells originally demonstrated undetectable levels of KCC2 mRNAs (Payne et al. 1996; Williams et al. 1999). However, KCC2 was detected in adrenal chromaffin cells at the protein level (Xie, et al. 2003), at the RNA level in NG-108 cells (Gagnon et al. 2013) and even in the microglial cell-line BV-2 (Zierler, et al. 2008). KCC2 expression has also been demonstrated in several other non-neuronal cells. For instance, fetal human lens epithelial cell line FHL124 (Lauf et al. 2012; Misri, et al. 2006), a finding that was not extended to rat lens tissues (Chee et al. 2006). Interestingly, KCC2a but little if any KCC2b mRNAs were detected in FHL124 raising the possibility that the promoter region of the Slc12a5 gene commanding its expression and the machinery involved in the splicing of exon 2 (1b) to generate KCC2a may not be restricted in cells of ectodermal origin like the lenses (Lauf et al. 2012). However, this speculation needs further validation.

The hypertriploid human epithelial papilloma virus-infected cell line SiHa (Friedl, et al. 1970) also express KCC2 at the molecular and functional levels (Wei et al. 2011). Indeed, RT-PCR, immunofluorescence, biotinylation, Western blot and functional experiments demonstrated its expression. Interestingly, the Authors mentioned that KCC2b is the dominant variant of KCC2 expressed in SiHa, although data was not shown (Wei et al. 2011). Notably, KCC2 is expressed in this cell line despite robust
NRSF expression. In addition, comparable levels of KCC2 mRNA expression were observed in SiHa cells depleted of NRSF expression supporting the notion that this TF is not involved in KCC2 expression (Uvarov et al. 2006). An interesting observation of these studies was the fact that SiHa cells expressing the highest levels of KCC2 mRNAs showed different cell morphology when compared to SiHa cells expressing low levels of that transcript (Wei et al. 2011). SiHa cells expressing high levels of KCC2 showed increased actin formation, higher migration rates and invasion in response to insulin-like growth factor type 1 (IGF-1) when compared to SiHa cells with naturally low levels of KCC2. These effects appear to be related to KCC2 over-expression per se since forced expression of functionally inactive versions of KCC2 in low-expressing SiHa cells increased actin formation, migration rates and invasion in response to IGF-1. Therefore, KCC2 has the ability to modulate cell proliferation, migration, invasion of SiHa cells and potentially cervical cancer progression in a fashion independent of the cotransport ability of KCC2 (Wei et al. 2011).

The molecular and functional presence of KCC2 along with other KCCs was also reported in the human osteoblast-like cell line C1. Indeed, RT-PCR experiments demonstrated KCC2 transcripts [see Fig 7 in (Brauer, et al. 2003)] and a measurable Cl\(^-\) conductance in response to isotonic high KCl solution. Although these functional experiments cannot distinguish among KCCs, KCC2 could be responsible for such current, particularly when KCC2 is considered the only constitutively active KCC (Payne 1997). Similarly, KCC2 was detected at the protein level in bovine adrenal chromaffin cells [see Fig 7 in (Xie et al. 2003)], at lower levels relative to NKCC1, a finding consistent with the notion that these cells keep [Cl\(^-\)]\(_i\) above electrochemical equilibrium making possible the depolarizing effect of GABA observed in these catecholamine-
secreting cells (Xie et al. 2003), a phenomenon observed in other hormone secreting
cells, including insulin-secreting β-cells (Braun et al. 2010), lactotrophs, somatotrophs,
thyrotrophs and melanotrophs (Stojilkovic et al. 2010) or gonadotropin-releasing
hormone (GnRH)-secreting neurons (Watanabe et al. 2014). Although it could be argued
that KCC2 expression in cell lines does not reflect a similar situation in vivo, KCC2
mRNAs have been also detected in nasal mouse primary tissues containing olfactory
mucosa [see Fig 2 in (Nickell et al. 2007)], mouse testis [see Fig 3 in (Uvarov et al.
2007)], immature rat liver [see Fig 1 in (Balakrishnan, et al. 2003)], rat primary vascular
smooth muscle cells [see Fig 1 in (Di Fulvio et al. 2001)], primary chicken
cardiomyocytes (Antrobus et al. 2012) and ex-vivo human pancreatic islets [see Fig 1 in
(Taneera et al. 2012)].

11. Similarities between neurons and insulin-secreting β-cells

Pancreatic insulin-secreting β-cells and neurons share a similar excitable
mechanism in response to stimuli. Both, insulin secretion in response to glucose and
neurotransmitter release under neuronal stimuli occur after plasma membrane
depolarization, Ca$^{2+}$ influx and granule exocytosis (De Camilli and Jahn 1990). However,
similarities between these cell types go beyond electrical excitability. For instance: i) Cl$^-$
plays a key role in neuronal excitability in response to GABA or glycine (Ben-Ari, et al.
2012) whereas both neurotransmitters modulate insulin secretion in vivo and in vitro
(Braun et al. 2010; Braun et al. 2004; Franklin and Wollheim 2004; Geigerseder, et al.
2003; Gu, et al. 1993; Pizarro-Delgado et al. 2010; Rorsman et al. 1989; Satin and
secreting β-cells express typical "neuron-specific" genes such as tyrosine and dopamine
β-hydroxylase (Oomori, et al. 1994; Persson-Sjogren, et al. 2002; Takayanagi and
Watanabe 1996; Watanabe and Nagatsu 1991), glutamic acid decarboxylase (Kim, et al.
al. 2010), N-methyl-D-aspartate receptor-2 (Inagaki, et al. 1995), secrete GABA
(Geigerseder et al. 2003; Taneera et al. 2012) and possess synaptic-like microvesicles
(MacDonald, et al. 2005), and iii) neurons and β-cells share TFs as well as KCC2.
Indeed, neuronal TFs such as Islets-1 (Karlsson, et al. 1990), Pax6 (Turque, et al. 1994),
Beta2 (Naya, et al. 1995) or members of the EGR family (Muller et al. 2012; Thiel et al.
2014), the latter involved in the transcriptional regulation of KCC2 expression (Ludwig et
al. 2011b; Uvarov et al. 2006), are also expressed in β-cells.

12. Insulin secretion depends on [Cl\(^-\)]\(_i\) of β-cells

As mentioned, glucose stimulates insulin secretion by altering the electrical
activity of the β-cell of the islet of Langerhans in the pancreas (Rorsman and Braun
2013). Insulinotropic glucose concentrations i.e., 6-25 mM increase \(E_m\) from its basal
resting level of \(-70\) mV to \(-20\) mV [even to 0 mV, see (Henquin and Meissner 1982)] by
a phenomenon mostly due to the gradual closure of ATP-dependent K\(^+\) (\(K_{\text{ATP}}\)) channels
Overall, this depolarization reaches a threshold responsible for the opening of voltage-
dependent Ca\(^{2+}\) channels (VDCC) leading to massive Ca\(^{2+}\) entry and subsequent
exocytosis of insulin granules thus resulting in insulin secretion (Drews, et al. 2010).
However, the closure of \(K_{\text{ATP}}\) channels is not sufficient to depolarize the plasma
membrane indicating the presence of additional currents involved in plasma membrane
depolarization, most notably those mediated by electrogenic efflux of Cl\(^-\) ions (Best
2005; Best et al. 2010). The first report indicating the existence of this \(K_{\text{ATP}}\)-independent
mechanism involved in the first phase of insulin secretion was published more than 20
years ago (Best, et al. 1992). While glucose induces insulin secretion by inhibiting $K_{\text{ATP}}$ channels, a complete block of $K_{\text{ATP}}$ channels evidenced by $^{86}\text{Rb}^+$ fluxes after treatment with tolbutamide, a $K_{\text{ATP}}$ channel blocker, did not block insulin secretion in response to glucose (Best et al. 1992). The notion that $K_{\text{ATP}}$ channels are dispensable for insulin secretion, at least in rodents was reinforced eight years later when Seghers and collaborators created the first animal model lacking the receptor for sulphonylureas SUR1 (one of the two subunits of the $K_{\text{ATP}}$-channels) and was further observed that these mice were normoglycemic and their islets secreted insulin in response to glucose (Dufer, et al. 2004; Seghers, et al. 2000) a finding that was further demonstrated in mice lacking the inwardly-rectifying $K^+$ channel $\text{Kir}_{6.2}$ i.e., the additional subunit of the $K_{\text{ATP}}$-channel (Miki, et al. 1998; Seino, et al. 2000).

The demonstration that: $i$) β-cells exhibit $[\text{Cl}^-]$ above electrochemical equilibrium (Eberhardson, et al. 2000; Kozak and Logothetis 1997; Sehlin 1978; Sehlin and Meissner 1988), $ii$) volume-regulated anion channels (VRAC) mediate the electrogenic efflux of $\text{Cl}^-$ ions in β-cells (Best 2002; Best and Benington 1998; Best, et al. 1997; Best, et al. 1996a; Best, et al. 1996b; Kinard, et al. 2001; Kinard and Satin 1995), $iii$) the $\text{Cl}^-$ loader NKCC1 is expressed in β-cells (Majid et al. 2001) and its acute inhibition with BTD or furosemide transiently blocks insulin secretion (Sandstrom 1988, 1990; Sandstrom and Sehlin 1987, 1988a, b, c), and $iv$) depletion of $[\text{Cl}^-]$ inhibits insulin secretion (Best 2005) all led Best and coworkers to propose the "VRAC hypothesis" whereby the $K_{\text{ATP}}$ channel-independent mechanism of insulin secretion is the result of electrogenic depolarizing efflux of $\text{Cl}^-$ from β-cells (Best et al. 2010). This hypothesis resembles several of the established paradigms of $\text{Cl}^-$ regulation and function in immature or sensory neurons [reviewed in (Alvarez-Leefmans 2009)] and hormone
release from pituitary cells [reviewed in (Stojilkovic et al. 2010)]. Therefore, we propose the hypothesis that in addition to NKCCs, β-cells express KCC2 and that its expression is important for insulin secretion.
Hypothesis and Specific aims

**Hypothesis:** *KCC2 participates in insulin secretion*

**Specific aims:**

1. To characterize KCC2 expression in insulin secreting β-cells
2. To determine the role of KCC2 in Cl⁻ regulation in β-cells
3. To demonstrate a direct role of KCC2 in insulin secretion
Methods

1. Reagents, kits and immunochemicals

Restriction enzymes (XhoI, EcoRI, BamHI, SfiI, BglII, KpnI, NotI and XbaI), T4 DNA ligase and Antarctic Phosphatase were from New England Biolabs (Boston, MA). The QuikChange II XL Site-Directed Mutagenesis kit and Pfu Ultra II Fusion HS DNA polymerase were from Agilent-Stratagene (San Diego CA). Mammalian expression plasmids pCMV-myc and pCMV-HA and antibodies against tags (myc and HA) were from Clontech (La Jolla CA). Pfx DNA polymerase, SuperScript-III reverse transcriptase, Lipofectamine2000 transfection reagent and the Zero Blunt TOPO PCR Cloning kit were from Invitrogen (Carlsbad CA). dNTPs, Taq DNA polymerase and Taq buffer were from GenScript (Piscataway NJ). pGEM-T cloning vectors were from Promega (Madison, WI). DH5α competent cells were from Molecular Cloning Laboratories (South San Francisco, CA). Roswell Park Memorial Institute (RPMI) medium 1640, OptiMEM I medium, trypsin-EDTA, streptomycin and penicillin sulfate (stock 100x) were from Life Technologies (Carlsbad CA). HEPES buffer, bovine serum albumin (BSA), sodium bicarbonate and Histopaque solution were from Sigma (Saint Louis MO). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), 1kb/100bp DNA ladders, GeneJET gel extraction and plasmid miniprep kits, protease/phosphatase inhibitors and pre-casted Tris/HEPES 4-20% SDS-PAGE gels were from Thermo Scientific-Pierce (Rockford, IL). Collagenase was from Worthington (Lakewood, NJ). ExoSAP-IT enzymes, phenylmethylsulfonyl fluoride (PMSF), Tris-HCl 1M, pH 6.8, glycerol, bromophenol blue, dithiothreitol (DTT) and LB/agar were from USB/Affimetrix (Cleveland, OH). PVDF membranes were from Millipore (Billerica, MA). Radioimmunoprecipitation assay buffer
(RIPA), sodium dodecyl sulfate (SDS), Super Optimal Catabolite-repression (SOC), NZY broth bacteria media and LB/agar/antibiotics plates were from TEKnova (Waukegan, IL), Bradford reagents for protein determination were from BioRad (Hercules, CA). The source of primary and secondary of antibodies and their dilutions used in our experiments is indicated in Table 2. Antibodies were diluted in 1:1 (vol/vol) in glycerol and kept at −20°C to avoid freezing/thawing cycles.

2. Cell culture

All cell lines were cultured in a 5% CO₂ incubator at 37°C. The medium was changed every 3 days and stocking or propagation of cells were performed when they reached ~90% confluence. The mouse insulinoma β-cell line MIN6 was a kind gift of Dr. Jun-Ichi Miyazaki [Division of Stem Cell Regulation Research, Osaka University Medical School, Osaka Japan (Miyazaki, et al. 1990)]. MIN6 together with INS-1E β-cells (see below) are the most commonly used mammalian β-cell models to study mechanisms of insulin secretion (Skelin, et al. 2010). In addition, MIN6 cells have been extensively characterized throughout the years and have proven ideal for gene expression studies. MIN6 cells were cultured and propagated as originally described (Miyazaki et al. 1990). Briefly, cells were grown in DMEM containing 11.1 mM glucose supplemented with 10% FBS, 10 mM L-glutamine, 1 mM sodium pyruvate, 50 µM 2-mercaptoethanol and 1% antibiotics. MIN6 were grown in 75 cm² treated tissue culture flasks (T75), six- or 12-well plates according to the purpose of the experiments. For instance, T75 flasks were used for gene expression analysis whereas 6-well plates were used for immunofluorescence, protein extraction and determination of total chloride content. Insulin secretion experiments were performed in 12-well plates. MIN6 cells were passaged by gentle trypsinization at room temperature following usual methods. Briefly, 80-90% confluent
### Table 2. List of primary and secondary antibodies used

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Antigen</th>
<th>Host/isotype</th>
<th>Provider</th>
<th>Application</th>
<th>Dilution</th>
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<td>mouse/IgG</td>
<td>NeuroMab</td>
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<td>DSHB</td>
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<td>Abcam</td>
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<table>
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<td>rabbit IgG</td>
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<td>Jackson</td>
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<td>mouse IgG</td>
<td>donkey</td>
<td>Jackson</td>
<td>IF</td>
<td>1:1000</td>
</tr>
</tbody>
</table>
MIN6 cells growing in a T75 were carefully washed once with 5 ml phosphate buffered saline (PBS) before adding 3 ml of 0.05% (w/v) trypsin supplemented with 0.53 mM EDTA. Once cells were detached they were added into the desired volume of media to be re-seeded or they were stocked after gentle centrifugation and re-suspension in freezing medium consisting of 90% FBS plus 10% DMSO. The rat insulinoma β-cell line INS-1E, a kind gift of Dr. Pierre Maechler (Université de Genève, Faculté de Médecine, Switzerland), was used for comparative gene expression analysis. INS-1E cells were grown following the methods published by their developers (Merglen, et al. 2004). Briefly, INS-1E cells were grown in RPMI medium supplemented with 10% FBS, 10 mM L-glutamine, 1 mM sodium pyruvate, 50 µM 2-mercaptoethanol and 1% antibiotics. Cells were propagated in T75 flasks and stocked after confluence following similar procedures to that of MIN6 cells using 5% DMSO in basic medium as a frozen medium. The *Cercopithecus aethiops* kidney fibroblast-like cell line COS7 was obtained from ATCC (CRL1651, Manassas, VA). COS7 cells were cultured according to the provider's instructions. Cells were plated onto flasks or 6-well plates containing DMEM plus 11.1 mM glucose supplemented with 10% FBS and 1% antibiotics. Cells were propagated by trypsinization as indicated above for other cell lines and stocked in freezing media containing 40% DMEM, 10% DMSO and 50% FBS.

3. Islets extraction and purification

Islets were purified from enzymatically digested mice pancreases by using Histopaque gradient centrifugation. After mice had been euthanized using compressed CO$_2$ and according to the approved protocols of the Laboratory Animal Care and Use Committee (LACUC) at WSU, pancreases were collected, washed twice with ice-cold DMEM and finely minced by using surgical blades. Then cut tissues were incubated 10 min at 37°C in
4.3 mg of collagenase per ml of DMEM (1 ml per pancreas). After digestion was completed, indicated by a clear solution, ice-cold DMEM was added to stop collagenase activity and the digested tissues centrifuged 3 min at 900 rpm. The pellets were re-suspended in 5 ml RPMI, sieved through 0.22 μm pore filters, centrifuged 3 min at 0.9 rpm, re-suspended in 4 ml of Histopaque and further diluted by adding 5 ml of RPMI carefully through the tube's walls. Islets were purified by gradient-centrifugation at 4C, 5 min and 2000 rpm without rotor brake. To obtain purified pellets of islets without Histopaque contamination, islet layers located in the interface between separated Histopaque and RPMI were carefully retrieved with the aid of a Pasteur pipet and dispensed into 5 ml RPMI for centrifugation 3 min at 900 rpm. The final pallet of purified islets was used for RNA or protein extraction.

4. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cultured cells or purified mouse islets by using the GeneJET RNA Purification kit and following the instruction of the manufacturer. Total RNA from a panel of mouse tissues was purchased from Zyagen (San Diago, CA). Total RNA/protein extracts from human islets were kindly provided by Dr. Patrick McDonald (Canada Research Chair in Islet Biology, University of Alberta, Edmonton, AB, Canada). Total RNAs, irrespective of the source, were reverse transcribed into cDNA by using the SuperScript III first-strand synthesis system. PCR screening of transcript expression was performed by using 1-2 μl template cDNA from different sources in a 50 μl total volume reaction each containing 0.2 mM dNTPs, 1 mM MgSO₄, 0.5 pmol/μl of each primer set and 0.2 U of Platinum Pfx thermostable DNA polymerase. The thermal conditions followed were: one step of initial denaturation at 98C for 2 min followed by 40 cycles of three 30 sec steps consisting of denaturation, annealing and extension at 98C, 58C and
68°C, respectively, and a final extension step of 2 min at 68°C to allow full extension of incomplete amplicons. As positive controls of RT-PCR reactions, glyceraldehyde phosphate dehydrogenase (GAPDH) mRNAs were PCR-amplified by using specific and validated primer sets whereas H₂O instead of total RNA supplemented with GAPDH primer sets was used as negative control. Isoform- and species-specific PCR primers were designed by using the Primer3 algorithm of the suite Geneious R7 (Biomatters LTD, Auckland, NZ). Table 3 provides primer sequences and the mRNA nucleotide sequences of reference (RefSeq) used to design them. All primers were custom synthesized at Integrated DNA Technologies (IDT, Coralville, Iowa). The RT-PCR products obtained were subjected to 2% agarose-gel electrophoresis using 100 bp DNA ladders to estimate molecular weight (MW) in base pairs (bp). To confirm molecular identity, RT-PCR amplicons either depleted of contaminant single stranded DNAs by using ExoSAP-IT or gel-purified and directly cloned into pGEM plasmids were sequenced in both directions. In the latter cases, nucleotide sequences were obtained by PCR-amplification using universal primers e.g., SP6, T7, CMVf, M13f or M13r (Beckman Genomics, Beverly, MA). The nucleotide sequences obtained were cleaned from noise based on quality scores using Geneious R7 and aligned against relevant RefSeqs or plasmid DNAs by using the MAFFT v5 algorithm (Katoh, et al. 2002).

**5. Long-range PCR**

To amplify full open reading frames (ORFs) plus flanking untranslated regions (UTRs) of KCC2 transcripts, total RNA from cells were subjected to reverse transcription as described above and subsequently to long-range PCR by using Pfu Ultra II Fusion HS. PCR reactions were performed as follow: 2 µl of freshly obtained cDNAs reverse transcribed from 2 µg of total RNA were subjected to PCR amplification in a 50 µl
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<thead>
<tr>
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<th>antisense</th>
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<td>NM_020708</td>
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</table>

*Nucleotide positions are relative to the adenine nucleotide of the first coding ATG codon
reaction containing 400 µM dNTPs, 1 µM each transcript-specific primer (see Table 3) and 0.5 U Pfu DNA polymerase. The reactions were then denatured at 95C for 1 min and subjected to 40 cycles consisting of denaturation at 95C for 20 sec, annealing at 59C and extension at 72C for 2 min (30 sec per kb of template). The PCR products obtained were resolved in 1% agarose gels by electrophoresis and their length in bp estimated by using 1 kb DNA ladders.

6. Pfx/Pfu-generated amplicon purification, 3'-A overhanging and pGEM-T cloning

PCR products obtained with Pfx or Pfu are blunt-ended thus precluding regular cloning. To circumvent this limitation, purified blunted PCR fragments were subjected to Taq-mediated PCR in order to add a single 5'-adenine (A) overhang to the PCR products. Briefly, blunted PCR products were gel-purified by using GeneJET gel extraction kits following the instructions of the manufacturer. After estimation of the concentration of purified fragments using NanoDrop ND-1000 Spectrophotometer (Thermo Sci/NanoDrop Wilmington, DE), 5-10 ng of purified blunted amplicons were used as templates for PCR using Taq DNA polymerase. The PCR reaction settings were as follow: 0.2 mM dNTPs, 0.5 pmol/µl each primer, 1x Taq buffer, 2.5 U Taq DNA polymerase and nuclease-free water to 50 µl total volume. Cycling conditions were: denaturation for 3 min at 95C followed by 40 cycles of 95C/30sec, 55C/30sec and 68C/1min. Then a final extension period of 5 min at 68C was performed. After Taq PCR-amplification, amplicons were directly cloned into linearized pGEM-T vectors containing T-overhangs following the instruction of the manufacturer with modifications. Briefly, in a final volume of 10 µl, ligation buffer, T4 DNA ligase, ~50 ng of pGEM-T vector and 3 times molar excess of the
insert (PCR product) relative to the vector were combined and incubated overnight at 4C. After ligation, 2.5 µl of the reaction mixture were used to transform DH5α competent cells following the steps indicated below. Single ampicillin-resistant colonies harboring the recombinant plasmid were collected, grown in liquid media and characterized. Bacteria harboring the desired plasmids were stocked in 15% glycerol at −80C. Cloned inserts were sequenced in both directions by PCR using universal plasmid primers M13r and M13f located on the plasmid 5’ and 3’ of the insert, respectively.

7. Molecular cloning of long-range PCR products

Long-range PCR amplicons were purified as indicated in the previous section and non-directionally cloned into pCRII-Blunt-TOPO vectors (pCRB for short) by using the Zero Blunt TOPO PCR cloning kit and following the instructions of the manufacturer with modifications. Briefly, 4 µl of gel-purified PCR products plus 1 µl salt solution containing 200 mM NaCl and 10 mM MgCl₂, and 1 µl of pCRB vector were mixed and incubated 30 min at 4C. After that, 2 µl of the reaction were used to transform DH5α competent cells for plasmid cloning, purification and further characterization.

8. Transformation of competent E. coli bacteria

Transformation i.e., introduction of foreign DNA into cells in our case bacteria, was performed by incubating stocks of competent DH5α or dcm−/dam− E. coli on ice for 30 min in the presence of ~50 ng of plasmid DNA and heat-shocked at 42C exactly for 45 sec to allow cells to take foreign DNA. After this step, 250 µl of SOC growth medium were added per tube and cells were incubated at 37C for 1 hour at 230 rpm. The whole transformation reaction was spread onto LB/agar plates supplemented with the antibiotic
required to select bacteria containing recombinant plasmids and expressing resistance to that specific antibiotic i.e., kanamycin (50 µg/ml) or ampicillin (50 µg/ml). LB plates were incubated at 37C for 16-20 hours before 5-10 resistant colonies were collected and incubated in 5 ml LB plus resistance-specific antibiotic at 37C for additional 16-20 hours at 230 rpm in order to produce plasmids for purification and downstream applications.

9. Plasmid purification, screening, characterization and sequencing

Plasmid purification from antibiotic-resistant bacteria was performed by using the GeneJET plasmid miniprep kit and following the instructions of the manufacturer. Basically, bacteria from single isolated colonies growing in 5 ml LB/antibiotic were spun down, washed and lysed to release plasmids for subsequent purification from contaminating genomic DNA and proteins. Plasmids were filter-purified in mini-columns and eluted in water. Each eluate was subjected to electrophoresis in 1% agarose gels to confirm plasmid purification and estimate approximate yields. Then plasmids were digested with specific restriction enzymes based on the in silico predicted restriction maps to confirm insert orientation and vector/cDNA integrity. Only plasmids matching predicted DNA maps were sequenced. To further confirm identity, some plasmids were subjected to a round of PCR to amplify cloned inserts as small fragments by using Taq or as a whole by using Pfx or Pfu DNA polymerases. The PCR products and plasmid templates were sequenced on different days to secure molecular identity in at least two rounds of sequencing and to confirm the quality control of the sequence reactions performed outside of the laboratory. Once established, identified clones of bacteria harboring the expected, identified plasmids were stocked at –80C in 15% glycerol.
A unique SfiI restriction enzyme digestion site (5'-GGCCNNNNGGCC-3') was introduced in the 5'-UTR region of KCC2 cDNAs most proximal to the initiation codon ATG and cloned in pCRB plasmids to provide adequate frames for subsequent subcloning and tagging steps. To those ends, plasmids were subjected to PCR-based site directed mutagenesis by using the QuikChange II XL site-directed mutagenesis kit and primers containing the desired mutations. Mutagenesis primers were designed according to the usual guidelines, following the recommendations set forth in the manual of the mutagenesis kit and further optimized by using the QuikChange Primer Design Tool (www.genomics.agilent.com). The nucleotide sequences of the mutagenesis primers were as follows (5'-3'): GTG CGA TCC CGC GGC CCC GGA GGC C and its antisense GCG GCT CAT GGC CTC CGG GGC C GC GGG ATC GCA C (SfiI sites underlined). The PCR conditions for mutagenesis were as follows: 45 ng plasmid templates, 125 ng of each mutagenic primer (sense and antisense), 1 µl dNTP mix, 3 µl QuikSolution, ddH$_2$O to final volume 50 µl and 2.5 U Pfu Ultra HF DNA polymerase. The cycling parameters applied were as follows: 95C, 1 min for denaturation and 18 cycles of three steps each: denaturing at 95C/50 sec, annealing at 60C/50 sec and extension at 68C for 60 sec per 1 kb of template followed by a final single extension step of 7 min at 68C. After cycling mutagenesis conditions were finished, parental plasmids i.e., the wild-type (WT) template in the reaction, was eliminated by restriction enzyme digestion using 10 U of DpnI, which recognizes methylated 5'-ACGT-3' sites. This was performed incubating the reaction at 37C 1 hour. Then, 5 µl of the mutagenesis/restriction reaction were used to transform DH5α cells as indicated above. Single kanamycin-resistant colonies harboring the mutagenized plasmids were collected, grown in liquid
LB/kanamycin (50 µg/ml) to purify, identify and characterize plasmids. Bacteria harboring the desired plasmids were stocked in 15% glycerol and stored at 80°C.

11. Subcloning and tagging

To N-terminally label mKCC2 cDNAs cloned in pCRB with myc or HA tags, mutagenized inserts were excised from 5 µg of plasmid vectors by using the enzyme combination SfiI + XhoI or SfiI + BamHI and purified following the method explained below. In parallel, pCMV-myc and pCMV-HA vectors were cut with SfiI + XhoI or SfiI + BglII, respectively. The replacement of BamHI by BglII was to provide directional cloning and BamHI-compatible cohesive ends for subcloning of purified inserts. To this end, ~3 µg of co-digested plasmids were first dephosphorylated to prevent re-ligation and maximize cloning efficiency. For each µg of digested vector, 5 U of Antarctic Phosphatase were used in a final volume of 20 µl containing proper buffer. Dephosphorylation was allowed to proceed by incubating the reactions at 37°C for 15 min in the case of 5’ extensions or for 60 min for 3’ extensions, depending on the enzymes used. Digestion was performed by using 10-20 U of each enzyme per µg of plasmid in a suitable buffer and final volume of 50 µl at 37°C overnight. Positive controls for each enzyme alone were carried out in parallel digestion experiments under the identical conditions to that of double digestions to confirm both enzymes cut the plasmid. Once purified, the concentration of the inserts and vectors were estimated by using NanoDrop Spectrophotometer ND-1000.

12. DNA Ligation

To ligate digested and purified inserts and vectors, ligation reactions were performed by using 1 µl of T4 DNA ligase, 50 ng vector and a 3-5 molar excess of the insert relative to
the vector in a final reaction volume of 20 µl. Ligation was performed by incubating the reaction mixtures at 16C overnight followed by an enzyme inactivation step of 10 min at 65C. Aliquots (2-5 µl) of the ligation reactions were used to transform DH5α competent cells followed by colony selection, plasmid identification, sequencing and stocking, as explained in previous sections.

13. Western Blotting

Protein extract from cells or tissues were used in Western blot. In the case of cultured cells, protein extractions were performed on ~90% confluent cells growing in 6-well plates. Briefly, plates were placed on ice, and cells washed three times with ice-cold PBS and lysed by adding 50 µl/well of ice-cold lysis buffer (25 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0, 1 mM EGTA pH 8.0, 0.1% v/v 2-mercaptoethanol, 1% Triton 100X, 50 mM NaF, 150 mM NaCl, 1.5 mM MgCl₂ freshly supplemented with PMSF and protease/phosphatase inhibitors to prevent protein degradation) with the help of a syringe. For islets and tissues, protein extracts were obtained by using RIPA buffer. After tissue homogenization, 3 ml of RIPA was used per gram of tissue. RIPA buffer was also freshly supplemented with PMSF and protease/phosphatase inhibitors. The collected lysates from tissues or cells were incubated on ice for 30-60 min with vigorous vortex each 5 min. Protein concentration in lysates was determined by using the Bradford method. Results were compared against standard curves run in parallel and using known concentrations of BSA (0-5 µg/µl). A total of 50-100 µg of proteins in a maximum volume of 25 µl were mixed with an equal volume of loading buffer containing 8% SDS, 125 mM Tris HCl, pH 6.8, 20% glycerol, 0.02% bromophenol blue, 100 mM DTT and boiled 5 min in water. The total volume was then loaded onto pre-casted 4-20% SDS-PAGE gels and run for 1 h at 100 mV. The separated proteins were electro-transferred
onto PVDF membranes at 4C for ~2.5 hours at 72 mV. After that, membranes were cut and blocked for 1 hour at room temperature using 5% BSA in 1x TBST buffer (50 mM Tris, 150 mM NaCl and 0.05% Tween 20). Then membranes were incubated overnight at 4C with primary antibodies for KCC2 or β-actin diluted in blocking buffer. Excess primary antibodies were washed twice with TBST for 5 min each before being incubated for 1 hour at RT with relevant TBST-diluted horseradish peroxidase (HRP)-conjugated secondary antibodies, as indicated in Table 2. Then membranes were also washed twice with TBST 5 min each. The blotting signal was developed by chemiluminiscense and visualized using a BioRad image analyzer (Chemi-Doc MP Imaging system, Hercules, CA).

14. Immunofluorescence microscopy

Tissues were collected from perfused mice, placed in freshly prepared 4% p-formaldehyde at 4C overnight and then transferred to the same fixative containing 20% sucrose. After no longer than 15 days, post-fixed tissues were embedded in paraffin and thin-cut (5 µm) for further processing and analysis (AML Laboratories, Inc., Baltimore, MD). Briefly, paraffin was washed out from tissue slides by placing them in pure xylene 5 min twice followed by successive re-hydration incubations of 5 min each in ethanol solutions of decreasing concentrations i.e., 95%, 80%, 70% and 50%. Then, de-paraffinized and re-hydrated slides were rinsed in distilled water and placed in PBS for 10 min. To ensure antigen retrieval, slides were immersed in 10 mM sodium citrate buffer pH 6.0 containing 0.05% Tween-20 and incubated 30 min at 95C in a water bath. After cooling for 20 min at room temperature, tissue slides were permeabilized by incubating them in PBS-0.1% Triton X100 (PBS-T) 3 times 10 min each. Then slides were incubated in blocking solution consisting of PBS plus 10% serum corresponding to the
species on which secondary antibodies were raised. After 30 min blocking, slides were incubated overnight at 4C in using PBS-T plus primary antibodies at the dilutions indicated in Table 2. The next day, slides were carefully washed in PBS-T 3 times 10 min each before being incubated in PBS containing appropriate secondary antibodies for 2 hours at room temperature and in the darkness. After that incubation period, excess secondary antibodies was washed out by using PBS 3 times 10 min each at room temperature. Finally, immunostained slides were allow to dry before mounting media Vectashield (Vector Labs, Burlingame, CA) containing the DNA label 4',6- diamidino-2-phenylindole (DAPI) on top of the tissue slides and covered by acetone-cleaned glass coverslips. Digital images were taken by using an epi-fluorescence microscope (Olympus Corp, Tokio, Japan) connected to the digital camera Spot v5.1 (Imaging Solutions, Diagnostic Instruments, Inc. Sterling Heights, MI) at the Histocore Facility (WSU). Normal human pancreas tissue slides were obtained from Cell Marque Corporation (Rocklin, CA) or were kindly provided by Dr. Lydia Aguilar-Bryan (Pacific Northwest Diabetes Research Institute, Seattle, WA).

When cell lines were subjected to immunolabeling, a different procedure was followed. Briefly, cells were plated onto glass coverslips placed in 6-well (Fisher Scientific. #NC9130114). When cells reached ~70% confluence they were washed in cold PBS twice and then fixed in absolute methanol 15 min at 20C. Cells were then permeabilized in freshly prepared 4% p-formaldehyde containing 0.25% Triton X100 15 min at 4C and blocked in PBS containing 3% goat or donkey serum for 1 hour. Then, cells were incubated with primary antibodies overnight at 4C in blocking solution and at dilutions indicated in Table 2. Next day, cells were washed 4-5 times with PBS and incubated in PBS plus appropriate dilutions of secondary antibodies for 2 h at 4C in the
darkness. Finally, immunostained cells were washed 4 times in PBS before coverslips were taken carefully from the well plates, dried and placed upside-down onto 15 µl of Vectashield-DAPI placed on microscopy slides (24x50mm).

15. Cell transfection

Mammalian expression plasmids harboring mKCC2s either N-terminally tagged with myc or the viral hemaglutinin (HA) antigen were transfected into 50-70% confluent COS7 or MIN6 cells growing on coverslips in 6-well plates. Transfection was performed using lipofectamine in a 1:2.5 (plasmid to lipofectamine) ratio. Briefly, cells were washed 3 times with DMEM and PBS to eliminate traces of antibiotics. Then, cells were incubated in OptiMEM I without serum for 30 min at 37°C. Transfecting plasmids were prepared as follows: 5 µl Lipofectamine2000 were diluted in 150 µl OptiMEM I and incubated 5 min at room temperature. Then, 2 µg of plasmid diluted in 150 µl OptiMEM were added to the Lipofectamine mixture and incubated 20 min at room temperature. The total plasmid-Lipofectamine mixture (300 µl) was then added to each well. Cells were incubated with the transfection reaction 3-4 hours at 37°C. After that, cells were cultured in full medium without antibiotics. The next day this medium was replaced with full medium containing antibiotics. After 2-3 days, immunohistochemistry was performed as mentioned above using specific antibodies against myc or HA tags.

16. Potentiometric determination of total cellular Cl⁻ content

Total cellular Cl⁻ content was measured by using Cl⁻ ion selective electrodes and following the recommendations published (Northrop 1948; Sanderson 1952; Weinstein and Jennings 1959). The Cl⁻ electrode consists of a solid-state membrane selective to
the passage of Cl\textsuperscript{−} ions which generate a current establishing a change in the electrical potential relative to the internal electrode of reference. This change in electric potential is proportional to the concentration (activity) of Cl\textsuperscript{−} ions ([Cl\textsuperscript{−}]) in the sample, and both parameters are related by the Nernst equation:

\[ E = k + S \log [Cl^-] \]

Where \( E \) is the measured electrode potential in a 1000\textsuperscript{th} of a Volt (mV), \( k \) is a constant reference potential, Cl\textsuperscript{−} is the concentration of the anion in moles/L (M) and \( S \) is the slope of the equation, which under ideal conditions it takes a value of 56 mV every ten times molar change in [Cl\textsuperscript{−}]. The response in mV of the Cl\textsuperscript{−} electrode was calibrated against NaCl solutions of accurate and known concentrations ranging from 0.0001 to 500 mM. A shown in Figure 5, plots mV vs. \( p\text{Cl}^- \) (the negative logarithm of [Cl\textsuperscript{−}] in moles/L) demonstrated a constant slope of \( -54.00 \pm 0.69 \) mV within that range of [Cl\textsuperscript{−}]. The effect of ionic strength on the accuracy of the Cl\textsuperscript{−} electrode was also evaluated by using high and constant ionic strength provided by 1M NaNO\textsubscript{3}. There was no difference in electrode responses in the absence or presence of 1M NaNO\textsubscript{3} added to both standards and samples. Indicating that ionic strength effect on electrode potential was negligible. To determine Cl\textsuperscript{−} content in cultured cells, MIN6 were grown in 6-well plates until ~80% confluence. Then, they were washed and incubated in Cl\textsuperscript{−}-free isotonic media containing 130 mM Na\textsuperscript{+} gluconate, 5 mM K\textsuperscript{+} gluconate, 2 mM Ca\textsuperscript{2+} gluconate, 20 mM HEPES, 1 mM MgSO\textsubscript{4}, 0.83 mM Na\textsubscript{2}HPO\textsubscript{4} and 25 mM Mannose for one hour to deplete cells of endogenous Cl\textsuperscript{−}. Then, β-cells were incubated in isotonic solution containing physiological concentrations of Cl\textsuperscript{−} (ISO: 130 mM NaCl, 5 mM KCl, 2 mM CaCl\textsubscript{2}, 20 mM HEPES, 1 mM MgSO\textsubscript{4}, 0.83 mM Na\textsubscript{2}HPO\textsubscript{4} and 25 mM mannose) for variable periods of time to allow cells to re-accumulate the anion. At the end of each incubation period, cells
Figure 5. Calibration curve of the ion-selective electrode. The responses of the ion-selective electrode in mV to increasing concentrations of Cl$^-\,$ ions ranging from 0.0001 to 0.5 M were plotted following the relationship determined by the Nernst equation. The electrode responses for each pCl represents the mean ± range (n=12). The effective linear range of Cl$^-\,$ concentrations in which the electrode gives a linear response in mV with a slope of ~54 mV occurs from 0.0001 M to 0.5 M.
were placed on ice, washed twice with ice-cold Cl\(^-\)-free medium and lysed in 0.25N NaOH 30 min at RT. Glacial acetic acid was then added to neutralize the solution. Aliquots were taken to determine protein and Cl\(^-\) contents. Results are expressed as nmol of Cl\(^-\) per μg of total protein. The effects of VU or CLP257 on Cl\(^-\) uptake was investigated by adding these drugs to the ISO medium 5 min after maximal [Cl\(^-\)] uptake was reached.

17. Insulin Secretion

Basal or glucose-induced insulin secretion was studied in MIN6 cells after two hours incubation with non-insulinotropic 5.5 mM glucose (basal) or >7mM i.e., insulinotropic concentrations. The following procedure was followed: MIN6 β-cells were cultured in 12-well plates and once they reached ~90% confluence cells were incubated in DMEM containing low glucose (<5.5 mM) the night before the experiments. Then, cells were washed twice with sterile glucose-free Krebs Ringer Bicarbonate/HEPES buffer (KRBH: 135 mM NaCl, 3.6 mM KCl, 5 mM NaHCO\(_3\), 0.5 mM NaH\(_2\)PO\(_4\), 0.5 mM MgCl\(_2\), 1.5 mM CaCl\(_2\), 10 mM HEPES pH 7.4 and 0.1% BSA). Cells were then incubated in KBRH supplemented with different glucose concentrations ranging from 5.5 mM to 20 mM alone or in combination with drugs for 2 hours at 37°C. After that period, 800 μl of supernatants were collected and stored at 20°C overnight, centrifuged 10 min at 12000xg and used for the quantitative determination of insulin at a dilution of 1:20 or 1:50 (see below). The insulin content of attached cells was released by incubating them in acidified ethanol (75% ethanol + 1.5% HCl) overnight at -20°C. The next day, 800 μl of the acidified ethanol extracts were collected, centrifuged and the supernatant used for the quantitative determination of insulin at a dilution of 1:250-500.
Insulin released into the media and contained within cells was determined in the supernatant or acidified extracts, respectively, by using a commercially available enzyme-linked immuno-assay in accordance with the manufacturer’s directions (Alpco, Salem, NH # 80-INSMSU-E01). The insulin concentration in samples was extrapolated from insulin standard curves run in parallel (Figure 6). Insulin secretion was expressed as the ratio of secreted insulin into the culture media to the sum of secreted and intracellular content.

18. Statistics

Data are expressed as mean ± SEM. One-way analysis of variance (ANOVA) was used to compare data between multiple groups. Student’s two-tailed t-test was used for paired comparisons. A $p<0.05$ was used as criterion of statistical significance.
Figure 6. **Insulin standard curve.** Non-linear regression analysis performed on the relationship between absorbance determined at 450 nm and increasing concentrations of mouse insulin (0 - 6.9 ng/ml) as measured by ELISA. The standard curve generated is used to determine the concentration of insulin in cell culture supernatants, cell extracts or plasma samples. *Inset:* internal controls for ELISA using 10 different plasma samples with low or high levels of insulin (red and blue dots, respectively).
Results

1. Expression of the Slc12a family of Cl- extruders in β-cells

To determine the expression pattern of transcripts corresponding to Cl- extruders of the Slc12a family i.e., KCC1, KCC2, KCC3 and KCC4, total RNA obtained from MIN6 was subjected to RT-PCR. By using the species-, isoform- and variant-specific primer sets represented in Figure 7 and depicted in Table 3, RT-PCR experiments demonstrate amplicons of expected sizes for KCC1 and KCC2s or KCC3 and KCC4 in MIN6 β-cells, as shown in Figures 8A and 8B, respectively. To confirm molecular identity, these RT-PCR fragments were sequenced in both directions. Representative sequencing reactions are shown for RT-PCR fragments obtained with primer sets KCC2a-671 and KCC2b-602 (Fig 8C). Together, these results suggest that the main splice variants of KCC1, KCC2, KCC3 and KCC4 mRNAs are expressed in MIN6 β-cells.

The finding that KCC2 mRNAs are expressed in MIN6 raised the question as to what extent this "neuron-specific" gene product is found in β-cells. In other words, is it KCC2 exclusive of the clonal mouse β-cell line MIN6 or is it found in other β-cells lines or in primary β-cells of different species?

To begin to answer that question, KCC2 mRNA expression was determined in mouse and human islets where most of the endocrine cells are β-cells and in rat INS1-E cells. To this end, total RNA from those tissues and cells were reverse transcribed and PCR-amplified by using species- and isoform-specific primer sets directed against mouse, human or rat KCC2 transcripts (see Table 3). As shown in Figures 9A-C, PCR products of expected sizes corresponding to the two known splice variants of KCC2 i.e.,
Figure 7. KCC transcripts and their primer sets used for PCR. Schematic representation of KCC1, KCC2, KCC3 and KCC4 main transcripts, their coding regions (black arrowed boxes), exons (grey arrowed boxes), 5’- and 3’-UTRs (filled lines) and the relative positions of the primer sets (opposite arrowheads) used for transcript screening in RT-PCR experiments. The nucleotide sequences of these primer sets are indicated in Table 3.
Figure 8. Expression of KCC mRNAs in MIN6 β-cells. A. KCC1 and KCC2 mRNA expression was analyzed by RT-PCR using the mouse primer sets KCC1-440, -529 and -531 (left gel: first, second and third lanes, respectively) or KCC2-565, KCC2a-671, KCC2b-602 or -574 (right gel: first, second, third and fourth lanes, respectively). B. KCC3 and KCC4 mRNA expression by using RT-PCR and the following sets of mouse primers (from left to right): KCC3-589, KCC3a-401 (note a 356 bp fragment corresponding to mKCC3d), KCC3c-675, KCC3b-695, KCC4-571, -580, -663 and -691. As positive control of RT-PCRs, transcripts of GAPDH were routinely amplified (555 bp). As negative control, water was used instead of total RNA. C. Partial sequence chromatograms obtained from representative DNA sequencing reactions using purified KCC2a-671 and KCC2b-602 amplicons (green asterisks). The DNA sequence obtained is 100% identical to the spliced version of KCC2a and KCC2b, respectively therefore confirming molecular identity.
**Figure 9.** Expression of KCC2a and KCC2b mRNAs in rat β-cells and mouse or human islets. A-C. KCC2a and KCC2b mRNA expression in purified mouse islets (A), INS-1E β-cells (B) and human islets (C) was analyzed in parallel by RT-PCR using the following specific mouse, rat or human primer sets: mKCC2-565, mKCC2a-671, mKCC2b-574, rKCC2-550, rKCC2a-575, rKCC2b-755, hKCC2a-580 and hKCC2b-565 (see Table 3). As positive control of RT-PCRs, the mRNAs of GAPDH were amplified (555 bp). As negative control, water was used instead of total RNA. D. Representation of KCC2a and KCC2b transcripts, their coding regions, exons, 5′- and 3′-UTRs and the relative positions of the primer sets (opposite arrowheads connected by a line) used to amplify the whole ORF of KCC2 in overlapping fragments (E) or as a single amplicon (F). E. RT-PCR screening of exons 4-27 of KCC2s in MIN6 β-cells showing amplicons of expected sizes and number (i.e., no multiple bands indicative of gross splicing). RT-PCRs were performed by using primer sets KCC2-314, -337, -345, -408, -598, -693, -813 and -565. F. Representative reverse transcription experiment coupled to long-range PCR using total RNA from MIN6 β-cells and primer sets KCC2a-3591 or KCC2b-4024 (mKCC2a-1s + mKCC2-3as or mKCC2b-1s + mKCC2-5as, see Table 3). The bands of 3591 bp (KCC2a) and 4024 bp (KCC2b) were directly cloned in pCRB (see Fig 14A).
KCC2a and KCC2b are also detected in mouse and human islets as well as in INS-1E indicating that KCC2a and KCC2b mRNA expression is not a unique feature of MIN6 β-cells but rather a finding of β-cells in general. To discard the possibility of non-coding KCC2 transcripts partially transcribed in these cells, we made sure that the coding exons of KCC2 are simultaneously amplified in overlapping fragments as well as a whole ORF of mature KCC2 mRNAs. To this end, RT-PCR was performed using overlapping primer sets designed to cover most of the ORF of KCC2a and KCC2b transcripts (see Fig 9D). As shown in Figure 9E, RT-PCR experiments produced overlapping amplicons of predicted sizes suggesting that the ORFs of KCC2a and KCC2b are expressed in MIN6 β-cells. Further, absence of multiple bands per lane suggests no major splicing events in these KCC2a/b transcripts. To demonstrate that coding KCC2 exons including exons 1a (exon 1), 1b (exon 2), and 3-27 lay within single KCC2 transcripts, we performed RT coupled to long-PCR and primers sets designed to align 5'- and 3'-UTRs regions of KCC2a and KCC2b transcripts (see Fig 9D). As shown in Figure 9F, MIN6 β-cells express full-length KCC2a and KCC2b mRNAs of expected sizes excluding the possibility of short, non-translatable, unprocessed or aberrant KCC2 transcripts in these cells.

2. Expression and localization of KCC2 in insulin-secreting β-cells

To correlate the presence of full-length KCC2 transcripts with their protein products, we next confirmed KCC2 expression at the protein level in β-cells by Western blot and immunofluorescence microscopy. For these immunological studies, we first used a monoclonal antibody directed against unique residues 932-1043 of the C-terminus of rat KCC2 named N1/66. As shown in Figure 10A, N1/66 immunoblots of protein extracts obtained from MIN6 (100 µg), purified mouse islets (100 µg) or mouse
Figure 10. KCC2 protein expression analysis using monoclonal antibodies. A. Immunoblot analysis of MIN6, mouse islets and mouse brain protein extracts developed by using the monoclonal KCC2 antibody N1/66 (NeuroMab). Indicated are bands of predicted molecular weights (MWs) for KCC2 i.e., ranging from ~124 to 150 kDa. B-D. Immunostaining of KCC2 in fixed MIN6 cells (B), mouse pancreas and brain tissue slides (C and D, respectively) by using N1/66 coupled to Cy3-conjugated secondary antibodies.
brain (25 µg, positive control) revealed bands of ~124 kDa, the expected MW for core, high-mannose N-glycosylated KCC2 (Payne et al. 1996) in control samples, MIN6 β-cells or mouse islets. Notably, faint bands of ~135 kDa potentially representing low levels of different N-glycan types of the transporter are also detected. However, N-glycosylated KCC2 versions of higher MW could be observed in MIN6 or islets consistent with the very low levels of KCC2 expression relative to brain. To extend these results and confirm the notion that MIN6 or primary β-cells of the islet express KCC2 immunofluorescence microscopy experiments were performed. As shown in Figures 10B-D, N1/66 detects its antigen in fixed MIN6 β-cells as well as in mouse pancreas slides, in structures resembling the islet of Langerhans. Most notably, when brain and pancreas slides stained in parallel with this antibody are compared in hand with immunoblot data (Fig 10A), it can be clearly observed that KCC2 immunoreactivity is indeed present in the islets and in MIN6 β-cells.

To confirm and refine these results we performed similar experiments as those shown in Figure 10 but by using a widely characterized and thoroughly validated rabbit polyclonal antibody directed against KCC2 (Mao et al. 2012; Stil, et al. 2011; Stil, et al. 2009; Williams et al. 1999). To determine the specificity of this KCC2 antibody in our hands we first stained positive and negative controls i.e., mouse brain and spinal cord, the most abundant sources of KCC2 expression (Blaesse et al. 2009), and kidney and salivary gland, which express KCC1, KCC3 and KCC4 (Boettger et al. 2002; Chou, et al. 2008; Gillen et al. 1996; Liapis, et al. 1998; Mercado et al. 2005; Roussa, et al. 2002; Velazquez and Silva 2003) but not KCC2 (Chou et al. 2008; Melo et al. 2013a; Roussa et al. 2002; Uvarov et al. 2007). As shown in Figures 11A-D, KCC2 immunoreactivity is detected in central and spinal neurons, but not in the white matter or choroid plexus, as
**Figure 11.** KCC2 protein expression analysis using polyclonal antibodies. Immunostaining of KCC2 in tissue slides of mouse brain (A), spinal cord (B), kidney (C) and salivary gland (D) by using polyclonal antibodies against KCC2 coupled to Cy3-conjugated secondary antibodies. Note that the antibody detects its antigen in neurons of the hippocampus, but not in the white matter of corpus callosum, in the choroid plexus (inset A) or in the white matter of the spinal cord (B). Bars indicate 50 µm. Cell nuclei were counterstained by using DAPI.
previously demonstrated (Karadsheh et al. 2004; Williams et al. 1999). Along the same lines, staining is negative in kidney and salivary glands, where KCC2 has not been found so far suggesting that the antibody detects its antigen in neurons. Next, we stained rat, mouse and human pancreas slides. As shown in Figures 12A-C, KCC2 is clearly detected in the pancreatic islet but not in the exocrine pancreas strongly supporting the notion that KCC2 is expressed in islet of Langerhans and that the KCC2 antibody used does not cross-react or detect other proteins present in the exocrine pancreas. In addition, as shown in Figure 12D, immunoblots of protein extracts obtained from mouse islets or whole pancreas reveals discrete bands of MWs ranging from ~124 to ~240 kDa, the expected sizes for all major N-glycan versions of the transporter i.e., core, high-mannose N-glycosylated (~124 kDa), hybrid-type (130-135 kDa), complex N-glycosylated (~150-160 kDa) and the proposed dimers of KCC2 (~240-270 kDa) (Medina et al. 2014), a banding pattern consistent with low levels of KCC2 expression relative to brain. To further demonstrate KCC2 expression in the mouse pancreatic islet, KCC2 was co-stained with glucagon (GLC) and insulin (INS) in order to determine KCC2 localization in α- and/or β-cells of the islet, respectively. As shown in Figures 12E-H, KCC2 and INS clearly co-localize whereas less evident co-localization could be detected in GLC-positive cells demonstrating that insulin-secreting β-cells of the islet express most of KCC2. To extend these results, human pancreas slides were co-stained against KCC2 and INS and subjected to confocal immunofluorescence microscopy. As shown in Figure 13, KCC2 and INS co-localize in the human islet reinforcing the concept that KCC2 is expressed in mammalian insulin-secreting β-cells.

3. Cloning and characterization of KCC2a and KCC2b

As stated by Parker and Boron relative to the identification of Na⁺-dependent
Figure 12. KCC2 protein expression analysis in pancreas and islets using polyclonal antibodies. A-C. Immunostaining of KCC2 in human (A), mouse (B) and rat (C) pancreas slides demonstrating immunoreactivity in structures resembling the islet of Langerhans but not in peripheral exocrine pancreatic tissue. D. Immunoblot analysis of purified islets and the indicated amounts of mouse pancreas or brain protein extracts. E-H. Representative triple labeling in co-immunolocalization experiments performed in mouse pancreas using the following antibodies: KCC2 (E), glucagon (F), and insulin (G) and co-stained by using Cy3-, DyLight 408- and AlexaFluor 488-labeled secondary antibodies.
Figure 13. KCC2 protein expression analysis in mouse and human pancreas and islets using polyclonal antibodies. Confocal immunomicroscopy studies of KCC2 and insulin expression in mouse and human pancreas slides demonstrating co-immunolabeling of KCC2 and insulin in the islet of Langerhans but not in peripheral exocrine pancreatic tissue.
HCO₃⁻ transporters: "Also listed here are the major anatomical locations from which each variant has been cloned as a full-length cDNA (the only reliable demonstration of the presence of each in any preparation)." (Parker and Boron 2013), we therefore aimed at providing definitive proof of KCC2 expression by cloning KCC2a and KCC2b transcripts expressed MIN6 β-cells. To this end, the amplicons of 3591 bp and 4024 bp representing whole ORFs of KCC2a and KCC2b mRNAs, respectively (see Fig 9F) were purified and non-directionally cloned into pCRB plasmids following the methodology explained in the Methods section. The recombinant plasmids obtained were named and grouped according to the identity of the cDNA inserted i.e., pCRB-mKCC2a and pCRB-mKCC2b followed by a number (#) identifying the clone under study. Six pCRB-mKCC2a clones (#1-6) were recovered from solid plates, grown and purified to determine presence, orientation and identity of the inserts in order to design downstream subcloning strategies. The flow chart schematizing these procedures is shown in Figure 14. Basically, purified pCRB-mKCC2a plasmids (clones #1-6, shown in Fig 14B) were digested with XhoI (predicted to cut pCRB-mKCC2a once, resulting in a single 7 kb DNA band only if the insert of 3.6 kb was present, see map in Fig 14A). As shown in Figure 14C, clones #1, #4 and #5 produced the expected 7 kb fragments after XhoI digestion demonstrating the presence of the insert.

To determine the orientation of the insert, pCRB-mKCC2a #1, #4 and #5 were digested with KpnI (predicted to cut pCRB-mKCC2a twice: once within the insert at ~1.2 kb from the initiation codon ATG and once within the plasmid, see map in Fig 14A). Of note, if the insert was in the sense (forward) orientation i.e., from 5' to 3', KpnI is predicted to produce two bands, one of ~1.3 kb and another of ~5.7 kb. As shown in Figure 14D, pCRB-mKCC2a #4 produced the restriction fragments predicted for the
Figure 14. Cloning and characterization of KCC2a mRNAs in MIN6 β-cells. A. Representation of pCRB-mKCC2a plasmids obtained after direct insertion of KCC2a fragments of 3591 bp (see Fig 9F) and used to transform bacteria for cloning. B. Plasmid purification of six different colonies showing heterogeneity of plasmids. C. Restriction digestion of plasmids with XhoI predicted to linearize pCRB-mKCC2a. Note: only three clones contain the insert as demonstrated by a single band of ~7 kb. D. Restriction digestion of XhoI-positive plasmids with KpnI to determine orientation of the insert. Note: clones #1 and #2 have the insert in the forward orientation as demonstrated by the 2.3 kb restriction band. E. Sequence analysis of pCRB-mKCC2a #1, #4 and #5 showing a unique splicing event in clones #1 and #4.
sense orientation whereas *KpnI* digestion of pCRB-mKCC2a #1 and #5 produced bands of ~2.3 kb and 4.7 kb indicating that the orientation of the insert is antisense or reversed. To determine molecular identity, all pCRB-mKCC2a clones were fully sequenced and the nucleotide sequences obtained aligned against the *in silico* predicted, not yet cloned mKCC2a (*RefSeq* XM_006499943). As shown in Figure 14E, the insert sequences of pCRB-mKCC2a #1, #4 and #5 confirmed mKCC2 identity. However, to our surprise, sequence alignments also revealed that pCRB-mKCC2a #5 contains a previously unknown alternatively spliced event named here KCC2a-S25 in virtue of the missing 15 bp represented by exon 25 of the *Slc12a5* gene.

To characterize pCRB-mKCC2b, four independent clones were subjected to a similar strategy to that described for pCRB-mKCC2a in Figure 14. However, contrary to pCRB-mKCC2a, all pCRB-mKCC2b clones gave a single unique nucleotide sequence matching exactly the mKCC2b sequence of reference NM_020333. The predicted ORFs of the cDNA sequences corresponding to mKCC2a-S25, mKCC2a and mKCC2b are depicted in Figure 15. The nucleotide sequences of these three KCC2 clones have been submitted to GenBank and identified with accession numbers KJ535320 (mKCC2a), KJ535321 (mKCC2a-S25) and KJ535322 (mKCC2b). Notably, since KCC2b is the only KCC2 variant cloned from mouse tissues (represented by AK122460, AK147262, AF332063, AF332064 and BC054808, see Table 1) we provide for the first time two mouse KCC2 clones obtained from non-neuronal cells: KCC2a and KCC2a-S25. To demonstrate that cloned mKCC2a and mKCC2a-S25 mRNAs direct expression of their respective proteins, mutagenesis of pCRB-mKCC2a and pCRB-mKCC2a-S25 were performed by insertion of a *SfiI* site in the 5'-UTR region of both cDNAs in order to subclone them into HA- and myc-tagged expression vectors. The resulted pCMV-
Figure 15. Predicted primary structure of mKCC2a, mKCC2a-S25 and mKCC2b. \textit{In silico} translation of KJ535320 (mKCC2a), KJ535321 (mKCC2a-S25) and KJ535322 (mKCC2b) cDNAs cloned from MIN6 β-cells. Underlined are residues unique to KCC2a or KCC2b. Shaded boxes depict predicted transmembrane domains. The splicing event in KCC2a-S25 is also shown and predicted to lack residues 1077 to 1081 (EWENL, corresponding to residues 1037 to 1041 in mKCC2b).
HA.mKCC2a and pCMV-myc.mKCC2a-S25 vectors were transfected into MIN6 β-cells or COS7 and KCC2 expression followed by immunostaining using HA and myc antibodies. As shown in Figure 16, both HA-mKCC2a and myc-mKCC2a-S25 are expressed in these cell models demonstrating that KCC2a-S25 and KCC2a cDNAs originally cloned from insulin-secreting MIN6 β-cells are able to direct translation in COS7 and MIN6 cells.

4. Expression pattern of KCC2a, KCC2b and KCC2a-S25 in mouse tissues

Since KCC2a and KCC2a-S25 are the first splice variants of KCC2 to be cloned from non-neuronal tissues and KCC2a has been detected outside neurons (see Section 10), we tested the possibilities that: i) KCC2a has a wide tissue distribution, and ii) KCC2a-S25 as an "extra-neuronal" variant. To this ends, we first data-mined nucleotide databases using the splicing region of mKCC2a-S25 as a query and aligned them against KCC2 and KCC4 cDNAs from several species and tissues posted in GenBank. Figure 17A partially represents these findings, which can be summarizes as follows: i) mKCC2a-S25 exhibit near complete identity to a 3 kb partial KCC2 cDNA cloned from mouse embryonic brain (BC057624), and ii) a surprisingly similar splicing event occurs in full-length KCC4 cDNAs obtained from human tissues [AF105365 (Mount et al. 1999)], mouse testis [BC098390 (Strausberg, et al. 2002)] or partial KCC4 clones from human spleen (AK024493 and AK024494). These results suggest that mKCC2a-S25 is also expressed in the brain, that the mechanisms involved in splicing of exon 25 in KCC2a may be shared with KCC4 and that mKCC2a-S25 has a wide tissue distribution. To ascertain the latter, we screened KCC2a and KCC2b transcript expression in total RNA
Figure 16. Transient expression of HA.mKCC2a and myc.mKCC2a-S25 in MIN6 and COS7 cells. Immunodetection of HA.mKCC2a and myc.mKCC2a-S25 in MIN6 and COS7 cells transiently transfected with the indicated expression plasmids.
Figure 17. Expression pattern of KCC2 mRNAs and protein in mouse tissues. 
A. In silico alignment of the indicated KCC2 and KCC4 cDNAs showing the region corresponding to the last nucleotides of exon 24 and the first ones of exon 26 (KCC2) and their KCC4 homologous exons. Note the absence of exon 25 in KCC2 and KCC4 transcripts. B. RT-PCR performed on commercially available RNAs from the indicated mouse tissues. As negative control, water was used instead of total RNA and the GAPDH primer set (555 bp). Shown are representative 2% agarose gels where bands of predicted sizes for mouse KCC2a (671 bp) and KCC2b (574 bp) are detected. C. Representative images of immunoreactive KCC2 in the indicated mouse tissues and compartments using validated KCC2 polyclonal antibodies.
acquired from commercial sources. As shown in Figures 17B-C, KCC2a and KCC2b mRNAs are expressed among the tissue tested. To determine if some of these tissues express KCC2 at the protein level as well, we performed immunolocalization experiments in tissues where KCC2 expression has been detected at the mRNA or protein levels i.e., heart (Antrobus et al. 2012), adrenal medulla (Xie et al. 2003) or testis (Uvarov et al. 2007). As shown in the panels of Figure 17D, immunoreactive KCC2 is detected in those tissues indicating that KCC2 expression has a more wide distribution than originally thought.

To determine the expression pattern of KCC2a-S25 in mouse tissues we amplified KCC2 transcripts by RT-PCR using KCC2-565, a primer set predicted to co-amplify KCC2 mRNAs lacking and/or expressing exon 25 (15 bp). Then, we subjected these amplicons to digestion with MspI, a restriction enzyme predicted to cut in defined sites of the amplicon including the junction between exon 24 and exon 25, as depicted in Figure 18A. As shown in Figure 18B, digestion of KCC2-565 fragments with MspI produces a distinctive banding pattern corresponding to very low levels of KCC2 transcripts harboring splicing 25 (S25) in the brain or spinal cord whereas such splicing is clearly detected in all mouse tissues tested as well as MIN6 β-cells. Collectively, these results confirm for the first time that KCC2 has a wider expression pattern than originally thought and that KCC2a-S25 may represent in fact a non-neuronal variant of KCC2a.

5. Functional evidence of KCC2 in MIN6 β-cells and impact on insulin secretion

The unique functional and pharmacological properties of neuronal KCC2s i.e., a constitutively active Cl⁻ extruder with defined pharmacology (Delpire et al. 2012; Delpire
Figure 18. KCC2a/b-S25 are expressed in mouse endocrine tissues. A. Representation of KCC2a/b amplicons obtained by using KCC2-565 primer set. Indicated are the restriction sites for MspI and the predicted lengths of the digestion products in bp. Exon 25 is noted in red and its splicing eliminates an MspI site. B. Representative RT-PCR of the indicated β-cell lines and mouse tissues showing amplicons of expected sizes i.e., 565 bp. As negative control, water was used instead of total RNA. C. Digestion of PCR products with MspI and resolution of restriction fragments by 2% agarose gel electrophoresis. Shown is a representative experiment demonstrating KCC2a/b-S25 in MIN6 β-cells i.e., a fragment of 362 bp but minimally in mouse spinal cord or brain. D. Screening of KCC2a/b-S25 in mouse tissues. Shown is the expression of KCC2a/b mRNAs in the tissues tested. E. MspI digestion and analysis of restriction fragments. The representative experiment demonstrates undetectable levels of exon 25 in KCC2 mRNAs in all tissues tested except in pituitary where KCC2a/b mRNAs are expressed with or without exon 25, potentially reflecting mRNAs from the pars nervosa of the pituitary.
et al. 2009; Gagnon et al. 2013; Payne 1997; Uvarov et al. 2007) and our results supporting the concept that KCC2 is expressed in insulin-secreting β-cells prompted us to test the hypothesis that KCC2 regulates Cl⁻ fluxes in MIN6 β-cells. To this end, the impact of the selective KCC2 inhibitor VU (Delpire et al. 2012; Delpire et al. 2009) or the specific agonist CLP257 (Gagnon et al. 2013) on steady state total Cl⁻ content was determined in MIN6. First, MIN6 were depleted of endogenous Cl⁻ by incubating them in isotonic media (ISO) free of Cl⁻ ions. MIN6 cells were then allowed to re-accumulate Cl⁻ for 30 minutes in ISO containing physiological concentrations of Cl⁻. As shown in Figure 19A, MIN6 cells accumulates [Cl⁻]ᵢ as expected i.e., following its electrochemical gradient. However, when MIN6 cells accumulating Cl⁻ were challenged with a 5 minute pulse of VU or CLP257, the total Cl⁻ accumulated in these cells significantly increased or decreased, respectively, suggesting that KCC2 is functionally expressed in MIN6 β-cells.

If KCC2 regulates Cl⁻ fluxes in MIN6 and [Cl⁻], above levels predicted by Nertsian equilibrium in β-cells participates in insulin secretion (Di Fulvio, et al. 2014), it follows that regulation of KCC2 function modulates insulin secretion. To test this hypothesis, we analyzed the effect of VU and CLP257 on the ability of MIN6 cells to secrete insulin in response to glucose. As shown in Figures 19B-D, VU or CLP257 significantly increases or decreases, respectively, insulin secretion at all concentrations of glucose tested when compared to their respective controls suggesting that KCC2 function is implicated in insulin secretion.
Figure 19. Impact of KCC2 function on Cl− uptake and insulin secretion in MIN6 β-cells. A. Total Cl− uptake was potentiometrically determined in MIN6 cells depleted of endogenous Cl− after 30 minutes incubation in ISO media containing physiological Cl− concentrations plus vehicle or the indicated concentrations of VU, CLP257 or BTD (*p<0.05, n=7; **p<0.01, n=5 and ***p<0.001, n>10). B-C. Effects of VU (B) and CLP257 (C) on insulin secretion expressed relative to total insulin content under basal (5.5 mM) and stimulated conditions (7.5-20 mM) (*p<0.05 relative to basal, #p<0.05 relative to basal or respective untreated and w p<0.05 relative to respective untreated, n>6). D. Effects of VU and CLP257 on insulin secretion (as content ratio) normalized to basal (100%) (*p<0.05, n>6).
Discussion

The studies of Davies and collaborators were the first to demonstrate the presence of KCC1, KCC3a, KCC3b and KCC4 in the rat islet (Davies, et al. 2004). By using RT-PCR, we confirmed and extend those results by demonstrating that MIN6 β-cells express KCC1 and KCC4 as single variants whereas KCC3 is represented by four different splice variants i.e., KCC3a, KCC3b, KCC3c and KCC3d. Indeed, the mouse Slc12a4 gene may produce at least three transcripts, one of them predicted to lack exon 2 (KCC1b: XM_006530792) or exon 22 (AK164479). None of these splicing events were detected in our experiments designed to co-amplify KCC1 mRNAs within the region encompassing exons 2 or 22 (Fig 7). The absence of co-amplified bands of 531 bp and 436 bp [531−(95 bp of exon 2)], 440 bp and 255 bp [440−(185 bp of exon 22)] or single fragments of 436 bp or 255 bp in RT-PCR experiments using primer sets KCC1-531 or KCC1-440, respectively (Fig 8) minimizes the possibility of KCC1 splicing events involving exon 2 or exon and therefore, MIN6 β-cells may express a single variant of this ubiquitous and functionally characterized K⁺Cl⁻ co-transporter.

In humans, the SLC12A6 gene produces an unusual number of KCC3 transcripts, some of them demonstrated to direct expression of functional proteins (Mercado et al. 2005), others of unknown tissue distribution or functional properties (Gagnon and Di Fulvio 2013). In mice, molecular evidence in the form of full-length cDNAs support the existence of at least four different KCC3 mRNAs with unique 5'-ends (depicted in Fig 7) and therefore expected to produce N-terminally different KCC3 proteins. Our RT-PCR experiments using species- and variant-specific KCC3 primers sets suggest expression of the four known mouse KCC3 mRNAs: KCC3a (NM_133648),
KCC3b (NM_133649), KCC3c (XM_006498545) and KCC3d (XM_006498546).
Interestingly, KCC3d mRNAs appear expressed at relatively high levels in MIN6 β-cells when compared to the thoroughly characterized KCC3a. Indeed, RT-PCR experiments performed using the primer set KCC3a-401 resulted in the co-amplification of two amplicons: one of 401 bp, suggesting expression of KCC3a, and another of 356 bp indicating that 45 bp corresponding to exon 3 in \textit{Slc12a6} have been spliced out in this KCC3 mRNA (Fig 8). In the case of KCC4, single amplicons of predicted sizes were obtained when using four different primer sets designed to amplify the mouse KCC4 transcript originally cloned by Mount and collaborators \textit{i.e.}, AF087436 (Mount et al. 1999) which together with AK149750, AK143535, AK166215, BC059242, BC060133 and BC141107 make up the mouse KCC4 mRNA of reference NM_011390. Although these results suggest that KCCs are represented by several transcripts in MIN6, their functional significance remains to be determined. Notably, no $\text{K}^+\text{Cl}^-$ cotransport has been demonstrated in dissociated rat β-cells (Davies et al. 2004), raising the possibility that β-cells may not exhibit RVD under the conditions tested or that KCC1, KCC3 and KCC4 are inactive transporters in those cells. The latter conclusion is based on the finding that DIOA or NEM, known stimulators of KCCs (Lauf and Adragna 2000) had no effect of β-cell volume after 190 mOsm/kg hyposmotic shock (Davies et al. 2004). Interestingly, rat β-cells swell in response to glucose and shrink via VRACs (Davies, et al. 2007) indicating that β-cells may not require KCCs to modulate cell volume or KCCs do not regulate cell volume in these cells or that KCC cotransport may require higher hyposmotic challenges or NEM concentrations to be activated (Delpire et al. 2009). Clearly, more experiments may be needed to demonstrate a role of KCCs in β-cell volume regulation.
An important observation of the studies of Davies et al. is that the mRNAs for KCC2 and the cystic fibrosis conductance regulator (CFTR) were not detected by RT-PCR in dissociated rat β-cells (Davies et al. 2004), a finding consistent with the notion that KCC2 and CFTR are absent in the islets (Marino, et al. 1991; Trezise and Buchwald 1991). However, recent functional and immunological data demonstrates expression of CFTR in human and mouse β-cells (Boom, et al. 2007; Edlund, et al. 2014; Guo, et al. 2014), suggesting the possibility that KCC2 may have not been detected in rat β-cells due to low expression levels. Indeed, Taneera et al. demonstrated KCC2 expression in gene-chips of purified human islets and showed that KCC2 mRNA levels are one order of magnitude less abundant than those of NKCC1 (Taneera et al. 2012). Therefore, to maximize our chances of KCC2 detection in β-cells, we carried out a systematic approach: many RT-PCR experiments using a battery of species-specific primer sets (Table 3) and high-fidelity DNA polymerases, thermostable enzymes successfully used for the detection of low-abundance transcripts in unstable RNA preparations such as human neutrophils (Di Fulvio and Gomez-Cambronero 2005) or in samples were KCC2 mRNAs were ~150 times less abundant than NKCC1, such as primary sensory neurons (Mao et al. 2012). By these means, we demonstrated KCC2 mRNAs in human, mouse and rat β-cells (Figs 8A and 9A). We further extend these data by demonstrating that KCC2a and KCC2b mRNAs are expressed in MIN6 β-cells as full-length ORFs (Figs 9E-F) suggesting that these transcripts may also direct protein expression. Indeed, a specific monoclonal antibody directed against C-terminal residues 932-1043 encoded by exon 23 of the rat Slc12a5 gene detects its antigen in MIN6 β-cells, mouse islets and brain protein extracts (Fig 10A) confirming that KCC2 is expressed in β-cells/islets at the protein level. Interestingly, the banding pattern observed in immunoblots of protein extracts obtained from β-cells or islets was not identical as the one seen with brain
extracts (Fig 10A). In particular, the KCC2 antibody detected proteins in β-cells/islets distributed within the range of MWs encompassing ~124 kDa to ~140 kDa. This pattern is consistent with the expected one for core, high-mannose N-glycosylated KCC2 i.e., ~124 kDa (Payne et al. 1996) and intermediate N-glycan states between hybrid-type N-glycans to complex N-glycosylated versions of the transporter. Notably, the antibody used in these experiments did not detect KCC2 bands heavier than ~140 kDa in β-cells/islets suggesting differences in the N-glycan state of KCC2 relative to brain tissues where exceptionally abundant bands of ~150 kDa were detected (Fig 10A). This difference may be attributed to the fact that KCC2 is expressed in β-cells at much lower levels when compared to brain tissues and to the fact that complex N-glycosylation of proteins depends on the molar amount of substrate reaching the successive enzymatic steps of N-glycan biosynthesis (Stanley 2011). In addition, the low expression levels of KCC2 proteins observed in β-cells/islets may have compromised the specificity of the monoclonal antibodies used in these experiments. However, this valid argument is minimized by the fact that the antibody detects its antigen in cultured MIN6 cells (Fig 10B) and in mouse pancreas slides in structures resembling the endocrine islet, but not the exocrine tissue (Fig 10C) where KCC1, KCC3 and KCC4 have been abundantly detected (Boettger et al. 2002; Chou et al. 2008; Gillen et al. 1996; Liapis et al. 1998; Mercado et al. 2005; Roussa et al. 2002; Velazquez and Silva 2003), but not KCC2 (Chou et al. 2008; Melo et al. 2013a; Roussa et al. 2002). Further, we have confirmed KCC2 expression and localization to the islet by using a second antibody that has been extensively characterized and is widely used and regarded as specific for the neuron-specific KCC2 (Williams et al. 1999). Indeed, this polyclonal antibody stained neurons of the mouse brain and spinal cord but not in the white matter of corpus callosum or choroid plexus (Figs 11A-B) consistently reproducing previous results (Baldi, et al. 2010;
Karadsheh et al. 2004; Lu et al. 1999; Markkanen et al. 2013; Phan and Pflieger 2013; Williams et al. 1999). In addition, the antibody failed to produce immunoreactive signaling in slides of mouse kidney or salivary glands where KCCs, except KCC2, have been detected (Boettger et al. 2002; Chou et al. 2008; Gillen et al. 1996; Liapis et al. 1998; Melo et al. 2013a; Mercado et al. 2005; Roussa et al. 2002; Velazquez and Silva 2003). Furthermore, these antibodies labeled mouse, rat and human pancreatic islets but not the exocrine pancreas (Figs 12A-C) in a pattern similar to that obtained with KCC2 monoclonal antibodies (Fig 10C). Therefore, these results are compatible with the conclusion that the immunoreactive neuron-specific KCC2 is also expressed in the islet of Langerhans. This is further substantiated by the following observations: i) the high-affinity KCC2 polyclonal antibody detects its antigen in blots of protein extracts of purified islets and several amounts of total pancreas as a banding pattern ranging from ~125 kDa to ~240 kDa and consistent with low expression levels of core, high-mannose N-glycosylated (~124 kDa), hybrid-type (130-135 kDa), complex N-glycosylated (~150-160 kDa) and dimerized KCC2 (Fig 12D), and ii) immunoreactive KCC2 is co-detected with insulin in mouse and human pancreas slides (Figs 12E-H and Fig 13, respectively) whereas KCC2 co-localized minimally with glucagon (Figs 12E-H), demonstrating that KCC2 locates mostly in insulin-secreting β-cells of the islet of Langerhans.

Conclusive proof of KCC2 expression in β-cells comes in the form of three cDNA clones obtained from MIN6 β-cells whose nucleotide sequences have been submitted to GenBank under accession numbers KJ535320 (KCC2a), KJ535321 (KCC2a-S25) and KJ535322 (KCC2b) (Fig 15). One of these clones i.e., KJ535322 contained a full ORF of 3348 bp predicted to encode a protein of 1115 residues and ~124 kDa MW. The nucleotide sequence of KJ535322 matched full-length mouse KCC2b cDNA clones.
obtained from brain and posted in GenBank i.e., AF332063, AF332064, AK122460, AK147262, BC054808, KJ535322 and AK165769 [all of them represented by the RefSeq NM_020333 (Table 1)]. The ORFs of KJ535320 and KJ535321 showed similarity to several mouse expressed sequence tags (mESTs) but did not match full-length mouse cDNA clones, consistent with the fact that no mKCC2a has been cloned and posted in GenBank from any mouse source, until now. Therefore, based on similarity and homology against human KCC2a ESTs and the only full-length KCC2a cloned and posted in GenBank i.e., rKCC2a [EF641113 (Uvarov et al. 2007)], RefSeq database annotates mKCC2a as a predicted transcript under the accession number XM_006499943. The cDNA represented by KJ535321 shares 94.9% and 99.5% nucleotide and amino acid identity, respectively, to rKCC2a EF641113 therefore these data provide definitive proof of the existence of KCC2a in mouse β-cells. The cloning process also produced KJ535320, which when analyzed in silico and aligned against KJ535321 (mKCC2a) demonstrated a unique difference: absence of the nucleotides GGA GTG GGA AAA CTT , a 100% match to exon 25 of the mouse Slc12a5 gene (Figs 14-15).

When taken together, the results thus far discussed demonstrate that the "neuron-specific" KCC2 is also expressed in β-cells. This finding cannot be taken as a surprise, as several lines of evidence suggest the concept that KCC2 has a wide tissue distribution including published results (see Section 10), in silico data (Fig 17A) and our results demonstrating KCC2a and KCC2b mRNAs in mouse adrenal, heart, thyroid, ovary and testis (Fig 17B). It could be argued that expression of KCC2 in those mouse tissues is the result of contamination from neural tissues. Indeed, adrenal, heart, thyroid, ovary and testis are innervated. However, when specific and validated polyclonal
antibodies used to detect KCC2 in slides of brain, spinal cord and pancreas (Figs 11-13) were also used in slides of those tissues, the immunoreactive presence of KCC2 was mainly located in anatomically defined tissue compartments such as chromaffin cells of the adrenal medulla, spermatogonia and interstitial cells of the testis or heart muscle fibers (Fig 17C). These results confirm the original observations of Uvarov, Xie and Antrobus et al., the first to found KCC2 in those tissues (Antrobus et al. 2012; Uvarov et al. 2007; Xie et al. 2003). We further extend these results by demonstrating that splicing 25 is abundantly expressed in all mouse tissues examined (Fig 18) whereas brain and spinal cord express minimal levels of this splicing event (Fig 18C). However, these results cannot be interpreted as proof of extra-neuronal expression of mKCC2-S25. Indeed, a partial KCC2 cDNA cloned from mouse embryonic brain (BC057624) also harbors such splicing event. Further, the finding that exon 25 is also spliced out in human and mouse full or partial KCC4 cDNAs obtained from several tissues (Fig 17A), suggest a common mechanism involved in mKCC2-S25 and KCC4 expression.

A key functional characteristic of KCC4 is its robust activation under hypotonic conditions, however, contrary to KCC2a or KCC2b, KCC4 is inactive under isotonic conditions (Mercado et al. 2006; Mercado et al. 2001; Mercado et al. 2000). These observations suggest that mKCC2a-S25 and KCC4 may share functional properties. Elimination of exon 25 in mKCC2a predicts the absence of the C-terminal amino acid sequence EWENL whereas C-terminal truncation of other members of the Slc12a family impairs their trafficking, stability or localization (Ding, et al. 2013; Zaarour, et al. 2009; Zaarour, et al. 2012). Therefore, we hypothesized that mKCC2a-S25 may have a different localization pattern within the cell relative to mKCC2a. However, tag immunostaining of MIN6 β-cells or COS7 cells transiently transfected with HA.mKCC2a
or myc.mKCC2-S25 did not result in obvious miss-localization of the tagged proteins. In fact, these two proteins were found distributed in intracellular compartment and in much less relative abundance towards the periphery of the cells (Fig 16). These findings are by no means surprising; membrane proteins including co-transporters are expected to some extent in endoplasmic reticulum, Golgi cisternae, recycling endosomes and other intracellular compartments, particularly when expressed at low levels in β-cells (Alshahrani et al. 2012; Alshahrani and Di Fulvio 2012). Although the functional properties of mKCC2a-S25 remain unknown, elimination of EWENL in the intracellular C-terminus of KCC2a may impact its function. This speculation is based on the following facts: i) the functional domain responsible for the constitutive KCC2 activity under isotonic conditions i.e., the "ISO domain" (1043PSPVSSEG1KDFFSMKP1059) is encoded in the last residues of exon 24 (Mercado et al. 2006), ii) several putative phosphorsites including Y926, T929, S963, T1029, S1056 and Y1109 are involved in the modulation of KCC2 basal activity (Chamma et al. 2012; Medina et al. 2014), and iii) human KCC2-R952H and KCC2-R1049C mutant are functionally deficient and exhibit impaired plasma membrane insertion and phosphorylation of the distant S940 (Kahle et al. 2014; Puskarjov et al. 2014) corresponding to S963 in mKCC2a. These results indicate that changes involving single amino acids within the C-terminal residues encoded by the last nucleotides of exons 21 to the first ones of exon 26 do impact KCC2 function. Regardless of predictions, the discovery of mKCC2a-S25 form β-cells warrant further experiments to determine its functional, pharmacological and molecular properties.

Although the functional impact of KCC2 in extra-neuronal tissues remains unknown, our results indicate that KCC2 participates in Cl− regulation and insulin secretion in MIN6 β-cells (Fig 19) despite its low expression levels relative to neurons.
Treatment of MIN6 cells with VU (25 µM), a selective KCC2 inhibitor, increased total cellular Cl\(^{-}\) content by a small albeit significant \(~10\%\) when compared with non-treated cells, an effect that was further increased to \(~16\%\) relative to basal when 50 µM of VU were used. These results suggest that KCC2 is actively involved in Cl\(^{-}\) extrusion from β-cells under basal isotonic conditions, the hallmark of KCC2 function. The role of KCC2 in Cl\(^{-}\) homeostasis in β-cells is further supported by the finding that the KCC2 agonist CLP257 (Gagnon et al. 2013) significantly decreased Cl\(^{-}\) content by \(~18\%\) in MIN6 β-cells (Fig 19A). However, caution must be paid when using pharmacological tools to address transport function in any cell type. Indeed, VU significantly inhibits other targets such as adenosine A1 receptors, L-type Ca\(^{2+}\) channels, the K\(^{+}\) channel Kv11.1 also known as ether-à-go-go-related gene (ERG) (Delpire et al. 2009) as well as NKCC1 and KCC3, although at concentrations higher than 50 µM (Delpire et al. 2012). Notably, inhibition of ERG channels increases insulin secretion only at low levels of glucose \(i.e., 1.1\ \text{mM}\) (Rosati, et al. 2000) whereas inhibition of A1 receptors improves the second phase of insulin secretion (Johansson, et al. 2007; Salehi, et al. 2009). Therefore, VU-induced insulin secretion observed in MIN6 β-cells could be attributed, at least in part, to inhibition of those targets. However, the role of ERG requires the assumption that this K\(^{+}\) channel modulates insulin secretion independently of L-type Ca\(^{2+}\) channels, as inhibition of these channels blocks insulin secretion (Rorsman and Braun 2013). Inhibition of A1 receptors by VU could explain the increased insulin secretion observed in our end-point secretion experiments. Indeed, insulin secretion was assayed 2 hs after treatment, a condition well within the margin of the second phase of insulin secretion. The participation of NKCC1 or KCC3 on VU-stimulated insulin secretion is minimized by the facts that BTD, an NKCC inhibitor also inhibits insulin secretion (Di Fulvio et al. 2014) whereas KCC3 is an inactive cotransporter under isotonic conditions (Melo et al. 2013b).
Interestingly, no effect of VU (75 µM) was observed on GABA-mediated depolarization of vasopressin-secreting neurons where NKCC1 is the predominant mechanism of \([\text{Cl}^-]\) regulation whereas the same dose of VU provoked GABA-induced depolarization in oxytocin-secreting neurons where KCC2 is the main regulator of \([\text{Cl}^-]\) (Haam, et al. 2012).

Although the use of VU cannot be interpreted as KCC2-mediated, the fact that VU increases \(\text{Cl}^-\) content whereas CLP257 reduced \(\text{Cl}^-\) content in \(\beta\)-cells (Fig 19A) and inhibited insulin secretion at all concentrations of glucose tested (Figs 19C-D) altogether, these data suggest the involvement of KCC2 in \(\beta\)-cell hormone release. Indeed, it was reported that CLP257 does not interfere with the activities of other KCCs or NKCC1 (Gagnon et al. 2013). Further, 100 µM CPL257 efficiently and completely restored impaired KCC2 activity in neuronal slides expressing abundant NKCC1 (Gagnon et al. 2013). Nevertheless, the existence of several alternatively spliced variants of each KCC in \(\beta\)-cells cannot discard the possibility of additional targets participating in insulin secretion. Although further studies are needed to determine the role of CLP257 on \([\text{Cl}^-]\) regulation and insulin secretion in \(\beta\)-cells, within the frame of the aforementioned considerations and limitations, the impact of CLP257 on insulin secretion may be interpreted as regulated by KCC2 and therefore, our results suggest that \([\text{Cl}^-]\) and one of its constitutive regulators \(i.e.,\) KCC2 is involved in insulin secretion indicating for the first time that targeting KCC2 expressed in \(\beta\)-cells may have important therapeutic application toward controlling glucose level and improving the prognosis of T2DM.
**Conclusions**

We have presented evidence supporting the notion that KCC2 is not a neuron-specific cotransporter; it is expressed in all mouse tissues tested including the pancreas, more precisely in insulin secreting β-cells. In addition, we provide evidence for the existence of a new alternative variant of KCC2a *i.e.*, KCC2a-S25 expressed most abundantly in extra-neuronal tissues. Furthermore, pharmacological stimulation of KCC2 with recently developed agonists results in decreased Cl⁻ content and impaired insulin secretion *in vitro*. 
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