Western University
Scholarship@Western

Digitized Theses | Digitized Special Collections

2011

CHARACTERISTICS AND FUNCTION OF CONNEXIN43 MUTANTS LINKED TO OCULODENTODIGITAL DYSPLASIA

Robert James Lorentz

Follow this and additional works at: https://ir.lib.uwo.ca/digitizedtheses

Recommended Citation

Lorentz, Robert James, "CHARACTERISTICS AND FUNCTION OF CONNEXIN43 MUTANTS LINKED TO OCULODENTODIGITAL DYSPLASIA" (2011). *Digitized Theses*. 3286.
https://ir.lib.uwo.ca/digitizedtheses/3286

This Thesis is brought to you for free and open access by the Digitized Special Collections at Scholarship@Western. It has been accepted for inclusion in Digitized Theses by an authorized administrator of Scholarship@Western. For more information, please contact wlswadmin@uwo.ca.

CHARACTERISTICS AND FUNCTION OF CONNEXIN43 MUTANTS LINKED TO OCULODENTODIGITAL DYSPLASIA

(Spine title: Cx43 mutants associated with bladder defects)

(Thesis format: Integrated-Article)

By

Robert Lorentz

Graduate Program
in
Pharmacology and Toxicology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

© Robert Lorentz 2011

THE UNIVERSITY OF WESTERN ONTARIO

School of Graduate and Postdoctoral Studies

CERTIFICATE OF EXAMINATION

Supervisor	Examiners
Dr. Dale Laird	Dr. Doug Jones
Supervisory Committee	Dr. Donglin Bai
Dr. Michael Poulter	Dr. Dong Hamilton
Dr. Gerald Kidder	
Dr. Peter Chidiac	

The thesis by

Robert James Lorentz

Entitled:

CHARACTERISTICS AND FUNCTION OF CONNEXIN43 MUTANTS LINKED TO OCULODENTODIGITAL DYSPLASIA

Is accepted in partial fulfillment of the
requirements for the degree of
Master of Science

Date

Chair of the Thesis Examination Board

ABSTRACT:

Oculodentodigital dysplasia (ODDD) is a rare developmental disease that results from any one of over sixty known autosomal-dominant or autosomal-recessive mutations in the gene *GJA1*, which encodes for connexin43 (Cx43). In the current study, we assessed the characteristic of six Cx43 mutants, including two autosomal-dominant mutants which have been associated with bladder defects (D3N and G143S), two autosomal-dominant mutants which have not been associated with bladder defects (G2V and I130T) and two autosomal-recessive mutants (R33X and R76H). Collectively, we revealed no differences in the molecular or Cx43 channel function of the bladder disease –associated or –unassociated mutants suggesting that bladder defects linked to ODDD may be associated with other factors such as aging and/or co-morbidities. We also found that the R76H mutant could form functional channels while the R33X was functionally dead. These studies provide molecular insights into how autosomal dominant or recessive mutations affect Cx43 channel function and how Cx43 function may be linked to disease severity.

Keywords: gap junctions, connexins, Cx43, autosomal-dominant, autosomal-recessive, bladder, mouse model of human disease, GJIC, ODDD, hemichannel

CO-AUTHORSHIPS:

All experiments were performed by Robert Lorentz.

Dr. Cindy Shao provided guidance on performing all techniques employed in this study.

Chapter 2:

Entitled "Characteristics and Functions of Connexin43 Mutants linked to Oculodentodigital Dysplasia with Respect to Bladder Tissue" was written by Robert Lorentz with suggestions from Dr. Dale W. Laird

Chapter 3:

Entitled" Characteristics and Functions of Connexin43 Recessive Mutants linked to Oculodentodigital Dysplasia" was written by Robert Lorentz with suggestions from Dr. Dale W. Laird

DEDICATION:

To my family, for their love and support throughout my education.

ACKNOWLEDGMENTS:

I would like to thank the following people for their contributions to my studies:

- My supervisor, Dr. Dale Laird for his open door policy providing guidance and support not only towards my research but also in developing my writing and presentation skills
- Dr. Cindy Shao for her support and expertise in the laboratory
- My supervisory committee, Dr. Michael Poulter, Dr. Gerald Kidder and Dr. Peter Chidiac for their guidance
- The entire Laird Laboratory and Gap Junction Research Group members for their support and friendship over the past two years

TABLE OF CONTENTS

CERTIFICATE OF EXAMINATION ii
ABSTRACT iiii
CO-AUTHORSHIP iii
DEDICATION iii
ACKNOWLEDGEMENTS vi
TABLE OF CONTENTS viiii
LIST OF FIGURES xi
LIST OF APPENDICES xiiii
LIST OF ABREVATIONS iv
CHAPTER 1: Literature Review 1
1.1 Introduction 2
1.2 Connexins 2
1.3 Connexons, Hemichannels and Gap Junctional Plaques 3
1.4 Channel Diversity 6
1.5 Gap Junction Function 7
1.6 Connexin Mutations and Disease 8
1.6.1 Oculodentodigital Dysplasia 11
1.6.2 ODDD and Bladder Abnormailities 12
1.6.3 ODDD Mutant Mouse Models 3
1.7 Bladder Structure and Function 7
1.7.1 The Urothelium 7
1.7.2 The Lamina Propria 8
1.7.3 The Detrusor 8
1.8 Bladder Abnormalities 19
1.9 Connexins in the Bladder and Their Response to Bladder Syndromes 20
1.9.1 The Urothelium 20
1.9.2 The Lamina Propria 21
1.9.3 The Detrusor 22
1.10 Hypothesis 23
1.11 Objectives 23
1.12 Reference List 24

CHAPTER 2: Characteristics and Function of Connexin43 Mutants Linked to Oculodentodigital Dysplasia with Respect to Bladder Tissue 30
2.0 Abstract 31
2.1 Introduction 33
2.2 Methods 37
2.2.1 Cell Culture 37
2.2.2 Generation of Untagged and GFP-Tagged Cx43 Mutants (G2V, D3N, I130T and G143S) 37
2.2.3 Transient Transfections 38
2.2.4 Immunocytochemistry 38
2.2.5 Microinjection Dye Transfer Assay 39
2.2.6 ATP Release Assay 40
2.2.7 Animals 40
2.2.8 Tissue Harvesting and Preparation 41
2.2.9 Histology 42
2.2.10 Immunofluorescence 43
2.2.11 Western Blot Analysis 43
2.2.12 Statistics 44
2.3 Results 45
2.3.1 Cx43 mutants localize to sites of cell to cell apposition in HeLa and NRK cells 45
2.3.2 Gap junction channels formed by Cx43 mutants are impermeable to Lucifer yellow… 45
2.3.3 Cx43 mutants act as dominant-negatives on the channel function of co-expressed endogenous Cx43 52
2.3.4 The untagged Cx43 mutants, G2V and D3N, facilitate ATP release in Cx43-negative HeLa cells 52
2.3.5 G60S and I130T bladders have similar histology and detrusor thickness as wild-type littermates 55
2.3.6 G60S and I130T bladders have reduced levels of highly phosphorylated Cx43 species 58
2.3.7 G60S mouse bladders have altered Cx43 distribution in the lamina propria and detrusor layers… 61

2.3.8 G60S and I130T bladders have similar Cx26 levels as wild-type littermates 61
2.3.9 Cx26 is localized to an intracellular compartment in the basal urothelium of mutant and wild-type littermates 68
2.4 Discussion 71
2.4.1 Characterization of Cx43 mutants reveal no distinguishable difference between bladder disease -associated and -unassociated Cx43 mutants ... 72
2.4.2 Characterization of Cx43 in the bladders of G60S and I130T mutant mice.... 75
2.4.3 Differential Cx43 phosphorylation and subsequent distribution may disrupt bladder signaling and contractile stimulus propagation in mutant mice. 76
2.4.4 Cx26 unique basal urothelial expression profile may contribute to bladder function. 78
2.4.5 Conclusion. 79
2.5 References 80

CHAPTER 3: Characteristics and Function of Connexin43 Recessive Mutants Linked to Oculodentodigital Dysplasia 85
3.0 Abstract 86
3.1 Introduction 87
3.2 Methods 90
3.2.1 Transient Transfections 90
3.2.2 Immunocytochemistry 90
3.2.3 Microinjection Dye Transfer Assay 91
3.2.4 ATP Release Assay 92
3.2.5 Statistics 92
3.3 Results 93
3.3.1 The Cx43 R76H mutant localized to sites of cell to cell apposition in HeLa and NRK cells..... 93
3.3.2 Gap junction channels formed from the R76H mutant are partially functional 93
3.3.3 The recessive mutants produced similar levels of ATP release as wild-type HeLa and NRK cells 98

3.4 Discussion 101
3.4.1 Characterization of recessive Cx43 mutants reveal R76H mutant contains residual channel function 101
3.4.2 Conclusion 103
3.4 References 104

CHAPTER 4: General Discussion 106
4.1 Summary 107
4.1.1 Chapter 1 - Objective #1: Characterize the localization and function of bladder disease -associated (D3N and G143S) and -unassociated (G2V and I130T) Cx43 mutants when expressed in reference cell models 107
4.1.2 Chapter 1 – Objective #2: Determine the expression and distribution of Cx43 and Cx26 in the bladder of G60S and I130T Cx43 mutant mice and their control littermates 107
4.1.3 Chapter 2 – Objective #1: Characterize the localization and function of two recessive Cx43 mutants (R33X and R76H) when expressed in reference cell models 109
4.2 Contribution of Research 110
4.2.1 ODDD Patients and Bladder Abnormalities 110
4.2.2 Recessive R76H Mutant Retains Residual Gap Junction Channel Function 111
4.3 Future Studies 112
4.3.1 Determine if the Cx43 mutant mice possess a bladder phenotype 112
4.3.2 Use primary cultured smooth muscle and urothelial cells to assess the gap junction channel activity in mutant and wild-type mice 113
4.4 Conclusion 114
4.5 References 115
APPENDIX 118
A1. Animal Protocol 19
A2. Curriculum Vitae 120

LIST OF FIGURES

Figure		Page
1.1	Schematic representations of a connexin, hemichannels and gap junctions	5
1.2	Composite of Cx43 mutations linked to ODDD	10
1.3	Summary of the reported connexin expression in the bladder	15
2.1	Cx43 mutants localized to sites of cell to cell appositions in HeLa cells	47
2.2	Cx43 mutants localize to sites of cell to cell appositions in Cx43-positive NRK cells	49
2.3	Cx43 mutants form functionally compromised channels and act as dominant-negatives to the channel function of co-expressed endogenous Cx43	51
2.4	Cx43 mutants differentially affect ATP release depending on endogenous connexin status	54
2.5	G60S and I130T mouse bladders have similar histology and detrusor thickness as their wild-type littermate controls	57
2.6	G60S and I130T mouse bladders exhibit a reduction in the phosphorylated species of Cx43	60
2.7	G60S mouse bladders have altered Cx43 distribution in the lamina propria, while I130T bladders display similar Cx43 distribution as wild-type littermates	63
2.8	G60S and I130T mice and their wild-type littermates display distinct Cx43 detrusor localizations	65

2.9 G60S and I130T mouse bladders have similar Cx26 levels as wild-type littermates 67

2.10 Cx26 was localized to intracellular compartments of basal urothelial cells of mutant and wild-type mice 70

3.1 The R76H, but not the R33X, mutant forms gap junction-like plaques at sites of cell-cell appositions 95

3.2 The R76H Cx43 mutant can be assembled into gap junction channels and acts as a dominant-negative to the function of wild-type Cx43 97

3.3 Recessive mutants R76H and R33X did not alter ATP release in HeLa or NRK cells 100

APPENDICES

Page

A1. University of Western Ontario Animal Care and Use Protocol Approval 119

A2. Curriculum Vitae 120

LIST OF ABREVATIONS

AT	Amino Terminus
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
bFGF	Basic Fibroblast Growth Factor
BOO	Bladder Outlet Obstruction
BSA	Bovine Serum Albumin
cDNA	Complementary Deoxyribonucleic Acid
CL	Cytoplasmic Loop
Cx	Connexin
DCF	Divalent-Cation-Free
ddH_2O	Double Distilled Water
DMEM	Dulbecco's Minimal Essential Medium
EL1	Extracellular Loop 1
EL2	Extracellular Loop 2
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GFP	Green Fluorescent Protein
GJIC	Gap Junctional Intercellular Communication
HBSS	Hank's Balanced Salt Solution
HeLa	Human Cervical Cancer (Henrietta Lacks)

IL	Intercellular Loop
kD	Kilo-Dalton
NRK	Normal Rat Kidney
NO	Nitric Oxide
ODDD	Oculodentodigital Dysplasia
OAB	Overactive Bladder
P0, P1, P2	Phosphorylated Species of Connexin43
P2X	Purinoreceptor Family
PBS	Phosphate-Buffered Saline
s.e.m	Standard Error of the Mean
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

CHAPTER 1

Literature Review

1.1 Introduction

The ability of cells to interact with their neighbours is an important cellular feature with implications in many processes from development to normal tissue function (Dahl et al. 1995, Rossello and Kohn 2010). One mechanism in which adjacent cells are able to interact is through gap junctional intercellular communication (GJIC). Almost every cell in the body expresses the protein(s) required for the production of gap junction channels. The presence of gap junction proteins enables cells of an organ to form a syncytium through GJIC, allowing the organ to function as a cohesive unit. This specialized form of communication is highly regulated and can be quickly altered depending on the cells physiological needs. Gap junction channels allow the movement of various cellular components; such as second messengers, ions, metabolites and nutrients, between connected cells (Alexander and Goldberg 2003, Veenstra 1996). Therefore, as a result of the ubiquitous use of GJIC, it may come as no surprise that an alteration in the ability of cells to directly communicate can produce a number of human diseases and pathologies (Cronier et al.. 2009).

1.2 Connexins

Vertebrate gap junctions are formed by specialized integral membrane proteins called connexins (Cxs) (Goodenough et al.. 1996). There are currently 21 human and 20 mouse Cx isoforms which range from 23 to 62 kilo-Daltons (kD), whose names are largely based on the connexins molecular weight (Eiberger et al. 2001, Sohl and Willecke 2003). Connexins are categorized based on sequence homology and cytoplasmic tail length (Berthoud et al. 2004). Each connexin member shares a similar topology,

consisting of four transmembrane domains (TM 1 – 4), two extracellular loops (EL1 and EL2), an intracellular loop (IL) and cytoplasmically exposed amino and carboxyl termini (Figure 1.1 A) (Maeda et al. 2009, Sohl and Willecke 2004).

Connexins are temporally and spacially regulated in human physiology. For example, keratinocytes are known to express Cx26, Cx30, Cx30.3, Cx31, Cx31.1 and Cx43, at different stages of differentiation (Salomon et al. 1994). On the other hand, some tissues continuously express only a few connexins. The liver, for example, is known only to expresses Cx26 and Cx32 (Paul 1986). The differential tissue expression of connexins suggests that the properties associated with each connexin are unique and specific to the cells expressing them.

1.3 Connexons, Hemichannels and Gap Junctional Plaques

Six connexins oligomerize within the Golgi apparatus to form a connexon (Figure 1.1 B) which is delivered to the plasma membrane via transport vesicles (Lauf et al. 2002, Musil and Goodenough 1993). At the plasma membrane, the connexon may exist as a single undocked functional unit known as a hemichannel, or bind to a connexon from an adjacent cell to form a functional gap junction channel (Figure 1.1 C). In both cases, a 1.5 nm pore is formed allowing for the selective passage of molecules generally less than 1 kD in size (Alexander and Goldberg 2003, Veenstra 1996). Connexons and hemichannels were once thought to act exclusively as an intermediate step in gap junction channel formation (Li et al. 1996). More recent reports suggest that hemichannels, which are predominantly in a closed state, have the ability to open in response to mechanical stress, metabolic stress and low extracellular calcium

Figure 1.1 Schematic representations of a connexin, hemichannels and gap junctions. Each connexin consists of four transmembrane domains (TM 1-4), two extracellular loops (EL1 and EL2), an intracellular loop (IL) and cytoplasmically-exposed amino and carboxyl termini (A). Six connexin proteins oligomerize to form a connexon which is delivered to the plasma membrane (B). Within the plasma membrane, connexons can either dock with a connexon from an adjacent cell forming a gap junction channel, or exist as a hemichannel (C). Tens to thousands of gap junction channels coalesce to form a gap junction plaque (C)

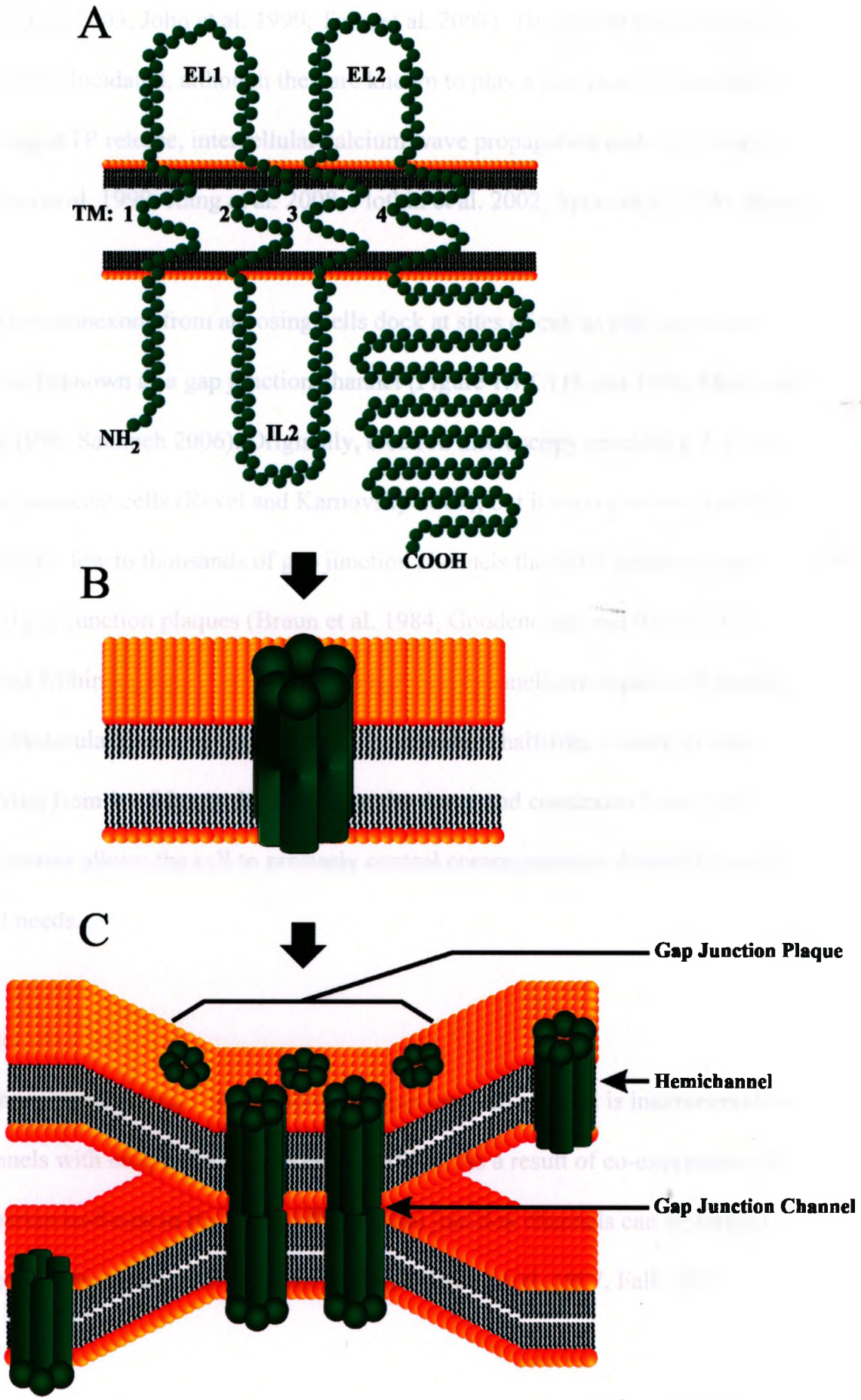
A
EL1
EL2
TM: 1
2
3
4
NH2
IL2
COOH
B
C
Gap Junction Plaque
Hemichannel
Gap Junction Channel

Figure 1.1

(Jiang and Cherian 2003, John et al. 1999, Tong et al. 2007). The role of hemichannels *in vivo* has yet to be elucidated, although they are known to play a part in several cellular events including ATP release, intercellular calcium wave propagation and regulation of cell death (John et al. 1999, Kang et al. 2008, Plotkin et al. 2002, Spray et al. 2006, Stout et al. 2002).

When two connexons from apposing cells dock at sites of cell to cell apposition they form what is known as a gap junction channel (Figure 1.1 C) (Laird 1996, Musil and Goodenough 1990, Salameh 2006). Originally, electron microscopy revealed a 2-3 nm space between adjacent cells (Revel and Karnovsky 1967), but it is now known that this space consists of a few to thousands of gap junction channels that have coalesced into tightly packed gap junction plaques (Braun et al. 1984, Goodenough and Revel 1970). Once contained within a plaque, individual gap junction channels are capable of opening and allowing molecular passage. Gap junctions have a short half-life, relative to other proteins, varying from 1 – 5 hours depending on the tissue and connexin (Laird 2005). This quick turnover allows the cell to precisely control communication depending on it physiological needs.

1.4 Channel Diversity

The connexin composition of an individual gap junction channel is instrumental in creating channels with unique features (Veenstra 1996). As a result of co-expression of multiple connexin isoforms in the same cell type, gap junction channels can be formed through the co-oligomerization of different isoforms (Brink et al. 1997, Falk 2000).

However, not all connexin isoforms can co-oligomerize, allowing for further selectivity of the mixed connexin channels that can be assembled (Falk 2000). Ultimately, various hexameric combinations result in channel properties unique to the constituents of that gap junction channel (Cottrell and Burt 2005, Falk 2000, Valiunas et al. 2000). Connexons composed of one connexin isoform are termed homomeric; connexons composed of more than one connexin isoform are termed heteromeric. Further increasing the complexity and specificity of gap junction channels, the connexin composition of two docked connexons may be different. Homotypic channels are composed of identical connexons from apposing cells; whereas heterotypic channels are composed of two different connexons from apposing cells (Rackauskas et al. 2010).

1.5 Gap Junctional Function

Tens to thousands of gap junction channels coalesce into gap junction plaques at sites of cell to cell appositions (Goodenough and Revel 1970). The movement of a connexin within the plasma membrane is associated with its interaction with various proteins including microtubules and microfilaments (Laird 2010). The movement of a single Cx43-based gap junction channel into a gap junction plaque is associated with a change in the Cx43 phosphorylation state, allowing the channel to become functionally active (Musil and Goodenough 1991, Solan and Lampe 2007). The C-terminus of Cx43 has many consensus sites for protein phosphorylation. Typically, Cx43 exists as three distinct species that are distinguished as P0, P1 and P2, which is attributed to its increase states of phosphorylation (Solan and Lampe 2009). A reduction in the most

phosphorylated species (P2) has been shown to be associated with a reduction in GJIC (Koo et al. 1997).

Gap junction channels are not only regulated by phosphorylation, but also by cellular factors such as pH, voltage, and calcium, all of which affect channel function by regulating the extent by which the channel is either open or closed (Bukauskas and Weingart 1994, Peracchia and Wang 1997, Spray et al. 1984). For example, dual whole-cell patch clamp has revealed that an alteration in the transjunctional voltage results in a voltage-dependent gating effect, a property that has been localized to the amino terminus of the connexin (Bukauskas and Peracchia 1997, Kyle et al. 2009). In addition, intracellular [H^+] and [Ca^{2+}] are known to alter channel conductivity (Peracchia 2004). Intracellular pH is thought to regulate the interaction between the carboxyl terminus and channel forming amino acids, in a particle-receptor interaction (Morley et al. 1996). Also, the intracellular loop of Cx43 contains a calmodulin binding motif, located at residues 136-158, which may play a role in how the channel is gated (Zhou et al. 2007).

1.6 Connexin Mutations and Disease

Mutations in genes encoding various connexins are known to cause at least eight distinct human diseases, which range in severity (Laird 2006). Several deafness and skin diseases have been associated with Cx26, Cx30, Cx30.1, Cx31 and Cx43 mutants (Laird 2008, Lee and White 2009, Scott et al. 2011, White and Paul 1999), while mutations in the gene encoding Cx32 have been linked to X-linked Charcot-Marie Tooth disease, associated with demyelination of peripheral nerves (Bergoffen et al. 1993, White and Paul 1999).

Figure 1.2 Composite of Cx43 mutations linked to ODDD. To date, over 60 mutations spanning the gene encoding Cx43 have been linked to ODDD. The majority of these mutations result in single amino acid substitutions (red balls), while two frame shift (grey balls), one deletion (orange ball) and two recessive mutants (purple balls) have also been linked to ODDD. ODDD mutants that have been reported to cause bladder impairments are denoted in yellow while select mutants that have not been linked to bladder conditions are noted in orange. The green balls at positions 60 and 130 represent two mutant mouse models of ODDD, G60S and I130T, respectively

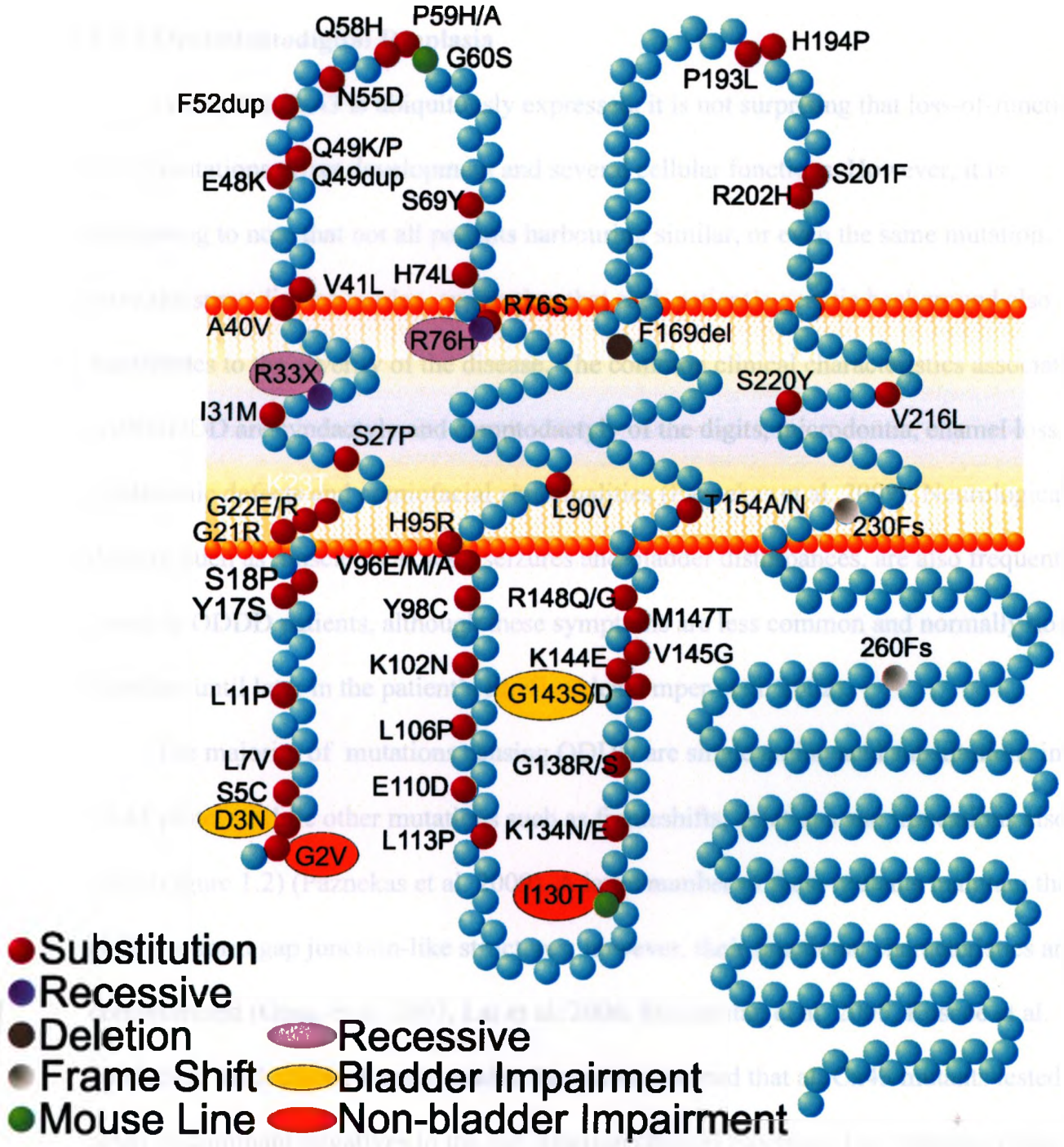
Q58H
P59H/A
G60S
N55D
F52dup
Q49K/P
E48K
Q49dup
S69Y
V41L
H74L
A40V
R76S
R76H
R33X
I31M
S27P
G22E/R
G21R
H95R
V96E/M/A
S18P
Y17S
Y98C
K102N
L11P
L106P
L7V
S5C
D3N
E110D
G2V
L113P
H194P
P193L
S201F
R202H
F169del
S220Y
V216L
L90V
T154A/N
230Fs
R148Q/G
M147T
K144E
V145G
260Fs
G143S/D
G138R/S
K134N/E
I130T
Substitution
Recessive
Deletion
Frame Shift
Mouse Line
Recessive
Bladder Impairment
Non-bladder Impairment

Figure 1.2

Currently, over 60 mutations in the gene encoding Cx43 have been linked to oculodentodigital dysplasia (ODDD) (Figure 1.2) (Paznekas et al. 2009).

1.6.1 Oculodentodigital Dysplasia

Given that Cx43 is ubiquitously expressed, it is not surprising that loss-of-function Cx43 mutations affect development and several cellular functions. However, it is interesting to note that not all patients harbouring similar, or even the same mutation, have the same disease burden, suggesting that each patient's genetic background also contributes to the severity of the disease. The common clinical characteristics associated with ODDD are syndactyly and camptodactyly of the digits, microdontia, enamel loss, ophthalmic defects and craniofacial abnormalities (Paznekas et al. 2009). Neurological defects, such as muscle weakness, seizures and bladder disturbances, are also frequently found in ODDD patients, although these symptoms are less common and normally do not manifest until later in the patient's life (Loddenkemper et al. 2002).

The majority of mutations causing ODDD are single amino acid substitutions in the Cx43 protein, while other mutations such as frameshifts, deletions and truncations also exist (Figure 1.2) (Paznekas et al. 2009). A large number of these mutants maintain the ability to form gap junction-like structures; however, their functional characteristics are compromised (Gong et al. 2007, Lai et al. 2006, McLachlan et al. 2005, Roscoe et al. 2005, Seki et al. 2004). Further studies have also reported that all Cx43 mutants tested acted as dominant-negatives to the gap junction channel function of co expressed wild-type Cx43 (Gong et al. 2007, McLachlan et al. 2005, Roscoe et al. 2005), suggesting that

despite retaining one copy of wild-type Cx43, the gap junctional intercellular communication is likely severely reduced in ODDD patients.

Mutations associated with ODDD are generally autosomal-dominant; however, there have been a few reported autosomal-recessive cases, including the mutants R76H and R33X (Figure 1.2) (Paznekas et al. 2003, Pizzuti et al. 2004, Richardson et al. 2006). The current information on the R76H and R33X mutants and patients harbouring them is limited to genetic screening and a description of the patient's health status (Pizzuti et al. 2004, Richardson et al. 2006). Studies employing Cx43 knock-out mice have revealed that Cx43 is required for normal tissue development and survival, and that the loss of Cx43 is lethal at birth (Flenniken et al. 2005, Manias et al. 2008, Reaume et al. 1995, Toth et al. 2010). In humans, the R33X autosomal recessive mutant in particular is effectively a Cx43 knockout as only 33 amino acids of Cx43 would be translated and no Cx43 channels would form. Patients harbouring the R33X mutant displays very severe clinical symptoms of ODDD (Richardson et al. 2006), and life expectancy is thought to be short. On the other hand, the R76H mutant may have residual gap junction channel function that could act to sustain life, although this has yet to be tested.

1.6.2 ODDD and Bladder Abnormalities

Along with the common clinical characteristics associated with ODDD, several less common syndromes have also been linked to Cx43 mutants. Neurological symptoms have been noted in ~30% of ODDD families, and these are known to progressively affect the lower half of the body (Loddenkemper et al. 2002, Paznekas et al. 2009). Interestingly, incontinence has been reported in ~12% of 177 ODDD patients studied,

although it should be noted that these symptoms generally do not present themselves until later in life (Paznekas et al. 2009). Therefore, it is possible that incontinence is a much larger problem and under-reported as many later life disease symptoms may not be archived as ODDD-associated diseases. Neurological symptoms have been proposed to be at the root of the majority of incontinence reports (Loddenkemper et al. 2002, Paznekas et al. 2009); however, this has not been tested or investigated and myogenic defects may also exist in these patients. As a result of Cx43 expression throughout the different layers of the bladder (See 1.1.10) (Figure 1.3), it is thought that the resulting bladder abnormalities may also be the result of altered function in any one of the three layers associated with the bladder.

1.6.3 ODDD Mutant Mouse Models

To further study ODDD and the role of Cx43, several disease-linked Cx43 mutant mice have been generated. The present study used $GJA1^{Jrt/+}$ (Flenniken et al. 2005) and the $GJA1^{I130T/+}$ (Kalcheva et al. 2007) (referred to as the G60S and I130T mice respectively) heterozygous mutant mice that carry one copy of the Cx43 mutant and one copy of wild-type Cx43. As assessed in several distinct tissues, including heart, skin, ovary and teeth, both mice exhibit reduced Cx43 levels and Cx43 function (Flenniken et al. 2005, Kalcheva et al. 2007, Langlois et al. 2007, Toth et al. 2010). The mouse harbouring the G60S mutant was generated at the Centre for Modeling Human Diseases (Toronto, Canada), in a N-ethyl-N-nitrosourea mutagenesis screen. It was made available to the UWO Gap Junction Group by Dr. Janet Rossant. This mutation lies in the first extracellular domain, a domain known for its importance in connexon docking

Figure 1.3 Summary of the reported connexin expression in the bladder. Hematoxylin and eosin stained bladder sections revealed the urothelium, the lamina propria, and the detrusor layers of the bladder. Higher magnification images revealed that the urothelium is composed of transitional epithelium while the lamina propria is composed of loose fibroelastic connective tissue, and the detrusor is composed of smooth muscle cell bundles. Previous studies have reported that the urothelium expresses Cx26 and Cx43, the lamina propria expresses Cx43, while the detrusor expresses Cx40, Cx43 and Cx45.

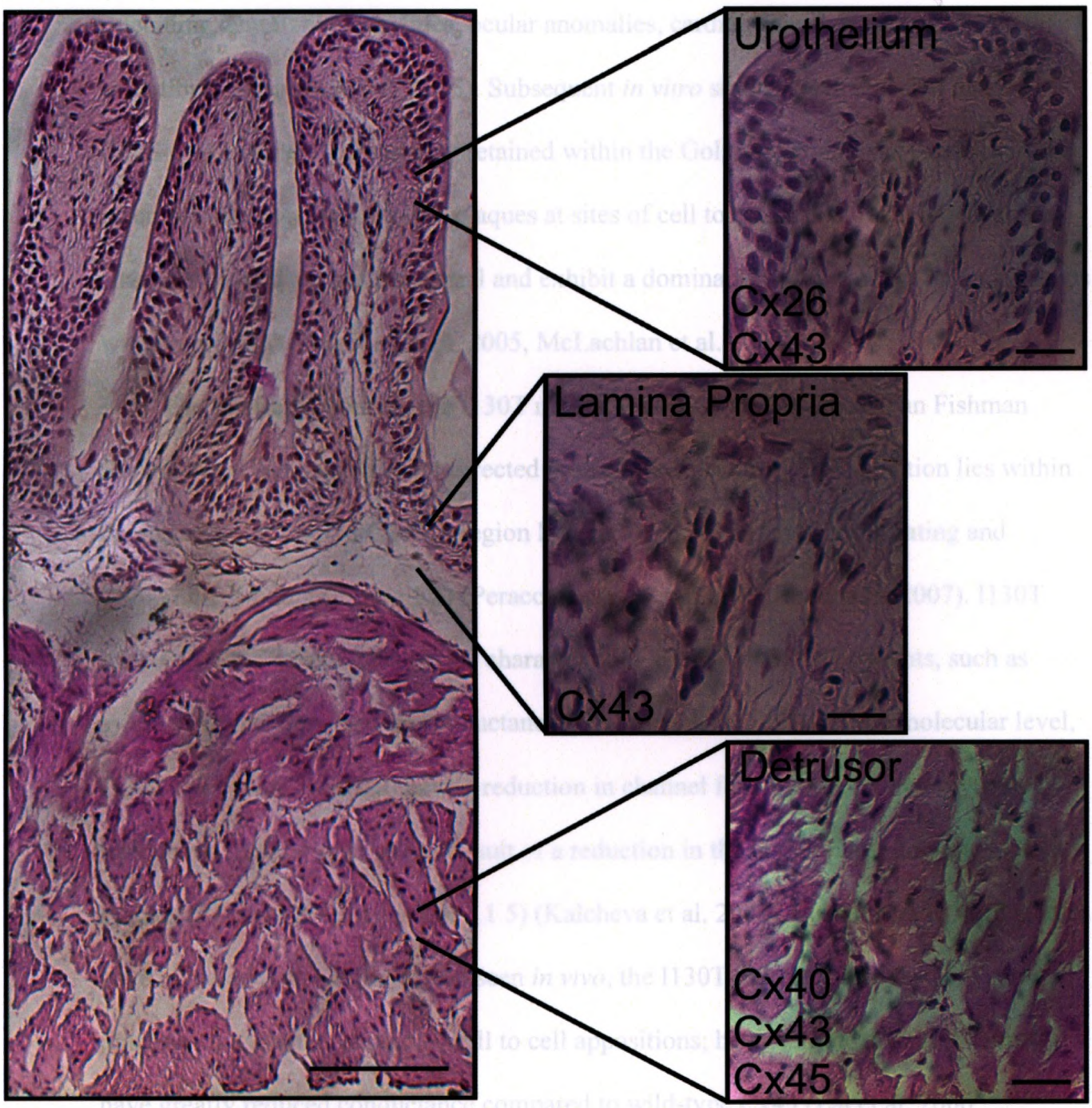
Urothelium
Cx26
Cx43
Lamina Propria
Cx43
Detrusor
Cx40
Cx43
Cx45

Figure 1.3

(Figure 1.2) (John and Revel 1991). While this mutation has yet to be reported in any human ODDD patients, the mice share phenotypic characteristics similar to ODDD patients. G60S mice exhibit syndactyly of the forelimb and hindlimb digits, craniofacial (including dental) abnormalities, ocular anomalies, cardiac disturbances and skeletal alterations (Flenniken et al. 2005). Subsequent *in vitro* studies have revealed that the G60S Cx43 mutant is frequently retained within the Golgi apparatus, but can escape and assemble into gap junction-like plaques at sites of cell to cell appositions; however, the channels formed are not functional and exhibit a dominant-negative effect on endogenous wild-type Cx43 (Flenniken et al. 2005, McLachlan et al. 2005).

The mouse harbouring the I130T mutant was generated by Dr. Glenn Fishman (New York, USA), using a site-directed mutagenesis protocol. This mutation lies within the intracellular loop of Cx43, a region known for its importance in pH gating and calmodulin binding (Figure 1.2) (Peracchia and Wang 1997, Zhou et al. 2007). I130T mice exhibited several phenotypic characteristics similar to ODDD patients, such as syndactyly and slow cardiac conductance (Kalcheva et al. 2007). At the molecular level, I130T mice were shown to have a reduction in channel function in cultured myocytes, which was thought to arise as a result of a reduction in the higher phosphorylated Cx43 species (P1 and P2 – see section 1.1.5) (Kalcheva et al. 2007). Subsequent in vitro studies have revealed that, similar to that seen *in vivo*, the I130T mutant assembles into gap junction-like plaques at sites of cell to cell appositions; however, the channels formed have greatly reduced conductance compared to wild-type Cx43 (Lai et al. 2006, Shibayama et al. 2005).

1.7 Bladder Structure and Function

The bladder is a hollow semicircular organ located in the pelvic region of the abdomen. Urine enters the bladder through two ureters, one extending from each kidney, that insert into the body of the bladder. Following bladder contractions, urine exits the bladder through the urethra, extending out of the base of the bladder. Histologically, the bladder is composed of three distinct layers; the urothelium, the lamina propria and the detrusor, each responsible for a specific function (Figure 1.3).

1.7.1 The Urothelium

The primary function of the urothelium was originally thought to be a passive barrier that prevents the entry of pathogens, but selectively controls the passage of water, ions and other molecules to the underlying tissue (Apodaca 2004). Composed of transitional epithelial tissue, the urothelium contains basal cells, intermediate cells and umbrella cells (Apodaca 2004, Birder and de Groat 2007). In order to maintain a passive barrier, urothelial cells are connected via tight junctions and contain a unique lipid-protein composition in the apical membrane of umbrella cells. (Lewis 2000). When the barrier is compromised, substances can pass into the underlying tissue causing urgency, frequency and pain (Birder 2001).

Recent studies have shown that the urothelium is more than just a passive barrier and actually plays an important role in sensing and responding to mechanical and chemical stimuli (Apodaca 2004, Birder and de Groat 2007, de Groat 2004). Several molecules are known to be released from the urothelium following physical or chemical stimulation, including ATP, NO, acetylcholine and cytokines. As well, receptors for

many molecules, such as purines, norepinephrine, acetylcholine and Na^+ are known to be expressed in the urothelium (Birder 2001). In particular, ATP release is thought to signal adjacent urothelial cells and/ or bladder nerves through P2X receptor stimulation within the underlying lamina propria; therefore, acting in both an autocrine and paracrine fashion (Apodaca 2004, Birder 2001, Cockayne et al. 2000)

1.7.2 The Lamina Propria

The lamina propria consists of loose fibroelastic connective tissue containing nerves, blood capillaries, a small amount of smooth muscle, and fibroblasts. There is a debate as to whether the fibroblasts are actually myofibroblasts or even interstitial cells, as seen in other tissues such as the stomach, prostate, liver and lungs (Schurch et al. 1998). Myofibroblasts contain features similar to both fibroblasts and smooth muscle cells (Wiseman et al. 2003). Their close proximity to afferent nerves and their ability to release and become activated by ATP suggest that these cells act as signal transducers within the bladder (Birder and de Groat 2007, Fry et al. 2007, McCloskey 2010, Wu et al. 2004).

1.7.3 The Detrusor

The detrusor is composed of smooth muscle cells sequestered into separate muscle bundles rich in nerve innervations. This layer has the function of maintaining bladder shape during filling and to providing the driving force behind bladder voiding (Andersson 1999, Kanai 2011). Shape is maintained via small tonic contractions of separate muscle bundles around the bladder. Voiding, however, is the result of a coordinated contraction

of the entire detrusor, made possible via the innervation of afferent and efferent nerves, the urothelium, and cells of the lamina propria (Kanai 2011, Neuhaus et al. 2005).

1.8 Bladder Abnormalities

The bladder functions as a storage and emptying vessel for urine. Disruption of either function may result in bladder disease or malfunction including incontinence, residual urine retention and pain (Christ et al. 2003). The most common type of bladder abnormality is detrusor overactivity, which is the result of an increase in smooth muscle contraction. However, the physiological mechanism that initiates this overactivity is not clearly understood (Christ et al. 2003). Controversy in this field surrounds the mechanistic origin of bladder overactivity and determination of whether or not the stimulus is of neuronal or myogenic origin (Christ et al. 2003, Haefliger et al. 2002). There are two main forms of incontinence: stress, which is the result of an increased abdominal pressure, and urge, which is involuntary leakage accompanied by, or immediately followed by, urgency. Urge can be further broken down into myogenic, thought to be of muscular origin, and neurological, thought to be of neuronal origin. (Christ et al. 2003). Although neurological defects are thought to represent the majority of abnormalities, there is a growing body of evidence to suggest a myogenic origin for at least some forms of incontinence (Haferkamp et al. 2004).

Bladder outlet obstructions (BOO), although not used in this study, are thought to be one of the best models for studying bladder abnormalities *in vivo*. In men, this condition usually arises as a result of benign prostatic hyperplasia, effectively reducing the diameter of the urethra (Miyazato et al. 2009, Mori et al. 2005). BOO models result in

phenotypes similar to those reported in humans, such as increase urine retention, detrusor hypertrophy and spastic bladder contractions (Imamura et al. 2009, Miyazato et al. 2009, Mori et al. 2005). It is thought that altered coupling of smooth muscle cells may be the cause, resulting in increased sensitivity to acetylcholine, or changes in how cells communicate (Imamura et al. 2009).

1.9 Connexins in the Bladder and Their Response to Bladder Syndromes

Various connexins are known to be expressed within the bladder, although controversy exists as to where they are localized. Overall, Cx26, Cx40, Cx43, and Cx45 have all been localized to some layer of the human bladder, using both immunohistochemistry and immunoblotting techniques (Haefliger et al. 2002, John et al. 2003a).

1.9.1 The Urothelium

The majority of reports suggest that Cx26 is the primary connexin localized in the urothelium (Gee et al. 2003, Grossman et al. 1994, Haefliger et al. 2002, Ikeda et al. 2007), although its function has yet to be elucidated. Cx26 is generally observed as punctate structures within the urothelium (Haefliger et al. 2002, Ikeda et al. 2007); however, one study has localized Cx26 to a more cytoplasmic profile in samples from bladder tumors (Gee et al. 2003). Several reports have indicated that Cx43 may also be localized to the urothelial layer in both humans and rats (Grossman et al. 1994, Haefliger et al. 2002); however, other reports have suggested that Cx43 is only localized to the

lamina propria and detrusor layers (see sections 1.1.10.2 and 1.1.10.3, respectively) (Fry et al. 2007, Hashitani et al. 2004, Neuhaus et al. 2005, Sui et al. 2002, Wang et al. 2006).

Cx26 levels have been reported to increase as a result of bladder dilation (filling), followed by a reduction after voiding in rat models of bladder outlet obstruction (Haefliger et al. 2002). Therefore, Cx26 within the urothelium is thought to be important in bladder signaling and possibly in bladder malfunction through various mechanisms (Apodaca 2004, Birder and de Groat 2007, Imamura et al. 2009, Sui et al. 2002).

1.9.2 The Lamina Propria

Currently, Cx43 is the only connexin that has been localized to the lamina propria in both human and rat bladders (Fry et al. 2007, Neuhaus et al. 2005, Sui et al. 2002). Cx43 forms punctate structures at sites of cell to cell apposition throughout the lamina propria in vimentin-positive cells, indicative of fibroblasts (Neuhaus et al. 2007, Sui et al. 2002). However, others have suggested that these same cells may in fact be myofibroblasts (Fry et al. 2007, Sui et al. 2002, Wiseman et al. 2003), which share characteristics of both fibroblasts and smooth muscle cells (Hinz 2010). Cx43 has been proposed to form a functional syncytium within the lamina propria, aiding in bladder contraction during voiding (Neuhaus et al. 2005, Sui et al. 2002).

There have been no reports of Cx43 changing within the lamina propria following bladder outlet obstructions. However, it is nearly impossible to separate this layer from the surrounding urothelium and detrusor layers which may hinder quantification of Cx43 levels in the lamina propria. It is still possible that an alteration in the amount of Cx43, or changes in Cx43 spatial localization, may be associated with some bladder malfunction.

Interestingly, basic fibroblast growth factor (bFGF) has been shown to be released from the urothelium in bladder outlet obstruction models (Imamura et al. 2009). Therefore, bFGF, which is known to increase Cx43 expression (Doble and Kardami 1995), may affect the lamina propria, altering its connexin status, resulting in a bladder malfunction.

1.9.3 The Detrusor

Various studies have indicated that Cx40, Cx43 and Cx45 are the only connexins present in the detrusor layer of human and rat bladders, although they are differentially localized within this layer (Hashitani et al. 2004, John et al. 2003b, Sui et al. 2003, Wang et al. 2006). Cx40 is thought to reside in bladder vasculature (Hashitani et al. 2004, John et al. 2003b), and therefore may not play a role in bladder function. Cx43 is localized to the border of smooth muscle cell bundles, suggesting a role in the propagation of Ca^{2+} wave and other chemical signals from one muscle bundle to another (Hashitani et al. 2004, Wang et al. 2006). Conversely, Cx45 is localized to the plasma membrane of smooth muscle cells, within the muscle bundles (John et al. 2003b, Sui et al. 2003). Both findings suggest that these connexins work together to form a syncytium, allowing for uniform bladder contractions. Upon BOO this layer has been reported to exhibit the most significant change in connexin status. Detrusor-localized Cx43 has been shown to increase in all models of BOO, suggesting it is critically important in the normal function of this layer (Christ et al. 2003, Imamura et al. 2009, Li et al. 2007, Mori et al. 2005).

1.10 Hypotheses

Chapter 2:

We hypothesized that both bladder disease –associated and –unassociated ODDD Cx43 mutants will disrupt gap junctional intercellular communication and hemichannel properties within reference cell lines. As well, the Cx43 localization and expression profile will be altered in the bladders of G60S and I130T Cx43 mutant mice.

Chapter 3:

We hypothesized that ODDD-associated recessive Cx43 mutants (R33X and R76H) are loss-of-function mutants.

1.11 Objectives

Chapter 2:

1. Characterize the localization and function of bladder disease –associated (D3N and G143S) and –unassociated (G2V and I130T) Cx43 mutants when expressed in reference cell models.
2. Determine the expression and distribution of Cx43 and Cx26 in the bladder of G60S and I130T Cx43 mutant mice and their control littermates.

Chapter 3:

1. Characterize the localization and function of two recessive Cx43 mutants (R33X and R76H) when expressed in reference cell models.

1.12 Reference List

Alexander DB, Goldberg GS (2003). Transfer of biologically important molecules between cells through gap junction channels. *Curr Med Chem* 10: 2045-2058.

Andersson KE (1999). Changes in bladder tone during filling: pharmacological aspects. *Scand J Urol Nephrol Suppl* 201: 67-72; discussion 76-99.

Apodaca G (2004). The uroepithelium: Not just a passive barrier. *Traffic* 5: 117-128.

Bergoffen J, Scherer SS, Wang S, Scott MO, Bone LJ, Paul DL *et al.* (1993). Connexin mutations in X-linked Charcot-Marie-Tooth disease. *Science* 262: 2039-2042.

Berthoud VM, Minogue PJ, Laing JG, Beyer EC (2004). Pathways for degradation of connexins and gap junctions. *Cardiovasc Res* 62: 256-256.

Birder LA (2001). Involvement of the urinary bladder urothelium in signaling in the lower urinary tract. *Proceedings of the Western Pharmacology Society* 44: 85-86

Birder LA, de Groat WC (2007). Mechanisms of Disease: involvement of the urothelium in bladder dysfunction. *Nature Clinical Practice Urology* 4: 46-54.

Braun J, Abney JR, Owicki JC (1984). How a gap junction maintains its structure. *Nature* 310: 316-318.

Brink PR, Cronin K, Banach K, Peterson E, Westphale EM, Seul KH *et al.* (1997). Evidence for heteromeric gap junction channels formed from rat connexin43 and human connexin37. *Am J Physiol* 273: C1386-1396.

Bukauskas FF, Weingart R (1994). Voltage-dependent gating of single gap junction channels in an insect cell line. *Biophys J* 67: 613-625.

Bukauskas FF, Peracchia C (1997). Two distinct gating mechanisms in gap junction channels: CO2-sensitive and voltage-sensitive. *Biophys J* 72: 2137-2142.

Christ GJ, Day NS, Day M, Zhao W, Persson K, Pandita RK *et al.* (2003). Increased connexin43-mediated intercellular communication in a rat model of bladder overactivity in vivo. *Am J Physiol Regul Integr Comp Physiol* 284: R1241-1248.

Cockayne DA, Hamilton SG, Zhu QM, Dunn PM, Zhong Y, Novakovic S *et al.* (2000). Urinary bladder hyporeflexia and reduced pain-related behaviour in P2X3-deficient mice. *Nature* 407: 1011-1015.

Cottrell GT, Burt JM (2005). Functional consequences of heterogeneous gap junction channel formation and its influence in health and disease. *Biochim Biophys Acta* 1711: 126-141.

Cronier L, Crespin S, Strale PO, Defamie N, Mesnil M (2009). Gap junctions and cancer: new functions for an old story. *Antioxid Redox Signal* 11: 323-338.

Dahl E, Winterhager E, Traub O, Willecke K (1995). Expression of gap junction genes, connexin40 and connexin43, during fetal mouse development. *Anat Embryol (Berl)* 191: 267-278.

de Groat WC (2004). The urothelium in overactive bladder: Passive bystander or active participant? *Urology* 64: 7-11.

Doble BW, Kardami E (1995). Basic fibroblast growth factor stimulates connexin-43 expression and intercellular communication of cardiac fibroblasts. *Mol Cell Biochem* 143: 81-87.

Eiberger J, Degen J, Romualdi A, Deutsch U, Willecke K, Sohl G (2001). Connexin genes in the mouse and human genome. *Cell Commun Adhes* 8: 163-165.

Falk MM (2000). Biosynthesis and structural composition of gap junction intercellular membrane channels. *Eur J Cell Biol* 79: 564-574.

Flenniken AM, Osborne LR, Anderson N, Ciliberti N, Fleming C, Gittens JE *et al.* (2005). A Gja1 missense mutation in a mouse model of oculodentodigital dysplasia. *Development* 132: 4375-4386.

Fry CH, Sui GP, Kanai AJ, Wu C (2007). The function of suburothelial myofibroblasts in the bladder. *Neurourol Urodyn* 26: 914-919.

Gee J, Tanaka M, Grossman HB (2003). Connexin 26 is abnormally expressed in bladder cancer. *J Urol* 169: 1135-1137.

Gong XQ, Shao Q, Langlois S, Bai D, Laird DW (2007). Differential potency of dominant negative connexin43 mutants in oculodentodigital dysplasia. *J Biol Chem* 282: 19190-19202.

Goodenough DA, Revel JP (1970). A fine structural analysis of intercellular junctions in the mouse liver. *J Cell Biol* 45: 272-290.

Goodenough DA, Goliger JA, Paul DL (1996). Connexins, connexons, and intercellular communication. *Annu Rev Biochem* 65: 475-502.

Grossman HB, Liebert M, Lee IW, Lee SW (1994). Decreased connexin expression and intercellular communication in human bladder cancer cells. *Cancer Res* 54: 3062-3065.

Haefliger JA, Tissieres P, Tawadros T, Formenton A, Beny JL, Nicod P *et al.* (2002). Connexins 43 and 26 are differentially increased after rat bladder outlet obstruction. *Exp Cell Res* 274: 216-225.

Haferkamp A, Mundhenk J, Bastian PJ, Reitz A, Dorsam J, Pannek J *et al.* (2004). Increased expression of connexin 43 in the overactive neurogenic detrusor. *Eur Urol* 46: 799-805.

Hashitani H, Yanai Y, Suzuki H (2004). Role of interstitial cells and gap junctions in the transmission of spontaneous Ca2+ signals in detrusor smooth muscles of the guinea-pig urinary bladder. *J Physiol* 559: 567-581.

Hinz B (2010). The myofibroblast: paradigm for a mechanically active cell. *J Biomech* 43: 146-155.

Ikeda Y, Fry C, Hayashi F, Stolz D, Griffiths D, Kanai A (2007). Role of gap junctions in spontaneous activity of the rat bladder. *Am J Physiol Renal Physiol* 293: F1018-1025.

Imamura M, Negoro H, Kanematsu A, Yamamoto S, Kimura Y, Nagane K *et al.* (2009). Basic fibroblast growth factor causes urinary bladder overactivity through gap junction generation in the smooth muscle. *Am J Physiol Renal Physiol* 297: F46-54.

Jiang JX, Cherian PP (2003). Hemichannels formed by connexin 43 play an important role in the release of prostaglandin E(2) by osteocytes in response to mechanical strain. *Cell Commun Adhes* 10: 259-264.

John H, Wang X, Hauri D, Maake C (2003a). [Gap junctions in the human urinary bladder]. *Aktuelle Urol* 34: 328-332.

John H, Wang X, Wehrli E, Hauri D, Maake C (2003b). Evidence of gap junctions in the stable nonobstructed human bladder. *J Urol* 169: 745-749.

John SA, Revel JP (1991). Connexon integrity is maintained by non-covalent bonds: intramolecular disulfide bonds link the extracellular domains in rat connexin-43. *Biochem Biophys Res Commun* 178: 1312-1318.

John SA, Kondo R, Wang SY, Goldhaber JI, Weiss JN (1999). Connexin-43 hemichannels opened by metabolic inhibition. *J Biol Chem* 274: 236-240.

Kalcheva N, Qu J, Sandeep N, Garcia L, Zhang J, Wang Z *et al.* (2007). Gap junction remodeling and cardiac arrhythmogenesis in a murine model of oculodentodigital dysplasia. *Proc Natl Acad Sci U S A* 104: 20512-20516.

Kanai AJ (2011). Afferent mechanism in the urinary tract. *Handb Exp Pharmacol*: 171-205.

Kang J, Kang N, Lovatt D, Torres A, Zhao Z, Lin J *et al.* (2008). Connexin 43 hemichannels are permeable to ATP. *J Neurosci* 28: 4702-4711.

Koo SK, Kim DY, Park SD, Kang KW, Joe CO (1997). PKC phosphorylation disrupts gap junctional communication at G0/S phase in clone 9 cells. *Mol Cell Biochem* 167: 41-49.

Kyle JW, Berthoud VM, Kurutz J, Minogue PJ, Greenspan M, Hanck DA *et al.* (2009). The N terminus of connexin37 contains an alpha-helix that is required for channel function. *J Biol Chem* 284: 20418-20427.

Lai A, Le DN, Paznekas WA, Gifford WD, Jabs EW, Charles AC (2006). Oculodentodigital dysplasia connexin43 mutations result in non-functional connexin hemichannels and gap junctions in C6 glioma cells. *J Cell Sci* 119: 532-541.

Laird DW (1996). The life cycle of a connexin: gap junction formation, removal, and degradation. *J Bioenerg Biomembr* 28: 311-318.

Laird DW (2005). Connexin phosphorylation as a regulatory event linked to gap junction internalization and degradation. *Biochim Biophys Acta* 1711: 172-182.

Laird DW (2006). Life cycle of connexins in health and disease. *Biochem J* 394: 527-543.

Laird DW (2008). Closing the gap on autosomal dominant connexin-26 and connexin-43 mutants linked to human disease. *J Biol Chem* 283: 2997-3001.

Laird DW (2010). The gap junction proteome and its relationship to disease. *Trends Cell Biol* 20: 92-101.

Langlois S, Maher AC, Manias JL, Shao Q, Kidder GM, Laird DW (2007). Connexin levels regulate keratinocyte differentiation in the epidermis. *J Biol Chem* 282: 30171-30180.

Lauf U, Giepmans BN, Lopez P, Braconnot S, Chen SC, Falk MM (2002). Dynamic trafficking and delivery of connexons to the plasma membrane and accretion to gap junctions in living cells. *Proc Natl Acad Sci U S A* 99: 10446-10451

Lee JR, White TW (2009). Connexin-26 mutations in deafness and skin disease. *Expert Rev Mol Med* 11: e35.

Lewis SA (2000). Everything you wanted to know about the bladder epithelium but were afraid to ask. *American Journal of Physiology-Renal Physiology* 278: F867-F874.

Li H, Liu TF, Lazrak A, Peracchia C, Goldberg GS, Lampe PD *et al.* (1996). Properties and regulation of gap junctional hemichannels in the plasma membranes of cultured cells. *J Cell Biol* 134: 1019-1030.

Li L, Jiang C, Hao P, Li W, Song C, Song B (2007). Changes of gap junctional cell-cell communication in overactive detrusor in rats. *Am J Physiol Cell Physiol* 293: C1627-1635.

Loddenkemper T, Grote K, Evers S, Oelerich M, Stogbauer F (2002). Neurological manifestations of the oculodentodigital dysplasia syndrome. *J Neurol* 249: 584-595.

Maeda S, Nakagawa S, Suga M, Yamashita E, Oshima A, Fujiyoshi Y *et al.* (2009). Structure of the connexin 26 gap junction channel at 3.5 A resolution. *Nature* 458: 597-602.

Manias JL, Plante I, Gong XQ, Shao Q, Churko J, Bai D *et al.* (2008). Fate of connexin43 in cardiac tissue harbouring a disease-linked connexin43 mutant. *Cardiovasc Res* 80: 385-395.

McCloskey KD (2010). Interstitial cells in the urinary bladder--localization and function. *Neurourol Urodyn* 29: 82-87.

McLachlan E, Manias JL, Gong XQ, Lounsbury CS, Shao Q, Bernier SM *et al.* (2005). Functional characterization of oculodentodigital dysplasia-associated Cx43 mutants. *Cell Commun Adhes* 12: 279-292.

Miyazato M, Sugaya K, Nishijima S, Kadekawa K, Machida N, Oshiro Y *et al.* (2009). Changes of bladder activity and connexin 43-derived gap junctions after partial bladder-outlet obstruction in rats. *Int Urol Nephrol* 41: 815-821.

Mori K, Noguchi M, Matsuo M, Nomata K, Suematsu T, Kanetake H (2005). Decreased cellular membrane expression of gap junctional protein, connexin 43, in rat detrusor muscle with chronic partial bladder outlet obstruction. *Urology* 65: 1254-1258.

Morley GE, Taffet SM, Delmar M (1996). Intramolecular interactions mediate pH regulation of connexin43 channels. *Biophys J* 70: 1294-1302.

Musil LS, Goodenough DA (1990). Gap junctional intercellular communication and the regulation of connexin expression and function. *Curr Opin Cell Biol* 2: 875-880.

Musil LS, Goodenough DA (1991). Biochemical analysis of connexin43 intracellular transport, phosphorylation, and assembly into gap junctional plaques. *J Cell Biol* 115: 1357-1374.

Musil LS, Goodenough DA (1993). Multisubunit assembly of an integral plasma membrane channel protein, gap junction connexin43, occurs after exit from the ER. *Cell* 74: 1065-1077.

Neuhaus J, Pfeiffer F, Wolburg H, Horn LC, Dorschner W (2005). Alterations in connexin expression in the bladder of patients with urge symptoms. *BJU Int* 96: 670-676.

Neuhaus J, Heinrich M, Schlichting N, Oberbach A, Fitzl G, Schwalenberg T *et al.* (2007). [Structure and function of suburothelial myofibroblasts in the human urinary bladder under normal and pathological conditions]. *Urologe A* 46: 1197-1202.

Paul DL (1986). Molecular cloning of cDNA for rat liver gap junction protein. *J Cell Biol* 103: 123-134.

Paznekas WA, Boyadjiev SA, Shapiro RE, Daniels O, Wollnik B, Keegan CE *et al.* (2003). Connexin 43 (GJA1) mutations cause the pleiotropic phenotype of oculodentodigital dysplasia. *Am J Hum Genet* 72: 408-418.

Paznekas WA, Karczeski B, Vermeer S, Lowry RB, Delatycki M, Laurence F *et al.* (2009). GJA1 mutations, variants, and connexin 43 dysfunction as it relates to the oculodentodigital dysplasia phenotype. *Hum Mutat* 30: 724-733.

Peracchia C, Wang XC (1997). Connexin domains relevant to the chemical gating of gap junction channels. *Braz J Med Biol Res* 30: 577-590.

Peracchia C (2004). Chemical gating of gap junction channels; roles of calcium, pH and calmodulin. *Biochim Biophys Acta* 1662: 61-80.

Pizzuti A, Flex E, Mingarelli R, Salpietro C, Zelante L, Dallapiccola B (2004). A homozygous GJA1 gene mutation causes a Hallermann-Streiff/ODDD spectrum phenotype. *Hum Mutat* 23: 286.

Plotkin LI, Manolagas SC, Bellido T (2002). Transduction of cell survival signals by connexin-43 hemichannels. *J Biol Chem* 277: 8648-8657.

Rackauskas M, Neverauskas V, Skeberdis VA (2010). Diversity and properties of connexin gap junction channels. *Medicina (Kaunas)* 46: 1-12.

Reaume AG, de Sousa PA, Kulkarni S, Langille BL, Zhu D, Davies TC *et al.* (1995). Cardiac malformation in neonatal mice lacking connexin43. *Science* 267: 1831-1834.

Revel JP, Karnovsky MJ (1967). Hexagonal array of subunits in intercellular junctions of the mouse heart and liver. *J Cell Biol* 33: C7-C12.

Richardson RJ, Joss S, Tomkin S, Ahmed M, Sheridan E, Dixon MJ (2006). A nonsense mutation in the first transmembrane domain of connexin 43 underlies autosomal recessive oculodentodigital syndrome. *J Med Genet* 43: e37.

Roscoe W, Veitch GI, Gong XQ, Pellegrino E, Bai D, McLachlan E *et al.* (2005). Oculodentodigital dysplasia-causing connexin43 mutants are non-functional and exhibit dominant effects on wild-type connexin43. *J Biol Chem* 280: 11458-11466.

Rossello RA, Kohn DH (2010). Cell communication and tissue engineering. *Commun Integr Biol* 3: 53-56.

Salameh A (2006). Life cycle of connexins: regulation of connexin synthesis and degradation. *Adv Cardiol* 42: 57-70.

Salomon D, Masgrau E, Vischer S, Ullrich S, Dupont E, Sappino P *et al.* (1994). Topography of mammalian connexins in human skin. *J Invest Dermatol* 103: 240-247.

Schurch W, Seemayer TA, Gabbiani G (1998). The myofibroblast: a quarter century after its discovery. *Am J Surg Pathol* 22: 141-147.

Scott CA, O'Toole EA, Mohungoo MJ, Messenger A, Kelsell DP (2011). Novel and recurrent connexin 30.3 and connexin 31 mutations associated with erythrokeratoderma variabilis. *Clin Exp Dermatol* 36: 88-90.

Seki A, Coombs W, Taffet SM, Delmar M (2004). Loss of electrical communication, but not plaque formation, after mutations in the cytoplasmic loop of connexin43. *Heart Rhythm* 1: 227-233.

Shibayama J, Paznekas W, Seki A, Taffet S, Jabs EW, Delmar M *et al.* (2005). Functional characterization of connexin43 mutations found in patients with oculodentodigital dysplasia. *Circ Res* 96: e83-91.

Sohl G, Willecke K (2003). An update on connexin genes and their nomenclature in mouse and man. *Cell Commun Adhes* 10: 173-180.

Sohl G, Willecke K (2004). Gap junctions and the connexin protein family. *Cardiovasc Res* 62: 228-232.

Solan JL, Lampe PD (2007). Key connexin 43 phosphorylation events regulate the gap junction life cycle. *J Membr Biol* 217: 35-41.

Solan JL, Lampe PD (2009). Connexin43 phosphorylation: structural changes and biological effects. *Biochem J* 419: 261-272.

Spray DC, White RL, de Carvalho AC, Harris AL, Bennett MV (1984). Gating of gap junction channels. *Biophys J* 45: 219-230.

Spray DC, Ye ZC, Ransom BR (2006). Functional connexin "hemichannels": a critical appraisal. *Glia* 54: 758-773.

Stout CE, Costantin JL, Naus CC, Charles AC (2002). Intercellular calcium signaling in astrocytes via ATP release through connexin hemichannels. *J Biol Chem* 277: 10482-10488.

Sui GP, Rothery S, Dupont E, Fry CH, Severs NJ (2002). Gap junctions and connexin expression in human suburothelial interstitial cells. *BJU Int* 90: 118-129.

Sui GP, Coppen SR, Dupont E, Rothery S, Gillespie J, Newgreen D *et al.* (2003). Impedance measurements and connexin expression in human detrusor muscle from stable and unstable bladders. *BJU Int* 92: 297-305.

Tong D, Li TY, Naus KE, Bai DL, Kidder GM (2007). In vivo analysis of undocked connexin43 gap junction hemichannels in ovarian granulosa cells. *Journal of Cell Science* 120: 4016-4024.

Toth K, Shao Q, Lorentz R, Laird DW (2010). Decreased levels of Cx43 gap junctions result in ameloblast dysregulation and enamel hypoplasia in Gja1Jrt/+ mice. *J Cell Physiol* 223: 601-609.

Valiunas V, Weingart R, Brink PR (2000). Formation of heterotypic gap junction channels by connexins 40 and 43. *Circ Res* 86: E42-49.

Veenstra RD (1996). Size and selectivity of gap junction channels formed from different connexins. *J Bioenerg Biomembr* 28: 327-337.

Wang HZ, Brink PR, Christ GJ (2006). Gap junction channel activity in short-term cultured human detrusor myocyte cell pairs: gating and unitary conductances. *Am J Physiol Cell Physiol* 291: C1366-1376.

White TW, Paul DL (1999). Genetic diseases and gene knockouts reveal diverse connexin functions. *Annu Rev Physiol* 61: 283-310.

Wiseman OJ, Fowler CJ, Landon DN (2003). The role of the human bladder lamina propria myofibroblast. *Bju International* 91: 89-93.

Wu C, Sui GP, Fry CH (2004). Purinergic regulation of guinea pig suburothelial myofibroblasts. *J Physiol* 559: 231-243.

Zhou Y, Yang W, Lurtz MM, Ye Y, Huang Y, Lee HW *et al.* (2007). Identification of the calmodulin binding domain of connexin 43. *J Biol Chem* 282: 35005-35017.

CHAPTER 2

Characteristics and Functions of Connexin43 Mutants linked to Oculodentodigital Dysplasia with Respect to Bladder Tissue

2.0 Abstract

Oculodentodigital dysplasia (ODDD) is a rare developmental disease resulting from germ line mutations in the *GJA1* gene, which encodes connexin43 (Cx43). Upon clinical examination, ODDD patients expressing the D3N and G143S mutants have reported impaired bladder function manifesting in some degree of incontinence. Conversely, ODDD patients harbouring topographically similar mutations, G2V and I130T, have yet to report any bladder impairments. Although the importance of Cx43 expression in the bladder is poorly understood, it is hypothesized that Cx43 plays a critical role in normal bladder function. To investigate the characteristics of bladder disease –associated (D3N and G143S) and –unassociated (G2V and I130T) ODDD-linked Cx43 mutants we first expressed the Cx43 mutants in connexin-positive NRK or connexin-negative HeLa cells. Immunofluorescence revealed that all mutants contained the ability to form gap junction-like plaques. However, microinjection of Lucifer yellow dye into mutant-expressing cells resulted in only a few incidents of dye transfer, suggesting that all Cx43 mutants failed to form functional gap junctions, and were also dominant-negative to the function of endogenous Cx43. In addition, in an ATP release assay, we noted that the G2V and D3N mutants exhibited more hemichannel activity in HeLa cells than the I130T or G143S mutants and wild-type Cx43, but none of the mutants altered ATP release in NRK cells. To assess the impact of Cx43 mutants on bladder histology and connexin distribution *in vivo,* we employed two heterozygous mutant mouse lines that express the G60S or I130T Cx43 mutants. The overall localization pattern of Cx43 in the lamina propria and detrusor appeared to be unaltered in bladder tissue of I130T and G60S mice, but the distribution pattern was more

intracellular in bladder tissue from the G60S mice. Both mutant mice exhibited a significant reduction in the highly phosphorylated P1 and P2 isoforms of Cx43. Interestingly, Cx26 exhibited an intracellular localization profile that was restricted to the basal cells of the urothelium in all mice suggesting that Cx26 may play a role in urothelial signaling. Our studies suggest that the reason only some ODDD patients have bladder defects is not rooted in clear differences in Cx43 mutant localization, channel or hemichannel function. Thus, either the molecular changes caused by specific Cx43 mutations have yet to be uncovered or any ODDD patient may be susceptible to developing bladder defects during aging or depending on the existence of other co-morbidities.

Keywords: Connexin, Cx43, mutants, bladder, mouse model of human disease, hemichannel, GJIC, ODDD

2.1 Introduction

Overactive bladder (OAB) is a syndrome that affects millions of men and women worldwide, generally manifesting itself in some degree of incontinence. In the United States alone, OAB is known to affect approximately 17% of the population (Abrams et al. 2003), with an estimated economic cost of 12.6 billion dollars (Hu et al. 2004). The current consensus is that there are two main forms of incontinence: stress and urge. Stress incontinence is caused by increased intra-abdominal pressure, resulting from physical activity or coughing/sneezing. The second form, urge, can be subdivided into neurogenic or idiopathic. Neurogenic is related to altered afferent and efferent neuronal bladder stimuli, which is common in conditions such as spinal cord injury, Parkinson's disease and multiple sclerosis (Christ et al. 2003). Idiopathic is associated with incontinence in which no pathological causation is known (Christ et al. 2003, Miller and Hoffman 2006). However, there is a growing body of evidence to suggest a myogenic origin for at least some forms of idiopathic incontinence (Brading 1997, Haferkamp et al. 2004). It has been proposed that altered gap junctional intercellular communication (GJIC) or cell signaling may be the cause of idiopathic incontinence, resulting in an increased sensitivity to acetylcholine, or changes in how cells communicate (Imamura et al. 2009, Kuhn et al. 2008a).

GJIC is an important cellular feature that allows cells to communicate with their neighbours, and has been implicated in a number of processes from tissue development to normal tissue function (Dahl et al. 1995, Rossello and Kohn 2010). Gap junctions are formed from specialized proteins known as connexins (Cxs). Six connexins that have oligomerized to form a connexon are delivered to the plasma membrane where they can

function as hemichannels or dock to a connexon from an adjacent cell forming a gap junction channel (Goodenough et al. 1996). Both active hemichannels and gap junction channels can selectively pass small molecules of less than 1 kD in size to the extracellular matrix or between connected cytoplasms, respectively (Alexander and Goldberg 2003, Li et al. 1996, Musil and Goodenough 1990). The selectivity of a gap junction channel or hemichannel is highly dependent on its connexin constituents. The 21 human and 20 mouse connexins all share similar membrane topologies and can selectively intermix to create a plethora of channel variations that exhibit unique characteristics (Eiberger et al. 2001, Maeda et al. 2009). Cx26, Cx40, Cx43 and Cx45 have all been reported to be expressed in one or more layers of the bladder (Haefliger et al. 2002, John et al. 2003a). Cx43, the most ubiquitously expressed connexin in the body, has been localized throughout the various layers of the bladder and is thought to mediate function.

In the bladder, the urothelial layer is composed of transitional epithelium which acts as a passive barrier and actively responds to urine composition and bladder distension (Apodaca 2004, Birder and de Groat 2007, de Groat 2004). ATP, known to be released from the urothelium, is thought to signal adjacent urothelial cells and/or bladder nerves through P2X receptor stimulation within the underlying lamina propria, therefore, acting in both an autocrine and paracrine fashion (Apodaca 2004, Birder 2001, Cockayne et al. 2000). Both Cx26 and Cx43 have been localized to the urothelium, although their roles in bladder function have yet to be elucidated (Grossman et al. 1994, Haefliger et al. 2002). The lamina propria, composed of loose fibroelastic connective tissue, expresses Cx43 which is localized to fibroblasts and myofibroblasts (Fry et al. 2007, Neuhaus et al. 2007, Sui et al. 2002, Wiseman et al. 2003). Myofibroblasts have been shown to be in

close proximity with both afferent and efferent neurons and are therefore thought to aid in bladder signaling (Sui et al. 2002, Wiseman et al. 2003). The detrusor layer of the bladder is composed of smooth muscle cells that express both Cx43 and Cx45 which collectively act to facilitate bladder contractions (Andersson 1999, Kanai 2011). Specifically, Cx43 has been localized to the border of smooth muscle cell bundles suggesting its importance in the transmission of electrical stimulus from one muscle bundle to the next, while Cx45 has been localized to the plasma membrane of cells within the muscle bundles (Hashitani et al. 2004, John et al. 2003b, Sui et al. 2003, Wang et al. 2006).

To date, over 60 mutations in the gene encoding Cx43 have been linked to the rare autosomal dominant disease oculodentodigital dysplasia (ODDD) (Paznekas et al. 2009). All mutants examined to date have been shown to have reduced channel function, and also act as dominant-negatives to the function of co-expressed wild-type Cx43 (Gong et al. 2007, McLachlan et al. 2005, Roscoe et al. 2005). Patients harbouring Cx43 mutants share common clinical characteristics, such as syndactyly and camptodactyly of the digit, microdontia, enamel loss, ophthalmic defects and craniofacial abnormalities (Paznekas et al. 2003). Bladder disturbances have been reported in ~12% of ODDD patients although the mechanism behind bladder defects remains poorly understood (Loddenkemper et al. 2002, Paznekas et al. 2009). Although, the percentage of ODDD patients suffering from bladder defects is lower than the general population at ~17% (Abrams et al. 2003), many of these patient develop bladder defect early in their life (Paznekas et al. 2009) a trait rarely seen in the general population. Since Cx43 is broadly distributed throughout the bladder and is thought to play a critical role in bladder contraction we suggested that the reason ODDD patients may suffer from bladder

problems may be rooted in how specific ODDD-linked mutants affect the distribution and function of Cx43-based gap junctions within the bladder.

To further study the physiological role(s) of Cx43 two heterozygous Cx43 mutant mouse lines harbouring the G60S or I130T mutants were generated and shown to mimic ODDD (Flenniken et al. 2005, Kalcheva et al. 2007). Upon examination of several cell types obtained from these mice, all were found to exhibit reduced Cx43-based GJIC and the mutant proteins were found to act as dominant-negatives to co-expressed wild-type Cx43 (Flenniken et al. 2005, Kalcheva et al. 2007, Lai et al. 2006, Manias et al. 2008, McLachlan et al. 2005, Shibayama et al. 2005). However, the status of Cx43 in the bladder of these mutant mice has not been examined.

In the present study, Cx43 mutants (D3N and G143S) that have been linked to incontinence and mutants (G2V and I130T) that have not been associated with incontinence were characterized. The G2V and D3N mutations are localized to the Cx43 amino-terminus, a region known to act as a transjunctional voltage sensor (Bukauskas and Peracchia 1997), while the I130T and G143S mutations are localized to the intracellular loop region, an important domain in pH gating and calmodulin binding (Peracchia 2004, Zhou et al. 2007). In addition, we examined the distribution and spatial localization of Cx43 in the bladders of two Cx43 mutant mouse lines to assess if Cx43 was perturbed in a manner that might suggest the bladder is functionally compromised.

2.2 Methods

2.2.1 Cell Culture

All cell culture reagents and labware were purchased from Invitrogen (Burlington, ON), and Becton-Dickenson (BD, Franklin Lakes, NJ). Human cervical cancer (HeLa) and normal rat kidney (NRK) cells, purchased from American Type Culture Collection (ATCC), were cultured in Dulbecco's Modified Essential Medium (DMEM) 4.5 g/L D-glucose supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine at 37°C and 5.0% CO_2. Prior to reaching confluency, cells were passed by enzymatic dissociation with 0.25% trypsin/EDTA solution at 37°C for 5-15 minutes.

2.2.2 Generation of Untagged and GFP-tagged Cx43 Mutants (G2V, D3N, G143S, I130T)

Human untagged and/or GFP-tagged G2V, D3N, G143S cDNA constructs were purchased from Norclone Biotech Industries (London, ON). I130T and G143S cDNA constructs were synthesized in-house using a QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) and sequenced for verification. Primer sequences used for I130T and G143S mutants are: I130T-forward 5'-gacatgcacttgaagcaga ctgagataaagaagttcaag-3', I130T-reverse 5'-cttgaacttctttatctcagtctgcttcaagtgcatgtc-3', G143S-forward 5'-tacggtattgaagagcatagtaaggtgaaaatgcgaggg-3', and G143S-reverse 5'-ccctcgcattttcaccttactatgctcttcaataccgta-3'

2.2.3 Transient Transfections

Transient transfection of cells was performed using a Lipofectamine 2000 reagent (Invitrogen) as recommended by the manufacturer and as previously described (Roscoe et al. 2005). Briefly, HeLa and NRK cells were grown to 50-70% confluency in 60 mm culture dishes. In cases where cells were used in the ATP release assay, HeLa and NRK cells were seeded in 12 well culture plates at 200,000 cells/well and 90,000 cells/well, respectively, one day prior to transfection. Transfection mixtures were prepared using OptiMEM (Invitrogen) with 5 μg of plasmid cDNAs and 2 μL of Lipofectamine 2000 reagent. Untagged plasmid cDNA was co-transfected with 1 μg DsRed plasmid cDNA. The transfection mixture was gently mixed and allowed to sit at room temperature for 15 minutes. The transfection mixture was then added to OptiMEM washed cells and incubated at 37°C for 5 hours. Finally, the transfection medium was removed, replaced with regular DMEM culture medium and the cells were incubated for another 24-48 hours.

2.2.4 Immunocytochemistry

Transfected cells were fixed and immunolabeled as previously described (Roscoe et al. 2005). Briefly, cells grown on 12 mm glass coverslips were fixed with precooled -20°C, 80% methanol -20% acetone mixture for 15 minutes at 4°C followed by several rinses with phosphate-buffered saline (PBS). Cells were then blocked with 3% bovine serum albumin (BSA) (Sigma-Aldrich, St Louis, MO) and 0.02% Triton-X100 in PBS for 45 minutes at room temperature. Cells were incubated with polyclonal rabbit anti-Cx43 primary antibody (2 μg/ml; Sigma C6219) for 1 hour at room temperature. After repeated

washing with PBS, cells were incubated with anti-rabbit AlexaFluor 555 (4μg/ml; Invitrogen A-21429) for 1 hour at room temperature. Nuclear staining was performed using Hoechst 33342 (10 μg/ml; Molecular Probes, Eugene, OR H3570) for 5 minutes at room temperature, followed by a 5 minute wash with ddH_2O. Coverslips were mounted on glass slides and allowed to sit overnight at 4°C. Labelled cells were imaged on a Zeiss LSM 510 Meta confocal microscope as previously described (Roscoe et al. 2005). Digital images were prepared using Zeiss LSM and CorelDraw 12 software.

2.2.5 Microinjection Dye Transfer Assay

Culture dishes, 60 mm, containing HeLa or NRK cells transiently transfected with cDNA encoding untagged or GFP-tagged Cx43, G2V, D3N, I130T or G143S were selected for microinjection. For GFP expressing mutants, cell clusters expressing GFP at sites of cell to cell appositions were selected for microinjection. For untagged mutants, cell clusters expressing DsRed in two adjacent cells were selected for microinjection. Single cells within transfected clusters were pressure injected using an Eppendorf Femtojet automated pressure microinjector with 2.5% Lucifer yellow dye (Invitrogen) until the cell brightly fluoresced. Three minutes after injection, digital images were collected using a Lecia DM IRE2 inverted epifluorescence microscope with a charged coupled camera (Hamamstsu Photonics, Japan) using OpenLab software. Dye transfer was recorded as being positive if the injected cell passed dye to a contacting cell expressing either GFP or RFP. Both untransfected Cx43-negative HeLa cells and Cx43-positive NRK were included for all studies. At least 80 microinjections were preformed for each mutant tested on 5 to 6 separate transfections.

2.2.6 ATP Release Assay

Culture plates (12 well plates containing one coverslip per well) containing HeLa or NRK cells transiently transfected with cDNA encoding untagged or GFP-tagged Cx43, G2V, D3N, I130T or G143S were selected. Transfection efficiency for all mutants was ~40-60% (data not shown). The ATP release assay was carried out as described previously (Tong et al. 2007), with the following changes. Briefly, for control conditions, all cells were washed twice with normal HBSS (Invitrogen) containing calcium and magnesium. For calcium-free conditions, cells were washed once with normal HBSS followed by a second wash with divalent-cation-free (DCF)-HBSS (Ca^{2+} and Mg^{2+} were replaced by 2 mM EGTA). Cells were then incubated in 500 μL of either normal HBSS or DCF-HBSS for 15 minutes at 37 °C. Following incubation, 10 μL of the incubation solution was removed and mixed with 90 μL of the reaction solution corresponding to the ATP Determination Kit (Invitrogen) instructions. ATP concentration was determined by measuring bioluminescence (at a wavelength of ~560 nm) with a luminometer (PerkinElmer, Waltham MA). Coverslips containing transfected cells were then fixed, labeled and imaged for Cx43 as mentioned in section 2.2.4. Four images per transfection were taken, after which images were assessed by a blind observer and the percent transfection efficiency was determined.

2.2.7 Animals

Animal studies were conducted on two mutant mouse lines harbouring either the G60S or I130T Cx43 mutants. Importantly, both mice are heterozygous for the gene

Gja1, which encodes Cx43. Therefore, the mice are expected to translate a 1:1 ratio of mutant to wild-type Cx43 protein and, thus genetically match human ODDD patients

G60S, also known as $Gja1^{Jrt/+}$, mice were supplied by the Centre for Modeling Human Disease, University of Toronto, on a mixed C57BL/6J and C3H/HeJ background (Flenniken et al. 2005). After confirming 100% penetrance of the syndactyly feature by PCR genotyping (Flenniken et al. 2005), subsequent genotypes determination was completed using visual inspection of pups. The mice were maintained under a constant photo period of 12 h light:dark cycle and received food and water ad libitum.

The I130T, also known as $Gja1^{I130T/+}$, mice were obtained from Dr. Glen Fishman, New York University School of Medicine, New York. These mice, which were on a mixed CD1/C57BL6 background, were further backcrossed onto a C57BL6 background for an additional 1-3 generations. A 100% incidence rate of syndactyly (n= 203) in the I130T mice was confirmed by PCR genotyping. However, while syndactyly was found on the front limbs of all mice, the back limbs did not always exhibit this phenotype.

2.2.8 Tissue Harvesting and Preparation

Whole bladder, heart and liver samples were collected from G60S, I130T and wild-type littermate mice at 3 months postnatal. All mice used in this study were euthanized using a carbon dioxide chamber followed by cervical dislocation, in accordance with the University of Western Ontario Guide for Care and Use of Laboratory Animals. Tissue samples assigned to hematoxylin and eosin staining were fixed overnight at 4°C in 10% neutral buffered formalin (NFB) (EMD, Mississauga, ON), dehydrated,

embedded in paraffin blocks, and sectioned longitudinally at 5μm intervals. Tissue samples assigned to immunohistochemistry were snap frozen in liquid nitrogen, embedded in a 10.24% polyvinyl alcohol, 4.26% polyethylene glycol Optimal Cutting Temperature Compound (Sakura, Torrance, CA), sectioned longitudinally at 5μm thickness, and stored at -80°C. Tissue samples assigned to immunoblotting were snap frozen in liquid nitrogen and stored at -80°C for future use.

2.2.9 Histology

To assess the histology of the mouse bladders, 5 μm paraffin-embedded sections from G60S and I130T mice and their wild-type littermates were stained with hematoxylin (0.4%) and eosin (0.5%). Briefly, paraffin-embedded bladder sections were deparaffinised in xylene, rehydrated in a descending gradient of ethanol baths and stained with hematoxylin (5 minutes), washed and stained with eosin (5 minutes). Sections were then dehydrated in an ascending gradient of ethanol baths followed by xylene and mounted with Cytoseal (Thermo Scientific, Rockford, IL). Bladder sections were imaged on a Lecia DM IRE2 inverted microscope equipped with a Micropublisher 5.0 RTV CCD colour, cooled camera. Linear measurements were made perpendicularly through the detrusor layer using ImageJ software. In total, 405 measurements were made on each of four bladders from G60S and I130T mice and their wild-type littermates, corresponding to 3 slides per mouse, 9 sections per slide and 15 measurements per sections. Statistical analysis included standard error, and comparison between G60S and I130T mice and their wild-type littermates.

2.2.10 Immunofluorescence

To assess the localization of Cx43 and Cx26 in mouse bladders, hearts, and livers, 5 μm cryo-sections from G60S and I130T mice and their wild-type littermates were first fixed in 10% NBF for 30 minutes at room temperature. Tissues sections were blocked with 3% BSA (Sigma-Aldrich) and 0.02% Triton-X100 in PBS for 45 minutes at room temperature. Cx43, Cx26 and actin were all detected using: rabbit anti-Cx43 (2 μg/ml; Sigma C6219), rabbit anti-Cx26 (0.5 μg/ml; Invitrogen 51-2800) and mouse anti-phalloidin (2 U/ml; Invitrogen A12379) respectively. All antibodies were diluted in 3% BSA and 0.02% Tween-20 and allowed to incubate on tissue sections for one hour. After repeated washings with PBS, tissues were incubated with secondary anti-rabbit AlexaFluor 555 antibody (4 μg/ml; Invitrogen A-21428), followed by repeated washings. Nuclear staining was performed using Hoechst 33342 (10 μg/ml; Molecular Probes) for 5 minutes at room temperature, followed by a 5 minute wash with ddH_2O. Tissues sections were mounted using glass coverslips and allowed to sit overnight at 4°C. Tissue sections were imaged on a Zeiss LSM 510 Meta confocal microscope as previously described (Roscoe et al. 2005). Digital images were prepared using Zeiss LSM and CorelDraw 12 software.

2.2.11 Western Blot Analysis

Tissue samples stored at -80°C were homogenized in Radio Immuno Precipitation Assay (RIPA) lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mN sodium orthovanadate, 1 mM sodium

fluoride), rocked for 1 hour at 4°C, and homogenates were removed from cell debris via a 10 minute, 6000 rpm centrifugation at 4°C.

Immunoblotting was preformed as previously described (Gehi et al. 2011) using 10 or 12% SDS-PAGE. Cx43, Cx26 and GAPDH were detected using polyclonal rabbit anti-Cx43 (0.02 μg/ml; Sigma C6219), polyclonal rabbit anti-Cx26 (0.05 μg/ml; Invitrogen 710500) and polyclonal mouse anti-GAPDH (1 μg/ml; Millipore, Temecula, CA), respectively. Primary antibodies were detected using either anti-rabbit AlexaFluor 680 (0.2 μg/ml, Invitrogen) or anti-mouse IRDye 800 (1:10000; Rockland, Gilbertsville, PA) antibodies. Membranes were developed using an Odyssey infrared imaging system (LiCor, Lincoln, NE) and analyzed under unsaturated conditions using Odyssey 2.0.4 software (Licor).

2.2.12 Statistics

All results were analyzed using the Student's two-tailed independent sample t-test, using a $P<0.05$ value to denote significance. All results were analyzed using GraphPad Prism 4.03 software, are presented as mean ± standard error.

2.3 Results

2.3.1 Cx43 mutants localize to sites of cell to cell apposition in HeLa and NRK cells

Cx43-negative HeLa cells and Cx43-positive NRK cells were transiently transfected with cDNA constructs encoding wild-type or mutant Cx43. Immunofluorescence revealed that GFP-tagged and untagged wild-type Cx43 assembles into gap junction-like plaque structures at sites of cell to cell apposition. As well, bladder disease –associated (D3N and G143S) and –unassociated (G2V and I130T) Cx43 mutants all assembled into gap junction-like plaques at sites of cell to cell apposition in both HeLa (Figure 2.1A,B) and NRK (Figure 2.2) cells. These localization patterns revealed that both sets of Cx43 mutants have the ability to make gap junctions when untagged or tagged with GFP.

2.3.2 Gap junction channels formed by Cx43 mutants are impermeable to Lucifer yellow

To assess if Cx43 mutants could form functional channels in gap junctional intercellular communication (GJIC)-deficient HeLa cells, mutant-expressing cells were microinjected with Lucifer yellow that has the capacity to transfer through gap junctions. As a control, the expression of Cx43-GFP was found to greatly increase (by ~85%) the incidence of dye transfer between microinjected HeLa cells (Figure 2.3A), as opposed to untransfected HeLa cells. However, the expression of bladder disease –associated and –unassociated Cx43 mutants resulted in only a few incidences of dye transfer (~6-18%) similar to that of untransfected HeLa cells (Figure 2.3A), suggesting that both sets of Cx43 mutants were not competent in assembling into functional gap junction channels.

Figure 2.1 Cx43 mutants localized to sites of cell to cell appositions in HeLa cells. HeLa cells were transfected with cDNA encoding GFP-tagged (A) and untagged (B) Cx43, G2V, D3N, I130T or G143S. Mutants assembled into gap junction plaques (arrowheads) at sites of cell-cell appositions in HeLa cells. Cells were immunolabeled for Cx43 (Red). Green = GFP, Blue = Hoechst Nuclear Stain. Bar = 20 μm

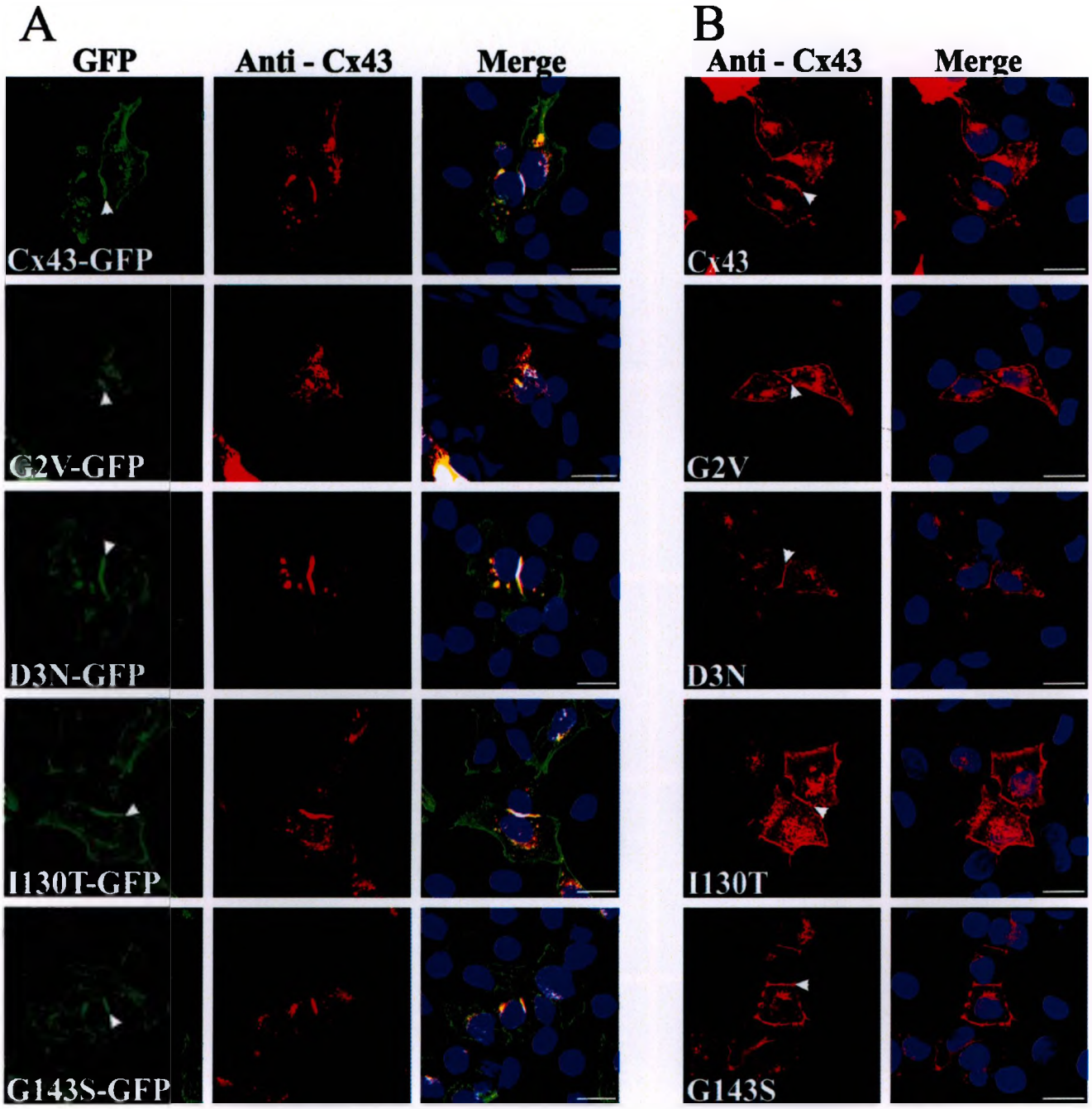
A
GFP
Anti - Cx43
Merge
Cx43-GFP
G2V-GFP
D3N-GFP
I130T-GFP
G143S-GFP
B
Anti - Cx43
Merge
Cx43
G2V
D3N
I130T
G143S

Figure 2.1

Figure 2.3 Cx43 mutants localize to sites of cell to cell appositions in Cx43-positive NRK cells. NRK cells were transfected with cDNA encoding GFP-tagged Cx43, G2V, D3N, I130T or G143S mutants. Cx43 mutants assembled into gap junction plaques (arrowheads) at sites of cell-cell apposition and appeared to co-oligomerize with endogenous Cx43 (yellow). Cells were immunolabeled for Cx43 (red). Green = GFP, Blue = Hoechst nuclear stain. Bar = 20 μm

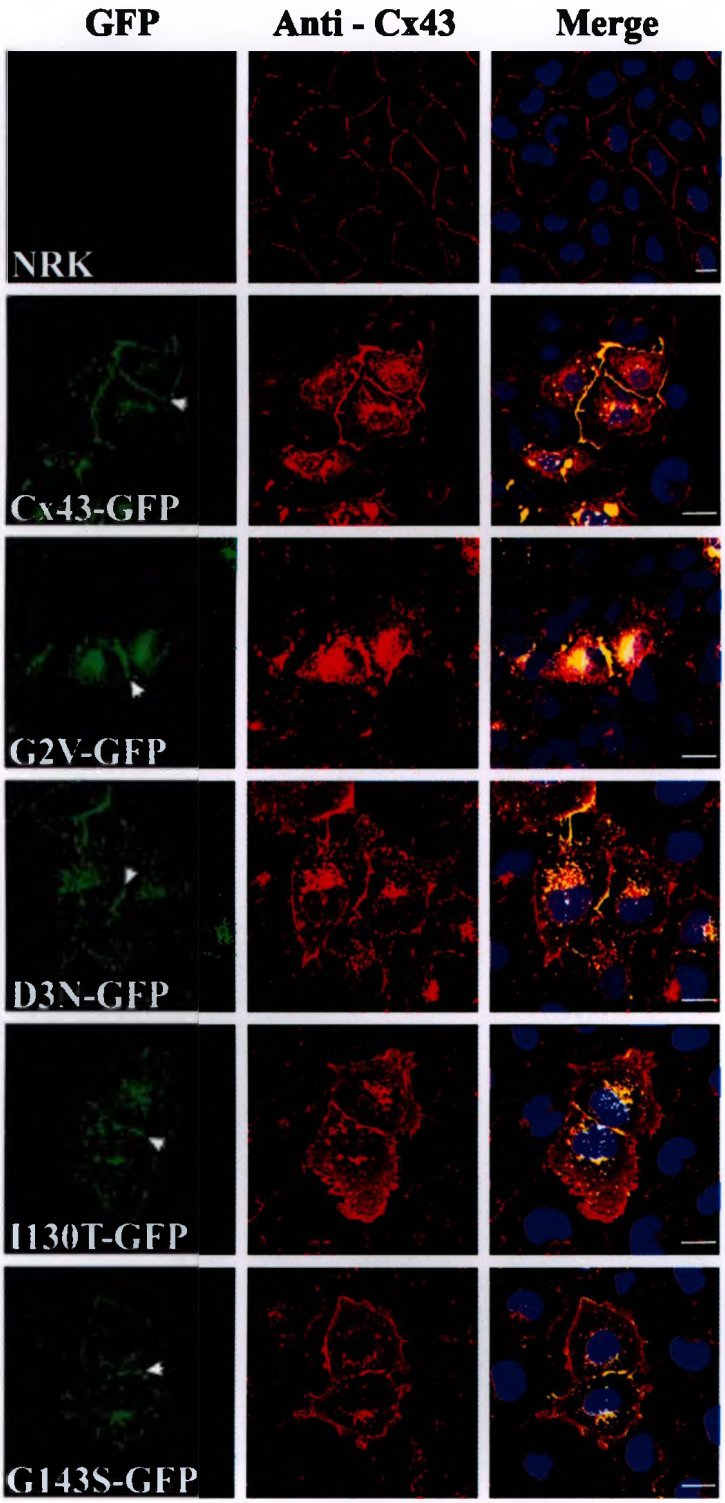
GFP
Anti - Cx43
Merge
NRK
Cx43-GFP
G2V-GFP
D3N-GFP
I130T-GFP
G143S-GFP

Figure 2.2

Figure 2.3 Cx43 mutants form functionally compromised channels and act as dominant-negatives to the channel function of co-expressed endogenous Cx43. HeLa and NRK cells expressing Cx43 mutant proteins were subjected to microinjection with Lucifer yellow and assessed for dye transfer. Microinjected HeLa cells expressing wild-type Cx43 most often transferred dye while cells expressing any of the mutants rarely passed dye (A). NRK cells expressing wild-type Cx43 also had a significant increase in the percent incidences of dye transfer. However, the Cx43 mutant proteins acted as dominant-negatives to the channel function of co-expressed wild-type Cx43 significantly reducing the incidence of dye transfer in NRK cells (B). A. Bars represent means ± s.e.m.; * $P<0.05$, N = 5-6 replicate transfections. B. Bars with different superscript letters are significantly different ($P<0.05$)

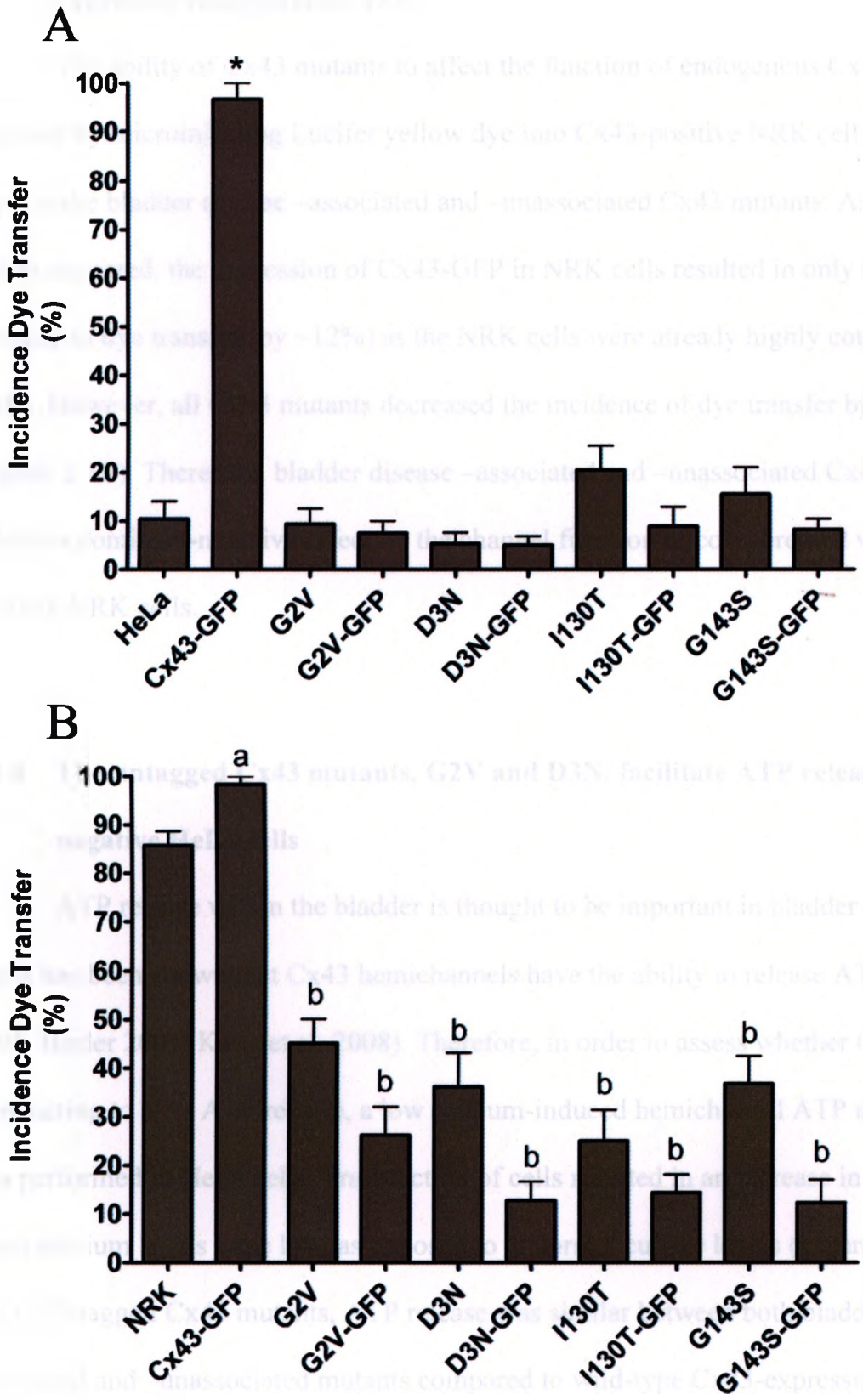
A
Incidence Dye Transfer (%)
100
90
80
70
60
50
40
30
20
10
0
*
HeLa
Cx43-GFP
G2V
G2V-GFP
D3N
D3N-GFP
I130T
I130T-GFP
G143S
G143S-GFP
B
Incidence Dye Transfer (%)
a
b
NRK
Cx43-GFP
G2V
G2V-GFP
D3N
D3N-GFP
I130T
I130T-GFP
G143S
G143S-GFP

Figure 2.3

2.3.3 Cx43 mutants act as dominant-negatives on the channel function of co-expressed endogenous Cx43

The ability of Cx43 mutants to affect the function of endogenous Cx43 was assessed by microinjecting Lucifer yellow dye into Cx43-positive NRK cell that co-express the bladder disease –associated and –unassociated Cx43 mutants. As a control, and as expected, the expression of Cx43-GFP in NRK cells resulted in only a slight increase in dye transfer (by ~12%) as the NRK cells were already highly coupled (Figure 2.3B). However, all Cx43 mutants decreased the incidence of dye transfer by ~41-73% (Figure 2.3B). Therefore, bladder disease –associated and –unassociated Cx43 mutants exhibit a dominant-negative effect on the channel function of co-expressed wild-type Cx43 in NRK cells.

2.3.4 The untagged Cx43 mutants, G2V and D3N, facilitate ATP release in Cx43-negative HeLa cells

ATP release within the bladder is thought to be important in bladder signaling, and it has been shown that Cx43 hemichannels have the ability to release ATP (Apodaca 2004, Birder 2001, Kang et al. 2008). Therefore, in order to assess whether Cx43 mutants were acting to alter ATP release, a low calcium-induced hemichannel ATP release assay was performed in HeLa cells. Transfection of cells resulted in an increase in ATP release when calcium levels were low, as opposed to at normal culture levels (Figure 2.4A-D). For GFP-tagged Cx43 mutants, ATP release was similar between both bladder disease –associated and –unassociated mutants compared to wild-type Cx43-expressing and untransfected HeLa cells (Figure 2.4A). However, it appeared as though the G2V-GFP

Figure 2.4 Cx43 mutants differentially affect ATP release depending on endogenous connexin status. HeLa and NRK cells transfected with cDNA encoding GFP-tagged or untagged Cx43, G2V, D3N, I130T or G143S were treated with HBSS with calcium ($+Ca^{2+}$) or without calcium ($-Ca^{2+}$) and assessed for extracellular ATP release. HeLa cells expressing GFP-tagged Cx43 mutants released similar levels of extracellular ATP concentrations as untransfected cells (A), whereas untagged G2V and D3N resulted in an increase in extracellular ATP concentrations compared to untransfected cells (B). However, when co-expressed with wild-type Cx43 all GFP-tagged and untagged Cx43 mutants resulted in similar extracellular ATP concentrations as untransfected NRK cells (C, D). Bars represent means ± s.e.m. * = ($P<0.05$). N = 3 replicate transfections

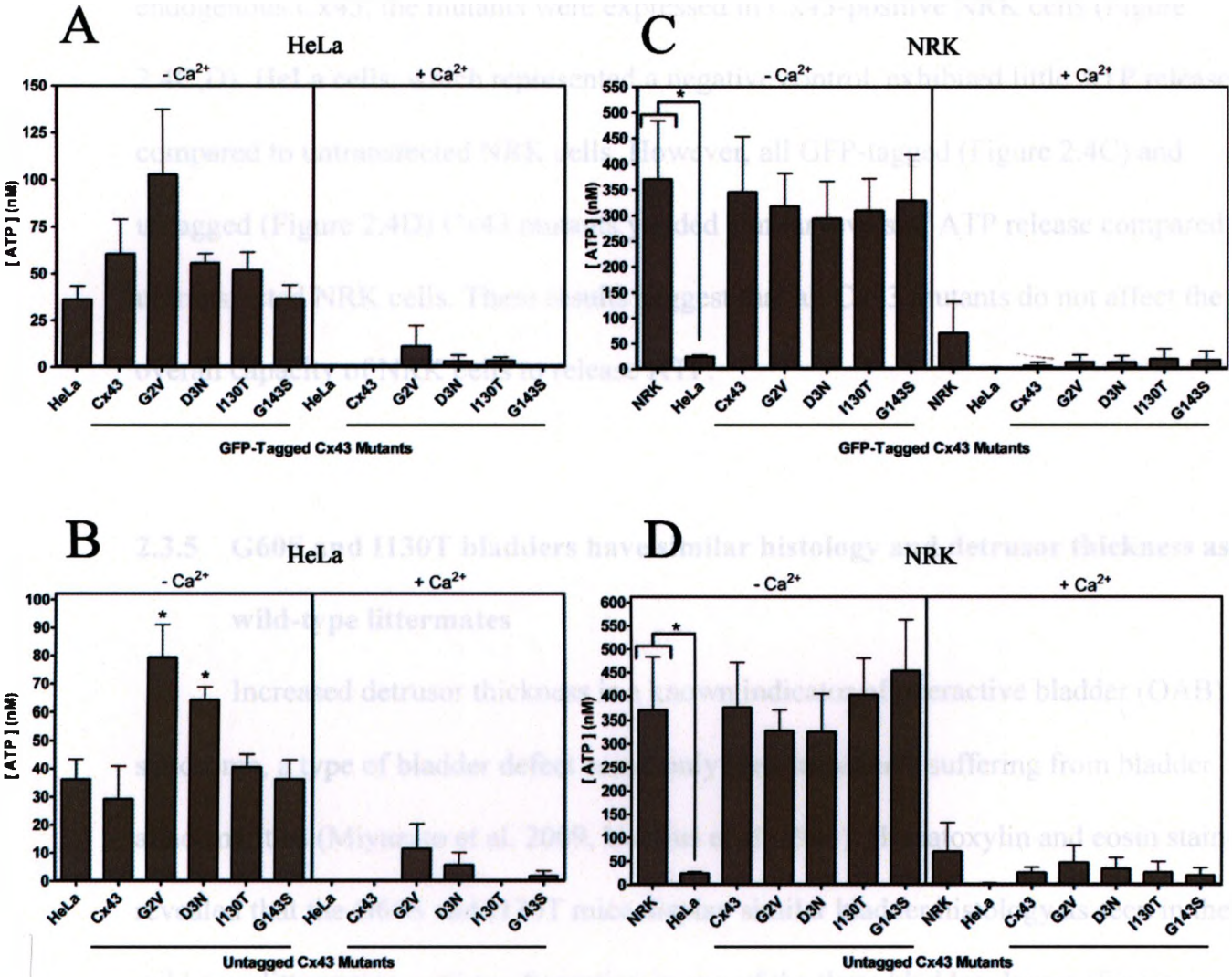
A
HeLa
- Ca2+
+ Ca2+
[ATP] (nM)
HeLa
Cx43
G2V
D3N
I130T
G143S
GFP-Tagged Cx43 Mutants
C
NRK
B
Untagged Cx43 Mutants
D

Figure 2.4

mutant was trending towards a marked increase (Figure 2.4A). Both untagged amino terminal Cx43 mutants, G2V and D3N, resulted in a significant increase in the amounts of ATP released compared to both untransfected HeLa cells and wild-type Cx43. In contrast, the I130T and G143S mutants did not facilitate ATP release (Figure 2.4B).

To determine if Cx43 mutants altered the ATP release capacity of co-expressed endogenous Cx43, the mutants were expressed in Cx43-positive NRK cells (Figure 2.4C,D). HeLa cells, which represented a negative control, exhibited little ATP release compared to untransfected NRK cells. However, all GFP-tagged (Figure 2.4C) and untagged (Figure 2.4D) Cx43 mutants yielded similar levels of ATP release compared to untransfected NRK cells. These results suggest that all Cx43 mutants do not affect the overall capacity of NRK cells to release ATP.

2.3.5 G60S and I130T bladders have similar histology and detrusor thickness as wild-type littermates

Increased detrusor thickness is a known indicator of overactive bladder (OAB) syndrome, a type of bladder defect commonly seen in patients suffering from bladder abnormalities (Miyazato et al. 2009, Uvelius et al. 1984). Hematoxylin and eosin staining revealed that the G60S and I130T mice display similar bladder histology as seen in their wild-type littermates, with no disruption in any of the three bladders layers (Figure 2.5A,B). Subsequent detrusor measurements revealed that detrusor thickness was similar in the G60S and I130T mice and their wild-type littermates.

Figure 2.5 G60S and I130T mouse bladders have similar histology and detrusor thickness as their wild-type littermate controls. Paraffin embedded bladders were sectioned, stained with H&E and measured for detrusor thickness. In all mice, the bladder structure was similar (A, B). Subsequent detrusor measurements revealed that G60S and I130T mouse bladders have similar thicknesses as wild-type littermates (C). Bar = 200 μm (A, B). Bars represent means ± s.e.m (C). N = 4 mice

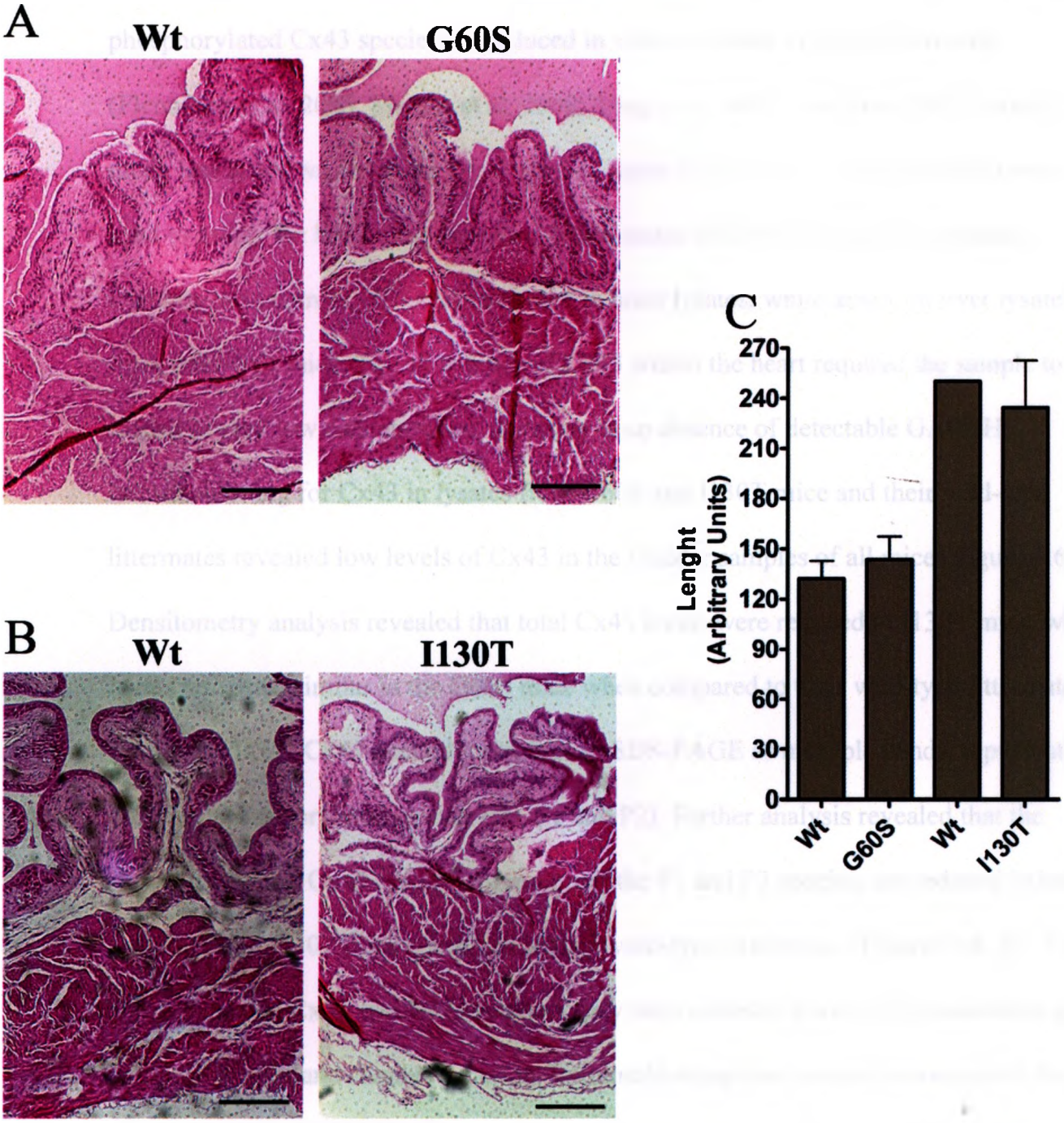
A
Wt
G60S
B
Wt
I130T
C
Lenght
(Arbitrary Units)
270
240
210
180
150
120
90
60
30
0
Wt
G60S
Wt
I130T

Figure 2.5

2.3.6 G60S and I130T bladders have reduced levels of the highly phosphorylated Cx43 species

Previous studies have indicated that the total Cx43 levels and the levels of highly phosphorylated Cx43 species are reduced in various tissues of the G60S mouse (Flenniken et al. 2005, Manias et al. 2008, Tong et al. 2009, Toth et al. 2010), while much less is known about the Cx43 species status in I130T mice. Western blots were used to assess the levels and phosphorylation status of Cx43 in the mutant mouse bladders. As controls, Cx43 was detected in heart lysates, while absent in liver lysates from wild-type mice. The abundance of Cx43 within the heart required the sample to be loaded at very low concentration, resulting in an absence of detectable GAPDH. Immunoblotting for Cx43 in lysates from G60S and I130T mice and their wild-type littermates revealed low levels of Cx43 in the bladder samples of all mice (Figure 2.6A). Densitometry analysis revealed that total Cx43 levels were reduced in I130T mice, while levels remained similar in the G60S mice when compared to their wild-type littermates (Figure 2.6B,D). Cx43 generally resolves in SDS-PAGE as multiple bands, representing different phosphorylated species (P0, P1 and P2). Further analysis revealed that the slower migrating Cx43 species, representing the P1 and P2 species, are reduced in both the G60S and I130T mice compared to their wild-type littermates (Figure 2.6C,E). These phosphorylated Cx43 species have previously been correlated with fully assembled gap junction plaques and channel function. Immunoblotting also revealed non-specific bands at ~47-50 kD and ~30 kD, in lysates from all bladder samples. Although these bands were present in only the bladder samples, it is unclear as to what these band represents and therefore they was not included in the densitometry analysis.

Figure 2.6 G60S and I130T mouse bladders exhibit a reduction in the phosphorylated species of Cx43. Cx43 normally resolves as a triplet banding pattern representing different phosphorylated species of Cx43 (P0, P1, and P2), as seen in heart tissue (A). G60S, I130T and wild-type littermate mouse bladders were all positive for Cx43 expression (A). Total Cx43 (P0, P1 and P2) expression was reduced in I130T mouse bladders (B), while remaining similar in the G60S mouse bladders (D) as compared to their wild-type littermates. The higher phosphorylated Cx43 species (P1 and P2) were reduced in the I130T (C) and G60S (E) mouse bladders compared to wild-type littermates. Non-specific bands were noted at ~37-50 kD and ~30 kD. Bars represent means ± s.e.m (B-E). * = ($P<0.05$). N = 5 (I130T) and 6 (G60S)

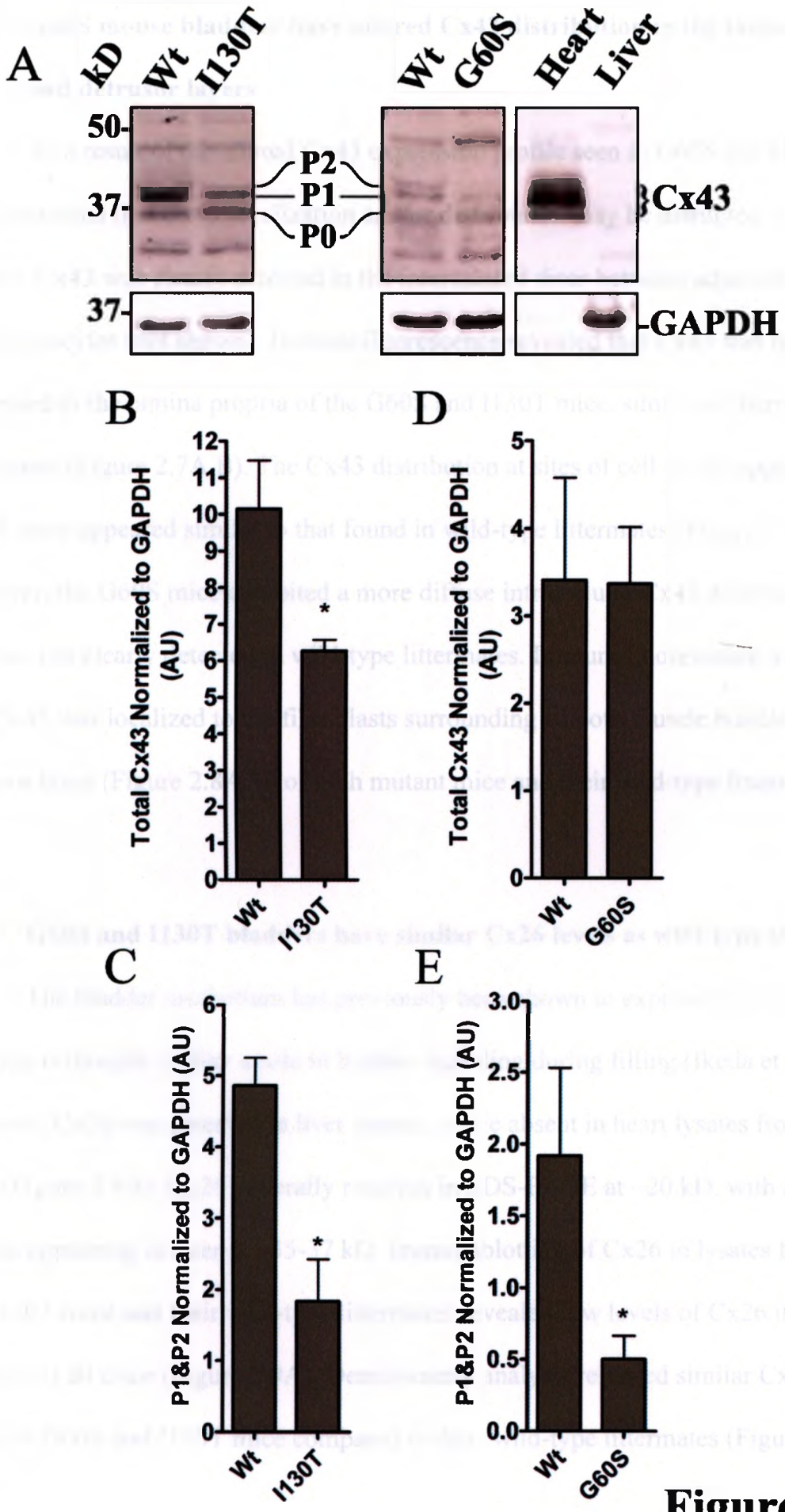
A
kD
Wt
I130T
Wt
G60S
Heart
Liver
50-
37-
P2
P1
P0
Cx43
37-
GAPDH
B
Total Cx43 Normalized to GAPDH (AU)
Wt
I130T
D
Total Cx43 Normalized to GAPDH (AU)
Wt
G60S
C
P1&P2 Normalized to GAPDH (AU)
Wt
I130T
E
P1&P2 Normalized to GAPDH (AU)
Wt
G60S

Figure 2.6

2.3.7 G60S mouse bladders have altered Cx43 distribution in the lamina propria and detrusor layers

As a result of the altered Cx43 expression profile seen in G60S and I130T mice, it was postulated that Cx43 localization and/or distribution may be disrupted. As a positive control, Cx43 was clearly detected in the intercalated discs between adjacent cardiomyocytes (not shown). Immunofluorescence revealed that Cx43 was highly expressed in the lamina propria of the G60S and I130T mice, similar to their wild-type littermates (Figure 2.7A,B). The Cx43 distribution at sites of cell to cell apposition in the I130T mice appeared similar to that found in wild-type littermates (Figure 2.7B). However, the G60S mice exhibited a more diffuse intracellular Cx43 distribution pattern that was not clearly detected in wild-type littermates. Immunofluorescence also revealed that Cx43 was localized to the fibroblasts surrounding smooth muscle bundles in the detrusor layer (Figure 2.8A,B) of both mutant mice and their wild-type littermates.

2.3.8 G60S and I130T bladders have similar Cx26 levels as wild-type littermates

The bladder urothelium has previously been shown to express Cx26, and its function is thought to play a role in bladder signaling during filling (Ikeda et al. 2007). As a control, Cx26 was detected in liver lysates, while absent in heart lysates from wild-type mice (Figure 2.9A). Cx26 generally resolves in SDS-PAGE at ~20 kD, with a dimer species appearing in liver at ~35-37 kD. Immunoblotting of Cx26 in lysates from G60S and I130T mice and their wild-type littermates revealed low levels of Cx26 in bladder samples of all mice (Figure 2.9A). Densitometry analysis revealed similar Cx26 bladder levels of G60S and I130T mice compared to their wild-type littermates (Figure 2.9B,C).

Figure 2.7 G60S mouse bladders have altered Cx43 distribution in the lamina propria, while I130T bladders display similar Cx43 distribution as wild-type littermates. Cryo-embedded bladder samples were sectioned and labeled for Cx43. Cx43 was localized to the lamina propria of the G60S, I130T and their wild-type littermate mouse bladders (A, B). G60S mouse bladders display a more diffuse Cx43 distribution (arrows), as opposed to the punctate distribution (arrowheads) seen in wild-type littermates (A). However, I130T mouse bladders display a similar punctate Cx43 distribution (arrowheads) pattern seen in wild-type littermates (B). Uro = Urothelium. LP = Lamina Propria. Red = Cx43, Blue = Hoechst Nuclear Stain. Bar = 20 μm

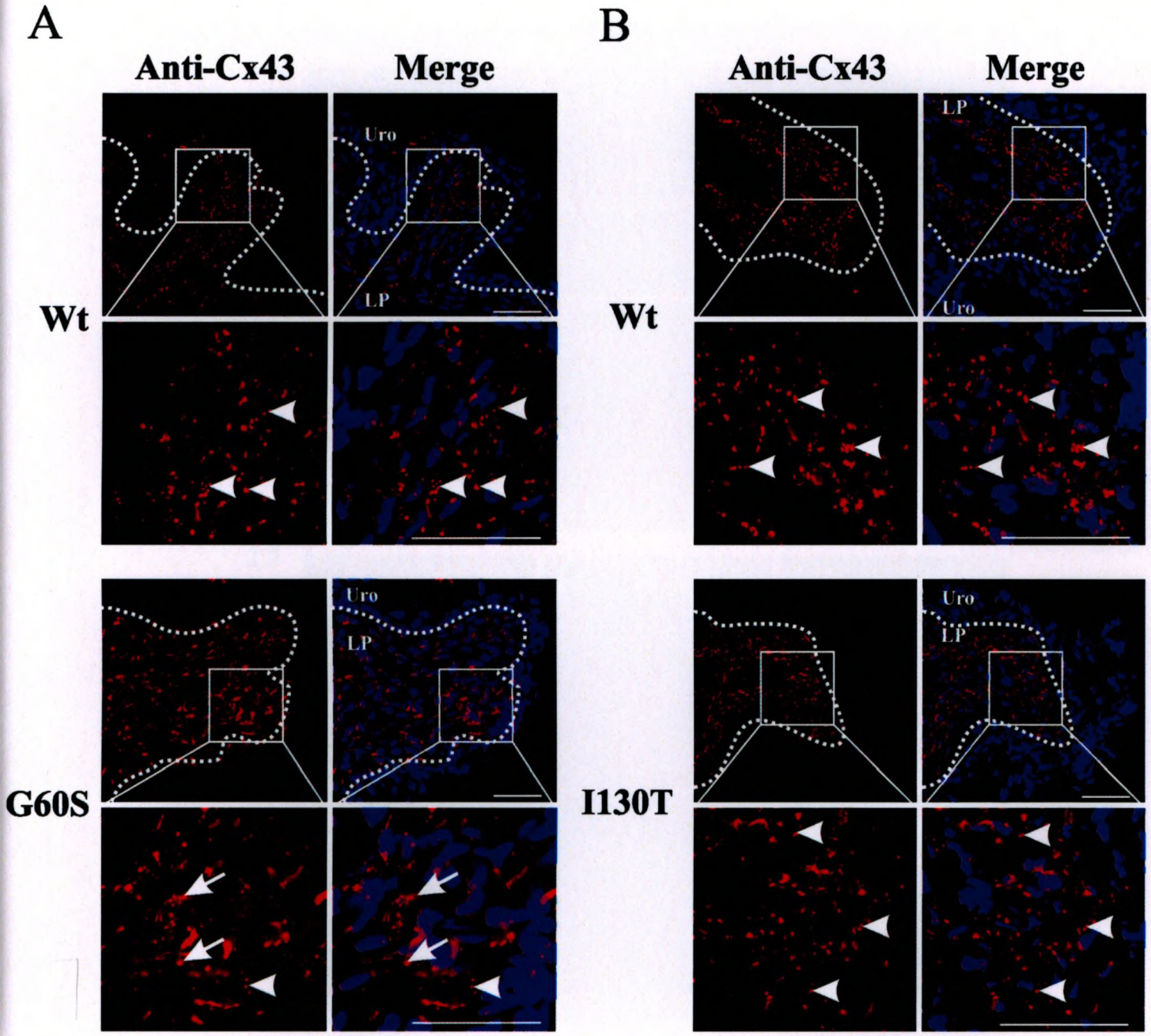
A
Anti-Cx43
Merge
Wt
Uro
LP
G60S
Uro
LP
B
Anti-Cx43
Merge
Wt
LP
Uro
I130T
Uro
LP

Figure 2.7

Figure 2.8 G60S and I130T mice and their wild-type littermates display distinct Cx43 detrusor localizations. Cryo-embedded bladder samples were sectioned and labeled for Cx43. Cx43 is predominantly localized to the connective tissue surrounding smooth muscle bundles in G60S, I130T and their wild-type littermate mouse bladders (A, B). Cx43 displays punctate structures (arrowheads) in G60S (A), I130T (B) and wild-type littermates. However, bladders from G60S mice appear to have a more diffuse Cx43 distribution profile (arrows), similar to the lamina propria, compared to wild-type littermates. Red = Cx43, Green = F-actin. Blue = Hoechst Nuclear Stain. Bar = 20 μm

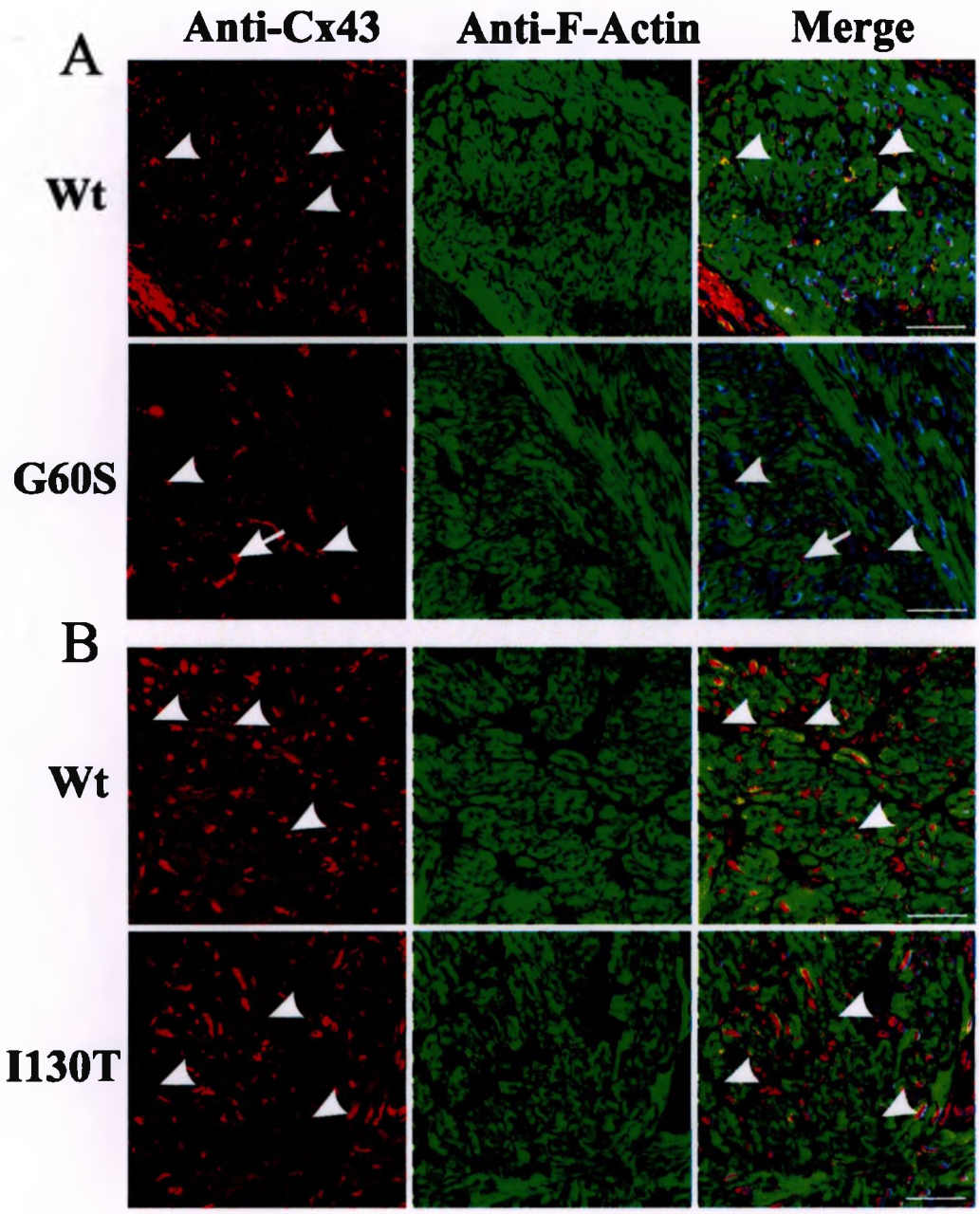

Figure 2.8

Figure 2.9 G60S and I130T mouse bladders have similar Cx26 levels as wild-type littermates. Cx26 generally resolves at ~21 kD with a dimer at ~37 kD as seen in liver lysates (A). G60S, I130T and wild-type littermate bladders were all positive for Cx26 expression (A). Total Cx26 expression was similar in I130T and G60S mice and their wild-type littermates (B, C). A non-specific band located at ~32-34kD was noted. Bars represent means ± s.e.m (B-E). N = 5 (I130T) and 6 (G60S)

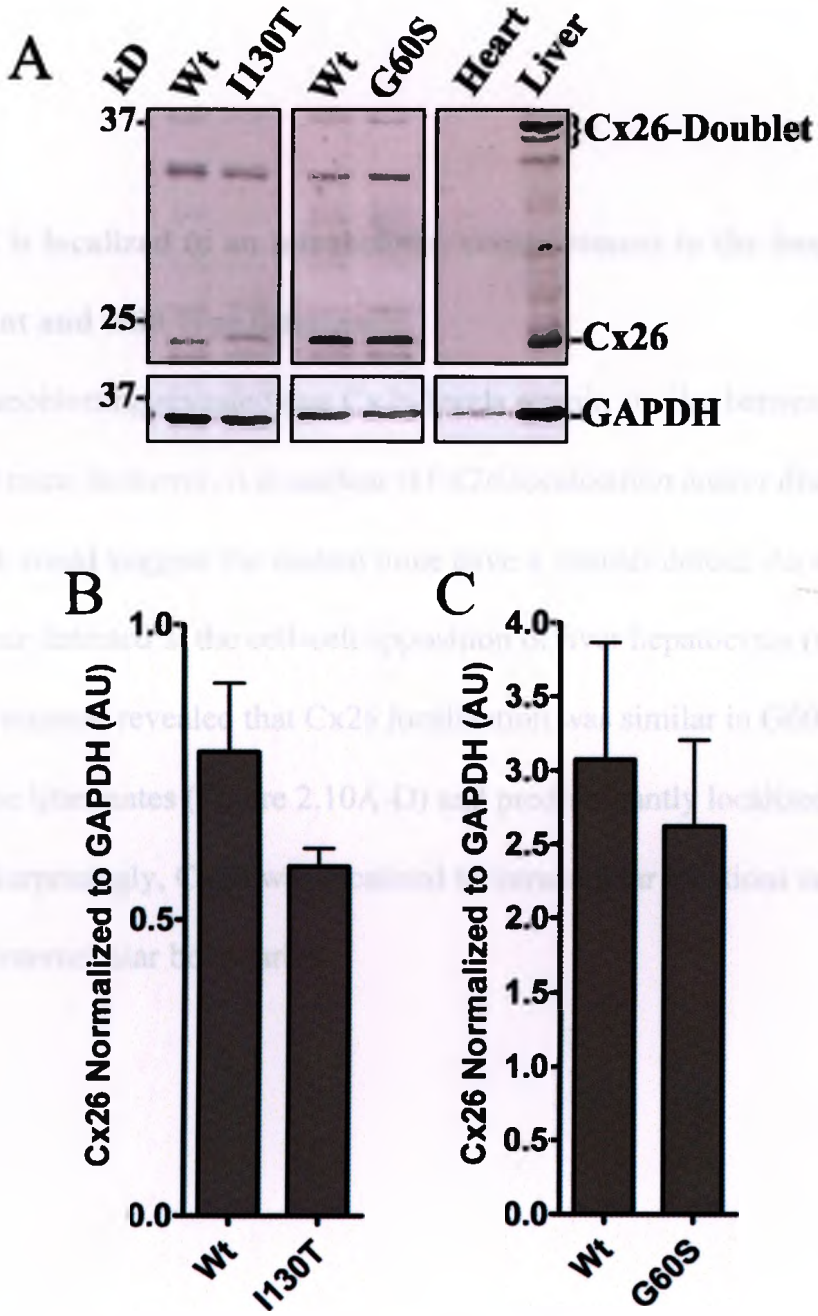
A
kD
Wt
I130T
Wt
G60S
Heart
Liver
37-
25-
37-
Cx26-Doublet
Cx26
GAPDH
B
Cx26 Normalized to GAPDH (AU)
1.0
0.5
0.0
Wt
I130T
C
Cx26 Normalized to GAPDH (AU)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Wt
G60S

Figure 2.9

Immunoblotting also revealed a nonspecific bands located at ~32-34kD in lysates from all bladder sample. Although the band was present in only the bladder samples, it is unclear as to what this band represents and therefore was not included in the densitometry analysis.

2.3.9 Cx26 is localized to an intracellular compartment in the basal urothelium of mutant and wild-type littermates

Immunoblotting revealed that Cx26 levels remain similar between wild-type and Cx43 mutant mice; however, it is unclear if Cx26 localization and/or distribution is altered, which could suggest the mutant mice have a bladder defect. As a positive control Cx26 was clear detected at the cell-cell apposition of liver hepatocytes (not shown). Immunofluorescence revealed that Cx26 localization was similar in G60S and I130T and their wild-type littermates (Figure 2.10A-D) and predominantly localized to the basal urothelium. Surprisingly, Cx26 was localized to intracellular locations and not, as expected, to intercellular boundaries.

Figure 2.10 Cx26 was localized to intracellular compartments of basal urothelial cells of mutant and wild-type mice. Cryo-embedded bladder samples were sectioned and labeled for Cx26. Cx26 displayed a strong basal urothelium expression and a unique localization pattern to an intracellular compartment in all mice (A, B). Uro = Urothelium. LP = Lamina Propria. Red = Cx43, Blue = Hoechst 32 Nuclear Stain. Bar = 20 μm

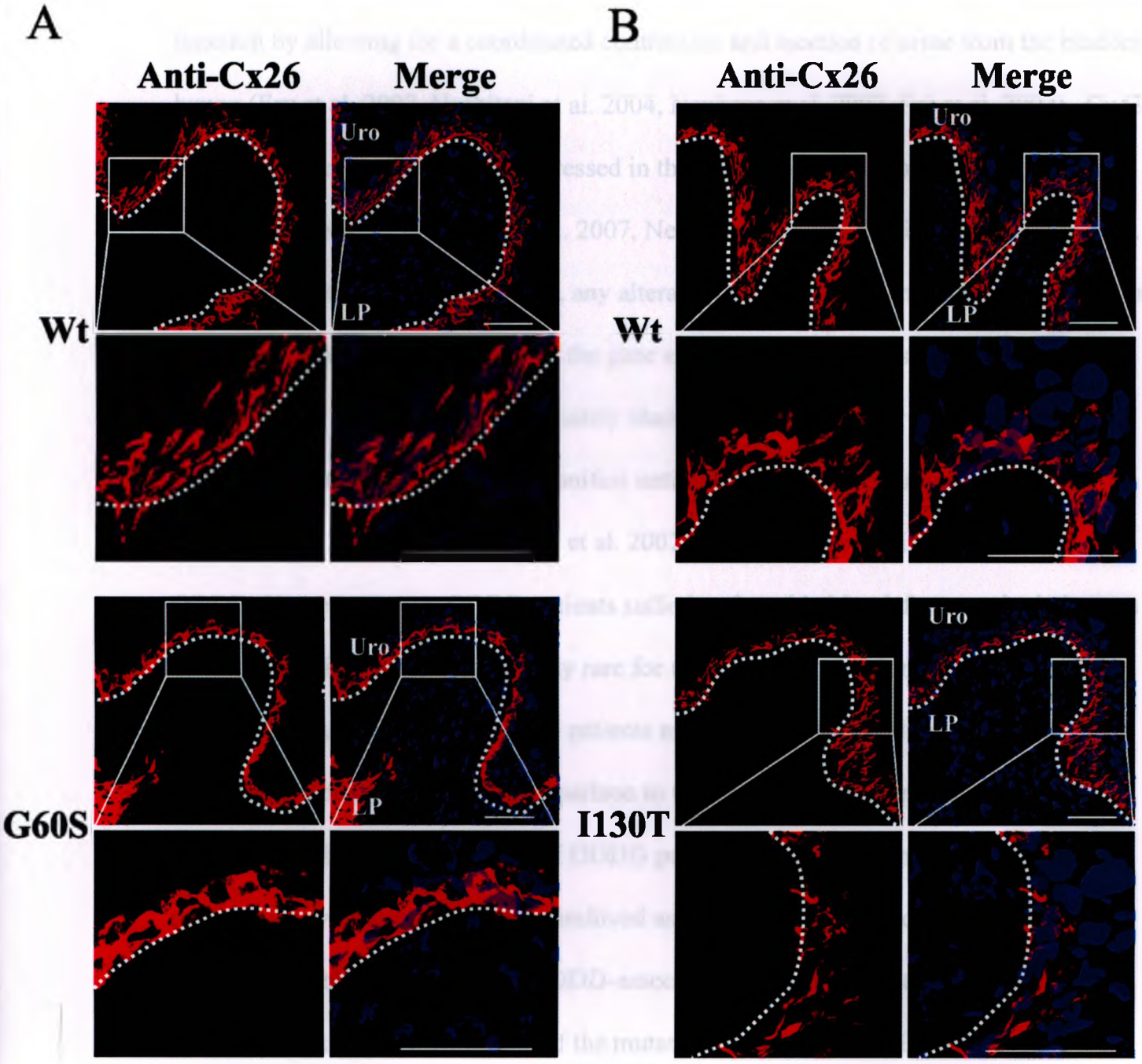
A
B
Anti-Cx26
Merge
Anti-Cx26
Merge
Wt
Wt
G60S
I130T
Uro
LP
Uro
LP
Uro
LP
Uro
LP

Figure 2.10

2.4 Discussion

GJIC is an important bladder feature thought to contribute to normal bladder function by allowing for a coordinated contraction and ejection of urine from the bladder lumen (Fry et al. 2007, Hashitani et al. 2004, Neuhaus et al. 2007, Sui et al. 2002). Cx43 is the most prevalent connexin expressed in the bladder as it is found in both the lamina propria and detrusor layer (Fry et al. 2007, Neuhaus et al. 2005, Sui et al. 2002, Sui et al. 2003, Wang et al. 2006). Therefore, any alteration in Cx43 function, such as that found in ODDD patients, where one copy of the gene encoding Cx43 carries a loss-of-function mutation may affect GJIC and ultimately bladder function. Bladder impairments in ODDD patients generally do not manifest until the second half of an individual's life (Goepel et al. 2010, Loddenkemper et al. 2002), long after the original diagnosis of ODDD. However, many ODDD patients suffering from bladder defects are in their teens or early twenties, which is extremely rare for the general population (Pazenkas et al. 2009). Thus suggesting that ODDD patients may have an increased propensity to developing a bladder defect in comparison to the general population. Therefore, it remains possible that the number of ODDD patients with bladder impairment is under-reported, as symptoms may not be archived as an ODDD-associated disease. It also remains possible that only some ODDD-associated mutations result in bladder impairments and that the location of the mutation, the resulting amino acid change and the patient's genetic background all contribute to the development of bladder impairment. Adding to the complexity, it is unclear if bladder abnormalities reported by ODDD patients are due to neurogenic or myogenic defects (Christ et al. 2003, Haefliger et al. 2002). In the present study we found no difference in the localization, dye transfer or

ATP release between Cx43 mutants which have been linked to bladder impairments (D3N and G143S) and those which have not (G2V and I130T). We also assessed the connexin status in the bladders of two genetically distinct Cx43 mutant mouse models of ODDD (G60S and I130T mice), and found the both mutant mouse lines have reduced levels of phosphorylated Cx43 compared to their wild-type littermates controls. Collectively, these results suggest that any Cx43 gene mutation, regardless of the mutation site, may contribute to the development of incontinence or other bladder abnormalities. Furthermore, mice harbouring systemic Cx43 mutants have an anatomical and histological normal bladder even though Cx43 may be spatially disorganized and exist primarily as species that are incompletely phosphorylated.

2.4.1 Characterization of Cx43 mutants reveal no distinguishable difference between bladder disease –associated and –unassociated Cx43 mutants

Previous studies investigating Cx43 mutants revealed that the majority of Cx43 mutants maintain the ability to assemble into gap junction plaques at sites of cell to cell apposition; as well, all mutants studied acted as dominant-negatives to the gap junction channel function of co-expressed endogenous Cx43 (Dobrowolski et al. 2007, Gong et al. 2007, McLachlan et al. 2005, Roscoe et al. 2005, Shibayama et al. 2005). The focus of our study was to determine if any differences exist between the bladder disease –associated (D3N and G143S) and –unassociated (G2V and I130T) Cx43 mutants. The current information regarding the G2V mutant is limited to its original identification in a patient screen (de la Parra and Zenteno 2007). Our study is the first to report that the G2V mutant contains no apparent assembly defect in both Cx43-negative (HeLa) and Cx43-

positive (NRK) cells. Furthermore, the G2V mutant exhibited loss-of-function channel activity, and was found to act as a dominant-negative to the gap junction channel function of co-expressed endogenous Cx43. These finding are in keeping with studies which revealed that Cx43 mutants D3N (Churko et al. 2011), I130T (Lai et al. 2006, Shibayama et al. 2005), and G143S (Dobrowolski et al. 2007) assembled into non-functional gap junction-like plaques and acted as dominant-negatives to co-expressed Cx43.

Cx43 proteins are also known to form hemichannels in the plasma membrane, which possess the ability to open and release ATP under specific mechanical and chemical stimuli, such as reduced extracellular calcium (Dobrowolski et al. 2007, Stout et al. 2002, Tong et al. 2007). One research group revealed that the G143S mutant has increased hemichannel activity when activated (Dobrowolski et al. 2007). Since, ATP is an important signaling molecule within the bladder (Apodaca 2004, Birder 2001, Cockayne et al. 2000) the potential gain-of-hemichannel function could theoretically contribute to a disease phenotype in the bladder. However, unlike Dobrowolski et al., (2007) we could not detect any G143S or I130T mutant gain-of-hemichannel function that would lead to ATP release. We have no immediate reasoning for these differences but speculate that it may be related to the sensitivity of our ATP release assays, as unexpectedly wild-type Cx43 did not significantly increase ATP release as previously shown (Dobrowolski et al. 2007, Stout et al. 2002, Tong et al. 2007). Two possible explanations exist to explain this discrepancy: Firstly, HeLa cells may be quite efficient at assembling cell surface hemichannels into gap junction channels, thus at any point in time the hemichannel content in HeLa cells that overexpress Cx43 may be quite low. Secondly, our cells may have been too confluent and therefore Cx43 was unable to form

hemichannels, as it was directly sequestered into gap junction channels. Interestingly, one report has suggested that the interaction between the Cx43 intracellular loop and cytoplasmic tail is important in controlling hemichannel activity (Ponsaerts et al. 2010). Therefore, we speculate that the G143S and I130T mutants may disrupt this interaction, resulting in non-functional hemichannels. Nevertheless, we did detect a gain-of-hemichannel function when we expressed the amino terminus localized G2V and D3N mutants. This gain-of-hemichannel function was not evident when the mutants were tagged with GFP suggesting that the bulky GFP may be sterically hindering the function of the Cx43 mutants. The amino terminus is known for its role in voltage-dependent channel gating (Bukauskas and Peracchia 1997, Kyle et al. 2009), and therefore it is possible that these mutants disrupt voltage-gating, allowing the hemichannel to remain in a partially open configuration. It was interesting that none of the Cx43 mutants examined altered the hemichannel activity of endogenous Cx43, suggesting that the mutants were not acting as dominant-negatives to hemichannel function of wild-type Cx43.

Collectively, we found no consistent difference between the bladder disease –associated (D3N and G143S) and –unassociated (G2V and I130T) Cx43 mutants in their intracellular localization, channel forming ability, ability to form hemichannels and ability to act as dominant-negatives on co-expressed endogenous Cx43. We speculate that the *in vitro* studies preformed may be insufficient to discriminate between bladder disease –associated and –unassociated Cx43 mutants, which led us to examine the connexin status in the bladders of two mutant mouse lines that mimic ODDD.

2.4.2 Characterization of Cx43 in the bladder of G60S and I130T mutant mice

Neurological defects are frequently found in ODDD patients, although they generally do not manifest until the second half of the patient's life (Loddenkemper et al. 2002). Bladder abnormalities have been reported in ~12% of ODDD patients and have, in a couple of cases, been linked to neurological defects; however, there is increasing evidence that suggests some bladder abnormalities are the result of myogenic defects (Christ et al. 2003, Haefliger et al. 2002). Many reports have localized Cx43 within the lamina propria and detrusor layers of the bladder and other studies have linked Cx43-based gap junctions to various bladder syndromes (Christ et al. 2003, Mori et al. 2005, Neuhaus et al. 2007, Sui et al. 2002). It therefore remains possible that ODDD mutants, which disrupt Cx43 function (Dobrowolski et al. 2007, Gong et al. 2007, McLachlan et al. 2005, Roscoe et al. 2005, Shibayama et al. 2005), may ultimately affect bladder function. In order to assess this possibility, we included in our study two Cx43 mutant mouse lines, G60S and I130T, both of which have been shown to phenotypically resemble ODDD patients (Flenniken et al. 2005, Kalcheva et al. 2007, Langlois et al. 2007, Toth et al. 2010), but it is unknown if they suffer from incontinence or any bladder abnormalities. Importantly, both mutant mice are predicted to possess a 1:1 ratio of mutant to wild-type Cx43, matching the phenotype of human ODDD patients. As well, the two mutant mouse lines were generated on different genetic backgrounds, more readily mimicking the diverse genetic background of ODDD patients. Previous studies have revealed that both mutant mouse lines have altered tissue function and/or development of the heart, bone, skin and teeth (Flenniken et al. 2005, Kalcheva et al. 2007, Langlois et al. 2007, Toth et al. 2010); however, no studies have assessed the

connexin status within the bladder. Here, we aimed to determine the connexin localization and expression profile, as possible indicators of altered bladder function.

2.4.3 Differential Cx43 phosphorylation and subsequent distribution may disrupt bladder signaling and contractile stimulus propagation in mutant mice

Previous studies have localized Cx43 to the rat lamina propria and detrusor layers of the bladder (Fry et al. 2007, Neuhaus et al. 2005, Sui et al. 2002, Sui et al. 2003, Wang et al. 2006). One of the simplest indicators of an overactive bladder syndrome is to look for detrusor hypertrophy (Miyazato et al. 2009, Uvelius et al. 1984). First, assessment of wild-type and mutant mice revealed an anatomically similar bladder and no differences were observed in the appearance or thickness of the detrusor layer. Within the lamina propria, we found Cx43-positive staining in all mice localized to what was assumed to be fibroblasts and myofibroblasts, based on vimentin-positive staining (Neuhaus et al. 2007, Sui et al. 2002). Myofibroblasts, in particular, are important in forming a functional syncytium for the rapid transmission of chemical and electrical stimuli, required for normal bladder function (Fry et al. 2007, Sui et al. 2002, Wiseman et al. 2003). Cx43 in the detrusor is thought to mediate the propagation of action potentials from one smooth muscle bundle to the next, allowing for a uniform bladder contraction (Hashitani et al. 2004). The results showed that while the wild-type and I130T mice displayed punctate gap junction-like structures, the G60S mutant mice displayed a more diffuse intracellular Cx43 expression profile in addition to several punctate structures. A similar diffuse localization profile of Cx43 was also observed within the detrusor layer of G60S mice, in which Cx43 has been localized to the border of smooth muscle bundles, as seen

previously (Hashitani et al. 2004, Wang et al. 2006). The more diffuse Cx43 expression profile seen in the G60S mice bladders is correlated with the reduction in the highly phosphorylated Cx43 species (P1 and P2), known to be important in of gap junction plaque formation and GJIC (Koo et al. 1997, Solan and Lampe 2009). Interestingly, the I130T mice bladders also exhibited a reduction in the highly phosphorylated Cx43 species, as well as reduced total Cx43 protein levels. Therefore, we conclude that in both mutant mouse lines Cx43 is not likely forming fully assembled gap junction plaques, which likely corresponds to non-functional gap junction channels in myofibroblasts and connected smooth muscle bundles, similar to that seen in other cell types (Flenniken et al. 2005, Kalcheva et al. 2007, Lai et al. 2006, Manias et al. 2008, McLachlan et al. 2005, Shibayama et al. 2005).

Even though bladder abnormalities are most commonly linked to Cx43 increases (Christ et al. 2003, Imamura et al. 2009, Li et al. 2007, Mori et al. 2005), it remains possible that a reduction in Cx43 levels and/or a resulting decrease in GJIC may also alter bladder function. Reduced GJIC may alter electrical and chemical stimulus propagation within the bladder, resulting in incomplete bladder contractions, increased residual urine levels and ultimately increased urinary frequency in both mutant mouse lines. Collectively, if GJIC is required for normal bladder function, this would suggest that the G60S and I130T mutants would possess the same propensity to develop a bladder phenotype. Additional behavioral studies are necessary to see if Cx43 mutant mice suffer from frequent urination and/ or bladder leakage.

2.4.4 Cx26 unique basal urothelial expression profile may contribute to bladder function

Even though our study revealed no Cx43 staining in the bladder urothelium, other groups have localized both Cx43 and Cx26 within this layer (Grossman et al. 1994, Haefliger et al. 2002). Therefore, as a result of Cx26 being localized to the urothelial layer as well (Gee et al. 2003, Grossman et al. 1994, Haefliger et al. 2002, Ikeda et al. 2007), we speculated that Cx26 may be upregulated in the urothelium to compensate for the overall reduction of Cx43 in the lamina propria and detrusor layers through potential signaling cross-talk mechanisms. The function of Cx26 within the urothelium is still unknown although it is thought to play a role in signal transduction during bladder filling (Ikeda et al. 2007). Cx26 localization in the urothelium has been reported to be typically at sites of cell to cell apposition (Haefliger et al. 2002, Ikeda et al. 2007); however, one group has reported an intracellular profile of Cx26 expressed throughout the entire urothelium (Gee et al. 2003). Other reports have also indicated that Cx26 hemichannels can release ATP into the extracellular environment (Huckstepp et al. 2010, Majumder et al. 2010), which may alter bladder function. In our study, the levels of Cx26 were similar between mutant and control groups. Interestingly, Cx26 displayed a unique intracellular urothelium expression profile localized to only the basal urothelium, suggesting that few, if any, gap junction channels or hemichannels are formed in this cell layer. Nevertheless, we found no evidence that Cx26 was being upregulated to compensate for the proposed loss-of-Cx43-function in the bladder.

2.4.5 Conclusion

In this study, we found no difference in the localization, dye transfer or ATP release between bladder disease –associated (D3N and G143S) and –unassociated (G2V and I130T) Cx43 mutants. Thus, the reason why only some ODDD patients suffer from incontinence is not likely linked to how a specific mutation changes the channel function but rather rooted in other mechanisms. It is possible that other defects exist in the bladder –associated mutants that have not been revealed in the present study such as alterations in their ability to interact with members of the gap junction proteome (Laird 2010), or alternatively, any ODDD-linked Cx43 mutant may have the capacity to contribute to the onset of bladder disease that is governed by additional genetic factors and the aging process. Future studies will require additional mutant mice that harbor specific mutations that have strong linkages to patients with bladder abnormalities.

2.5 References

Abrams P, Cardozo L, Fall M, Griffiths D, Rosier P, Ulmsten U *et al.* (2003). The standardisation of terminology in lower urinary tract function: report from the standardisation sub-committee of the International Continence Society. *Urology* 61: 37-49.

Alexander DB, Goldberg GS (2003). Transfer of biologically important molecules between cells through gap junction channels. *Curr Med Chem* 10: 2045-2058.

Andersson KE (1999). Changes in bladder tone during filling: pharmacological aspects. *Scand J Urol Nephrol Suppl* 201: 67-72; discussion 76-99.

Apodaca G (2004). The uroepithelium: Not just a passive barrier. *Traffic* 5: 117-128.

Birder LA (2001). Involvement of the urinary bladder urothelium in signaling in the lower urinary tract. *Proceedings of the Western Pharmacology Society* 44: 85-86

Birder LA, de Groat WC (2007). Mechanisms of Disease: involvement of the urothelium in bladder dysfunction. *Nature Clinical Practice Urology* 4: 46-54.

Brading AF (1997). A myogenic basis for the overactive bladder. *Urology* 50: 57-67; discussion 68-73.

Bukauskas FF, Peracchia C (1997). Two distinct gating mechanisms in gap junction channels: CO2-sensitive and voltage-sensitive. *Biophys J* 72: 2137-2142.

Christ GJ, Day NS, Day M, Zhao W, Persson K, Pandita RK *et al.* (2003). Increased connexin43-mediated intercellular communication in a rat model of bladder overactivity in vivo. *Am J Physiol Regul Integr Comp Physiol* 284: R1241-1248.

Churko JM, Shao Q, Gong XQ, Swoboda KJ, Bai D, Sampson J *et al.* (2011). Human dermal fibroblasts derived from oculodentodigital dysplasia patients suggest that patients may have wound-healing defects. *Hum Mutat* 32: 456-466.

Cockayne DA, Hamilton SG, Zhu QM, Dunn PM, Zhong Y, Novakovic S *et al.* (2000). Urinary bladder hyporeflexia and reduced pain-related behaviour in P2X3-deficient mice. *Nature* 407: 1011-1015.

Dahl E, Winterhager E, Traub O, Willecke K (1995). Expression of gap junction genes, connexin40 and connexin43, during fetal mouse development. *Anat Embryol (Berl)* 191: 267-278.

de Groat WC (2004). The urothelium in overactive bladder: Passive bystander or active participant? *Urology* 64: 7-11.

de la Parra DR, Zenteno JC (2007). A new GJA1 (connexin 43) mutation causing oculodentodigital dysplasia associated to uncommon features. *Ophthalmic Genet* 28: 198-202.

Dobrowolski R, Sommershof A, Willecke K (2007). Some oculodentodigital dysplasia-associated Cx43 mutations cause increased hemichannel activity in addition to deficient gap junction channels. *J Membr Biol* 219: 9-17.

Eiberger J, Degen J, Romualdi A, Deutsch U, Willecke K, Sohl G (2001). Connexin genes in the mouse and human genome. *Cell Commun Adhes* 8: 163-165.

Flenniken AM, Osborne LR, Anderson N, Ciliberti N, Fleming C, Gittens JE *et al.* (2005). A Gja1 missense mutation in a mouse model of oculodentodigital dysplasia. *Development* 132: 4375-4386.

Fry CH, Sui GP, Kanai AJ, Wu C (2007). The function of suburothelial myofibroblasts in the bladder. *Neurourol Urodyn* 26: 914-919.

Gee J, Tanaka M, Grossman HB (2003). Connexin 26 is abnormally expressed in bladder cancer. *J Urol* 169: 1135-1137.

Gehi R, Shao Q, Laird DW (2011). Pathways regulating the trafficking and turnover of pannexin1 and the role of the carboxyl terminal domain. *J Biol Chem.*

Goepel M, Kirschner-Hermanns R, Welz-Barth A, Steinwachs KC, Rubben H (2010). Urinary incontinence in the elderly: part 3 of a series of articles on incontinence. *Dtsch Arztebl Int* 107: 531-536.

Gong XQ, Shao Q, Langlois S, Bai D, Laird DW (2007). Differential potency of dominant negative connexin43 mutants in oculodentodigital dysplasia. *J Biol Chem* 282: 19190-19202.

Goodenough DA, Goliger JA, Paul DL (1996). Connexins, connexons, and intercellular communication. *Annu Rev Biochem* 65: 475-502.

Grossman HB, Liebert M, Lee IW, Lee SW (1994). Decreased connexin expression and intercellular communication in human bladder cancer cells. *Cancer Res* 54: 3062-3065.

Haefliger JA, Tissieres P, Tawadros T, Formenton A, Beny JL, Nicod P *et al.* (2002). Connexins 43 and 26 are differentially increased after rat bladder outlet obstruction. *Exp Cell Res* 274: 216-225.

Haferkamp A, Mundhenk J, Bastian PJ, Reitz A, Dorsam J, Pannek J *et al.* (2004). Increased expression of connexin 43 in the overactive neurogenic detrusor. *Eur Urol* 46: 799-805.

Hashitani H, Yanai Y, Suzuki H (2004). Role of interstitial cells and gap junctions in the transmission of spontaneous Ca2+ signals in detrusor smooth muscles of the guinea-pig urinary bladder. *J Physiol* 559: 567-581.

Hu TW, Wagner TH, Bentkover JD, Leblanc K, Zhou SZ, Hunt T (2004). Costs of urinary incontinence and overactive bladder in the United States: a comparative study. *Urology* 63: 461-465.

Huckstepp RT, Eason R, Sachdev A, Dale N (2010). CO2-dependent opening of connexin 26 and related beta connexins. *J Physiol* 588: 3921-3931.

Ikeda Y, Fry C, Hayashi F, Stolz D, Griffiths D, Kanai A (2007). Role of gap junctions in spontaneous activity of the rat bladder. *Am J Physiol Renal Physiol* 293: F1018-1025.

Imamura M, Negoro H, Kanematsu A, Yamamoto S, Kimura Y, Nagane K *et al.* (2009). Basic fibroblast growth factor causes urinary bladder overactivity through gap junction generation in the smooth muscle. *Am J Physiol Renal Physiol* 297: F46-54.

John H, Wang X, Hauri D, Maake C (2003a). [Gap junctions in the human urinary bladder]. *Aktuelle Urol* 34: 328-332.

John H, Wang X, Wehrli E, Hauri D, Maake C (2003b). Evidence of gap junctions in the stable nonobstructed human bladder. *J Urol* 169: 745-749.

Kalcheva N, Qu J, Sandeep N, Garcia L, Zhang J, Wang Z *et al.* (2007). Gap junction remodeling and cardiac arrhythmogenesis in a murine model of oculodentodigital dysplasia. *Proc Natl Acad Sci U S A* 104: 20512-20516.

Kanai AJ (2011). Afferent mechanism in the urinary tract. *Handb Exp Pharmacol*: 171-205.

Kang J, Kang N, Lovatt D, Torres A, Zhao Z, Lin J *et al.* (2008). Connexin 43 hemichannels are permeable to ATP. *J Neurosci* 28: 4702-4711.

Koo SK, Kim DY, Park SD, Kang KW, Joe CO (1997). PKC phosphorylation disrupts gap junctional communication at G0/S phase in clone 9 cells. *Mol Cell Biochem* 167: 41-49.

Kuhn A, Stadlmayr W, Monga A, Cameron I, Anthony F (2008). A pilot study of Connexin 43 (Cx43) in human bladder tissue in patients with idiopathic detrusor overactivity. *Eur J Obstet Gynecol Reprod Biol* 141: 83-86.

Kyle Jk DA *et al.* (2009). The N terminus of connexin37 contains an alpha-helix that is required for channel function. *J Biol Chem* 284: 20418-20427.

Lai A, Le DN, Paznekas WA, Gifford WD, Jabs EW, Charles AC (2006). Oculodentodigital dysplasia connexin43 mutations result in non-functional connexin hemichannels and gap junctions in C6 glioma cells. *J Cell Sci* 119: 532-541.

Laird DW (2010). The gap junction proteome and its relationship to disease. *Trends Cell Biol* 20: 92-101.

Langlois S, Maher AC, Manias JL, Shao Q, Kidder GM, Laird DW (2007). Connexin levels regulate keratinocyte differentiation in the epidermis. *J Biol Chem* 282: 30171-30180.

Li H, Liu TF, Lazrak A, Peracchia C, Goldberg GS, Lampe PD *et al.* (1996). Properties and regulation of gap junctional hemichannels in the plasma membranes of cultured cells. *J Cell Biol* 134: 1019-1030.

Li L, Jiang C, Hao P, Li W, Song C, Song B (2007). Changes of gap junctional cell-cell communication in overactive detrusor in rats. *Am J Physiol Cell Physiol* 293: C1627-1635.

Loddenkemper T, Grote K, Evers S, Oelerich M, Stogbauer F (2002). Neurological manifestations of the oculodentodigital dysplasia syndrome. *J Neurol* 249: 584-595.

Maeda S, Nakagawa S, Suga M, Yamashita E, Oshima A, Fujiyoshi Y *et al.* (2009). Structure of the connexin 26 gap junction channel at 3.5 A resolution. *Nature* 458: 597-602.

Majumder P, Crispino G, Rodriguez L, Ciubotaru CD, Anselmi F, Piazza V *et al.* (2010). ATP-mediated cell-cell signaling in the organ of Corti: the role of connexin channels. *Purinergic Signal* 6: 167-187.

Manias JL, Plante I, Gong XQ, Shao Q, Churko J, Bai D *et al.* (2008). Fate of connexin43 in cardiac tissue harbouring a disease-linked connexin43 mutant. *Cardiovasc Res* 80: 385-395.

McLachlan E, Manias JL, Gong XQ, Lounsbury CS, Shao Q, Bernier SM *et al.* (2005). Functional characterization of oculodentodigital dysplasia-associated Cx43 mutants. *Cell Commun Adhes* 12: 279-292.

Miller J, Hoffman E (2006). The causes and consequences of overactive bladder. *J Womens Health (Larchmt)* 15: 251-260.

Miyazato M, Sugaya K, Nishijima S, Kadekawa K, Machida N, Oshiro Y *et al.* (2009). Changes of bladder activity and connexin 43-derived gap junctions after partial bladder-outlet obstruction in rats. *Int Urol Nephrol* 41: 815-821.

Mori K, Noguchi M, Matsuo M, Nomata K, Suematsu T, Kanetake H (2005). Decreased cellular membrane expression of gap junctional protein, connexin 43, in rat detrusor muscle with chronic partial bladder outlet obstruction. *Urology* 65: 1254-1258.

Musil LS, Goodenough DA (1990). Gap junctional intercellular communication and the regulation of connexin expression and function. *Curr Opin Cell Biol* 2: 875-880.

Neuhaus J, Pfeiffer F, Wolburg H, Horn LC, Dorschner W (2005). Alterations in connexin expression in the bladder of patients with urge symptoms. *BJU Int* 96: 670-676.

Neuhaus J, Heinrich M, Schlichting N, Oberbach A, Fitzl G, Schwalenberg T *et al.* (2007). [Structure and function of suburothelial myofibroblasts in the human urinary bladder under normal and pathological conditions]. *Urologe A* 46: 1197-1202.

Paznekas WA, Boyadjiev SA, Shapiro RE, Daniels O, Wollnik B, Keegan CE *et al.* (2003). Connexin 43 (GJA1) mutations cause the pleiotropic phenotype of oculodentodigital dysplasia. *Am J Hum Genet* 72: 408-418.

Paznekas WA, Karczeski B, Vermeer S, Lowry RB, Delatycki M, Laurence F *et al.* (2009). GJA1 mutations, variants, and connexin 43 dysfunction as it relates to the oculodentodigital dysplasia phenotype. *Hum Mutat* 30: 724-733.

Peracchia C (2004). Chemical gating of gap junction channels; roles of calcium, pH and calmodulin. *Biochim Biophys Acta* 1662: 61-80.

Ponsaerts R, De Vuyst E, Retamal M, D'Hondt C, Vermeire D, Wang N *et al.* (2010). Intramolecular loop/tail interactions are essential for connexin 43-hemichannel activity. *FASEB J* 24: 4378-4395.

Roscoe W, Veitch GI, Gong XQ, Pellegrino E, Bai D, McLachlan E *et al.* (2005). Oculodentodigital dysplasia-causing connexin43 mutants are non-functional and exhibit dominant effects on wild-type connexin43. *J Biol Chem* 280: 11458-11466.

Rossello RA, Kohn DH (2010). Cell communication and tissue engineering. *Commun Integr Biol* 3: 53-56.

Shibayama J, Paznekas W, Seki A, Taffet S, Jabs EW, Delmar M *et al.* (2005). Functional characterization of connexin43 mutations found in patients with oculodentodigital dysplasia. *Circ Res* 96: e83-91.

Solan JL, Lampe PD (2009). Connexin43 phosphorylation: structural changes and biological effects. *Biochem J* 419: 261-272.

Stout CE, Costantin JL, Naus CC, Charles AC (2002). Intercellular calcium signaling in astrocytes via ATP release through connexin hemichannels. *J Biol Chem* 277: 10482-10488.

Sui GP, Rothery S, Dupont E, Fry CH, Severs NJ (2002). Gap junctions and connexin expression in human suburothelial interstitial cells. *BJU Int* 90: 118-129.

Sui GP, Coppen SR, Dupont E, Rothery S, Gillespie J, Newgreen D *et al.* (2003). Impedance measurements and connexin expression in human detrusor muscle from stable and unstable bladders. *BJU Int* 92: 297-305.

Tong D, Li TY, Naus KE, Bai DL, Kidder GM (2007). In vivo analysis of undocked connexin43 gap junction hemichannels in ovarian granulosa cells. *Journal of Cell Science* 120: 4016-4024.

Tong D, Colley D, Thoo R, Li TY, Plante I, Laird DW *et al.* (2009). Oogenesis defects in a mutant mouse model of oculodentodigital dysplasia. *Dis Model Mech* 2: 157-167.

Toth K, Shao Q, Lorentz R, Laird DW (2010). Decreased levels of Cx43 gap junctions result in ameloblast dysregulation and enamel hypoplasia in Gja1Jrt/+ mice. *J Cell Physiol* 223: 601-609.

Uvelius B, Persson L, Mattiasson A (1984). Smooth muscle cell hypertrophy and hyperplasia in the rat detrusor after short-time infravesical outflow obstruction. *J Urol* 131: 173-176.

Wang HZ, Brink PR, Christ GJ (2006). Gap junction channel activity in short-term cultured human detrusor myocyte cell pairs: gating and unitary conductances. *Am J Physiol Cell Physiol* 291: C1366-1376.

Wiseman OJ, Fowler CJ, Landon DN (2003). The role of the human bladder lamina propria myofibroblast. *Bju International* 91: 89-93.

Zhou Y, Yang W, Lurtz MM, Ye Y, Huang Y, Lee HW *et al.* (2007). Identification of the calmodulin binding domain of connexin 43. *J Biol Chem* 282: 35005-35017.

CHAPTER 3

Characteristics and Functions of Connexin43 Recessive Mutants linked to Oculodentodigital Dysplasia

3.0 Abstract

A rare developmental disease known as oculodentodigital dysplasia (ODDD) generally manifests as a result of one of over sixty known autosomal-dominant mutations in the gene *GJA1*, which encodes for connexin43 (Cx43). Recently, two autosomal-recessive mutations, resulting in the Cx43 mutants R76H and R33X (X represents a nonsense mutation resulting in a stop codon), have been reported in a genetic screen of patients suspected of having ODDD, but the characteristics of these mutants and how they cause disease only in the homozygous state remains unknown. To investigate the characteristics of the R76H and R33X recessive mutant we expressed both mutants in reference cell lines that lack or contain endogenous Cx43. Immunofluorescence revealed that only the R76H mutant exhibited the ability to form gap junction plaques at sites of cell to cell apposition, while the R33X mutant displayed a more diffuse intracellular expression profile. Lucifer yellow dye transfer studies revealed that the R76H mutant retained some residual gap junction channel function, which was not seen in the R33X mutant. In addition, the expression of either mutant did not affect the ability of cells to release ATP. We proposed that the R76H and R33X mutants are less potent at impairing gap junctional intercellular communication in the heterozygous state than other well studied autosomal-dominant mutants allowing heterozygous carriers of this mutation to be disease free. The residual gap junction channel forming ability of the R76H mutant and the inability of the R33X to oligomerize into gap junction channels is predicted to allow for carriers of this gene mutation to be disease-free with disease only manifesting in cases where a patient harbors the mutation on both alleles.

Keywords: Connexin, Cx43, mutants, recessive, GJIC, hemichannel, homozygous

3.1 Introduction

Gap junctions are specialized membrane channels that allow for the transfer of small molecules, ions, metabolites and second messengers between the cytoplasms of two connected cells (Alexander and Goldberg 2003, Musil and Goodenough 1990). Gap junctions are produced as the result of an oligomerization of six connexin (Cx) proteins, forming a connexon, which is delivered to the plasma membrane (Goodenough et al. 1996). A connexon can exist either as a singular unit within the plasma membrane, known as a hemichannel, or dock with a connexon of from an apposing cell forming a gap junction channel (Li et al. 1996, Musil and Goodenough 1990). There are 21 members of the human connexin family, all of which share a similar membrane topology consisting of four transmembrane domains, two extracellular loops, an intracellular loop and cytoplasmically exposed amino and carboxyl termini (Sohl and Willecke 2004).

Connexins are temporally and spacially regulated in human physiology, and many cell types are known to express one or more connexin isoforms. As a result, various connexin hexamers can exist, resulting in channel selectivity unique to its connexin constituents (Brink et al. 1997, Falk 2000). Connexons and gap junction channels composed of one connexin are termed homomeric and homotypic respectively, whereas those composed of two or more distinct connexins are termed heteromeric and heterotypic, respectively (Rackauskas et al. 2010). Therefore, as a result of the ubiquitous use of gap junctional communication it becomes apparent that an alteration in a cells ability to communicate can produced a number of human pathologies (Cronier et al. 2009).

Mutations in the gene encoding the almost ubiquitously expressed connexin, Cx43, results in the rare autosomal-dominant disease called oculodentodigital dysplasia (ODDD). ODDD is characterized by several common clinical phenotypes such as syndactyly and camptodactyly of the digits, microdontia, enamel loss, ophthalmic defects and craniofacial abnormalities (Paznekas et al. 2003). To date, over 60 known mutations, spanning the Cx43 gene sequence, have been linked to ODDD (Paznekas et al. 2009). The majority of the mutations associated with ODDD are autosomal-dominant and have been shown to reduce channel function and act as dominant-negatives to the function of co-expressed wild-type Cx43 (Gong et al. 2007, McLachlan et al. 2005, Roscoe et al. 2005). However, recent clinical presentations and genetic screening have revealed two autosomal-recessive mutations linked to ODDD, corresponding to the Cx43 mutants R76H and R33X (Paznekas et al. 2003, Pizzuti et al. 2004, Richardson et al. 2006). The current information on these patients is very limited. Interestingly, recessive Cx43 mutations provide a unique platform to study the function of Cx43 throughout various tissues and organs of the human body. Previous studies employing Cx43 knock-out mice revealed that Cx43 is required for normal tissue development and that the loss of Cx43 is lethal at birth (Reaume et al. 1995). In humans, the R33X autosomal recessive mutant, consisting of only the amino terminus and a partial fragment of the first transmembrane domain of Cx43 will not form gap junction channels, suggesting that these patients are essentially equivalent to a Cx43 knockout. Unlike knock-out mice, patients homozygous for the R33X mutant do survive past birth, although they display very severe clinical symptoms of ODDD including small deep set eyes, complete syndactyly, ophthalmological defects and dental abnormalities, (Richardson et al. 2006) and their life

expectancy is expected to be short. However, the patients harbouring the autosomal recessive R76H mutant appeared to share similarities to the autosomal dominant ODDD patients including hypotrichosis and dental abnormalities (Pizzuti et al. 2004). This raises the possibility that the R76H mutant may have residual gap junction channel function, although this has yet to be tested.

In this study we assessed the localization and function of two autosomal recessive Cx43 mutants, R33X and R76H. Collectively, the information gained may provide insight into why patients harbouring the autosomal recessive R33X mutant may possess greater morbidities than patients harbouring the R76H mutant.

3.2 Methods

3.2.1 Transient Transfections

All cell culture components were purchased from Invitrogen (Burlington, ON), and Becton-Dickenson (BD, Franklin Lakes, NJ). Human cervical cancer (HeLa) and normal rat kidney (NRK) cells were cultured as previously reported (Jordan et al. 1999, Thomas et al. 2005). Human untagged and/or GFP-tagged Cx43 mutant R76H and R33X cDNA constructs were purchased from Norclone Biotech Industries (London, ON). Transient transfected cells were generated using Lipofectamine 2000 reagent (Invitrogen) as recommended by the manufacturer and as previously described (Roscoe et al. 2005) with the following changes. Transfections were performed using 5 μg of plasmid cDNA and 2 μL of Lipofectamine 2000 reagent. Untagged Cx43 mutants used in the microinjection dye transfer assays required a co-transfection with 1 μg DsRed plasmid cDNA to discriminate between transfected and non-transfected cells. For ATP release studies, HeLa and NRK cells were seeded in 12 well culture plates, containing one glass coverslip per well, one day prior to transfection at 200,000 cells/well and 90,000 cells/well, respectively.

3.2.2 Immunofluorescence

Transfected cells were fixed and immunolabeled as previously described (Roscoe et al. 2005). Briefly, cells grown on 12 mm glass coverslips were fixed for 15 minutes with precooled -20°C 80% methanol-20% acetone at 4°C. All antibodies were diluted in 3% bovine serum albumin (BSA) (Sigma-Aldrich, St Louis, MO) and 0.02% Triton-X100 in PBS. Cells expressing untagged or GFP-tagged R76H and R33X Cx43 mutants

were incubated for one hour at room temperature with polyclonal rabbit anti-Cx43 primary antibody (2 μg/ml; Sigma). Cells were washed several times with PBS and incubated for 1 hour at room temperature with anti-rabbit AlexaFluor 555 antibody (4 μg/ml; Invitrogen). Hoechst 33342 nuclear stain (10 μg/ml; Molecular Probes, Eugene, OR) was applied for 5 minutes at room temperature followed by a 5 minute wash with ddH_2O. Labelled cells were imaged on a Zeiss LSM 510 Meta confocal microscope as previously described (Roscoe et al. 2005) and images were analyzed using a Zeiss LSM.

3.2.3 Microinjection Dye Transfer Assay.

HeLa or NRK cells clusters transiently expressing untagged or GFP-tagged Cx43, R76H, and R33X were selected for microinjection. For GFP tagged mutants, cell clusters with clear GFP signals in contacting cells were selected for microinjection. For untagged mutants, cell clusters expressing DsRed in contacting cells were selected for microinjection. A single transfected cell was pressure injected as previously described (Roscoe et al. 2005). Digital images were collected using a Lecia DM IRE2 inverted epifluorescence microscope with a charged coupled camera (Hamamstsu Photonics, Japan) using OpenLab software. If dye was successfully transferred to a GFP or RFP expressing cell contacting the microinjected cell, the test was recorded as a positive. A minimum of 50 microinjections were preformed for each mutant tested.

3.2.4 ATP Release Assay

HeLa or NRK cells transiently transfected with cDNA encoding untagged or GFP-tagged Cx43, R76H and R33X were selected. ATP release assays were performed as described previously (Tong et al. 2007), with the following changes. Cells were washed and incubated in either HBSS (Invitrogen) containing calcium and magnesium or divalent-cation-free (DCF)-HBSS (Ca^{2+} and Mg^{2+} were replaced by 2mM EGTA). Following a 15 minute incubation at 37°C, 10 µL of the incubation solution was mixed with 90 µL of the ATP Determination Kit (Invitrogen) reaction solution in a 96-well plate. Bioluminescence (at a wavelength of ~560 nm) was measured using a luminometer (PerkinElmer, Waltham MA) and ATP concentrations were determined. To determine transfection efficiency, coverslips containing transfected cells were fixed, labeled and imaged for Cx43 as previously described (Roscoe et al. 2005). Four images per transfection were assessed and the transfection efficiency was recorded by an observer blinded to the treatment conditions.

3.2.5 Statistics

Microinjection and ATP release assay results were analyzed using a Student two-tailed independent sample t-test, with significance denoted by $P<0.05$. All results were analysed using GraphPad Prism 4.03 software, and are presented as mean ± standard error.

3.3 Results

3.3.1 The Cx43 R76H mutant localized to sites of cell to cell appositions in HeLa and NRK cells

Cx43-negative HeLa cells and Cx43-positive NRK cell were transiently transfected with cDNA constructs encoding the autosomal recessive R76H and R33X Cx43 mutants. Immunofluorescence revealed that control wild-type GFP-tagged and untagged Cx43 expressed in HeLa (Figure 3.1A) or NRK (Figure 3.1B) cells resulted in the formation of gap junction-like plaque structures at sites of cell to cell apposition. Similarly, the R76H mutant assembled into gap junction-like plaques at sites of cell to cell appositions in both HeLa (Figure 3.1A) and NRK (Figure 3.1 B) cells. However, the mutant R33X failed to form any plaques and exhibited a homogenous cytoplasm and nuclear localization profile in both cell lines (Figure 3.1A,B). The R33X mutant was not stained with anti-Cx43 antibody (Figure 3.1A) as the Cx43 antibody used was developed using an epitope from the carboxyl terminus, and thus would not be able to bind to the 33 amino acid amino terminus fragment.

3.3.2 Gap junction channels formed from the R76H mutant are partially functional

To determine if the autosomal recessive mutants (R76H and R33X) formed functional channels, mutant expressing HeLa cells were microinjected with Lucifer yellow and dye transfer was assessed. The R76H mutant was found to exhibit a ~20-35% higher incidence of dye transfer compared to untransfected HeLa cells (Figure 3.2A). These results suggest that the channels formed by the R76H mutant can form functional

Figure 3.1 The R76H, but not the R33X, mutant forms gap junction-like plaques at sites of cell-cell appositions. HeLa and NRK cells were transfected with cDNA encoding Cx43, Cx43-GFP, R76H, R76H-GFP or R33X-GFP. The R76H mutant assembled into gap junction-like plaques (arrowheads) at sites of cell-cell appositions in both HeLa (A) and NRK (B) cells, while the R33X mutant displayed a cytoplasm and nuclear localization profile in both cell lines (A, B). Cells were immunolabeled for Cx43 (Red). Controls HeLa and HeLa-Cx43-GFP, NRK and NRK-Cx43-GFP are the same data set used in chapter 2, sections 2.3. Green = GFP, Blue = Hoechst Nuclear Stain. Bar = 20 μm.

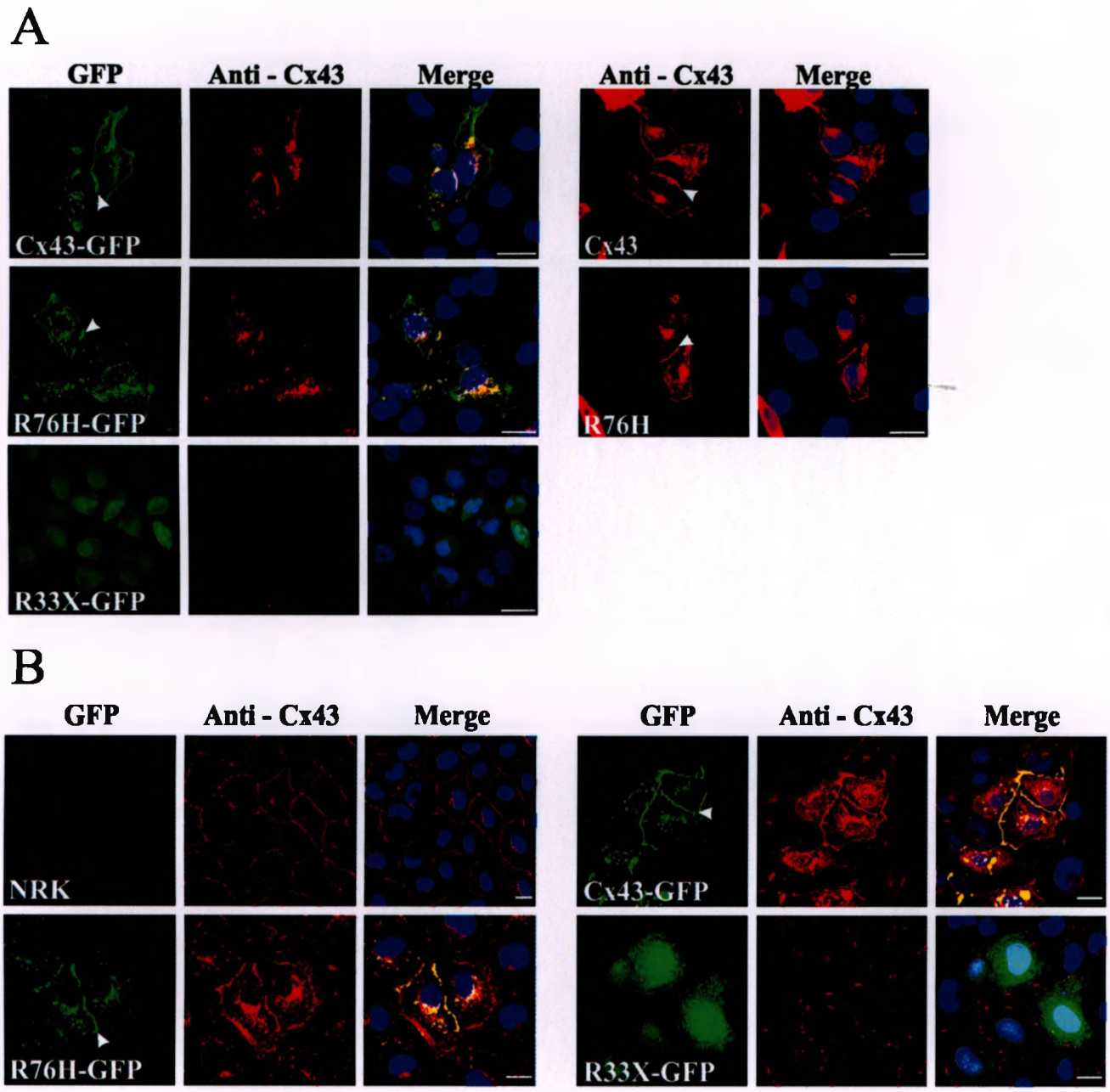
A
GFP
Anti - Cx43
Merge
Anti - Cx43
Merge
Cx43-GFP
Cx43
R76H-GFP
R76H
R33X-GFP
B
GFP
Anti - Cx43
Merge
GFP
Anti - Cx43
Merge
NRK
Cx43-GFP
R76H-GFP
R33X-GFP

Figure 3.1

Figure 3.2 The R76H Cx43 mutant can be assembled into gap junction channels and acts as a dominant-negative to the function of wild-type Cx43. HeLa and NRK cells expressing the Cx43 mutants were subjected to microinjection with Lucifer yellow and assessed for dye transfer. HeLa cells expressing the R76H or GFP-tagged R76H mutant exhibited a significant increase in the incidence of dye coupled cells while the R33X mutant expressing cells failed to pass Lucifer yellow (A). However, both recessive mutants acted as dominant-negatives to the gap junction function of endogenously expressed Cx43 (B). Controls HeLa, HeLa (Cx43-GFP), NRK and NRK (Cx43-GFP) are the same data set used in chapter 2, sections 2.3. Bars represent means ± s.e.m. *, a, b = ($P<0.05$). N = 5-6 replicate transfections

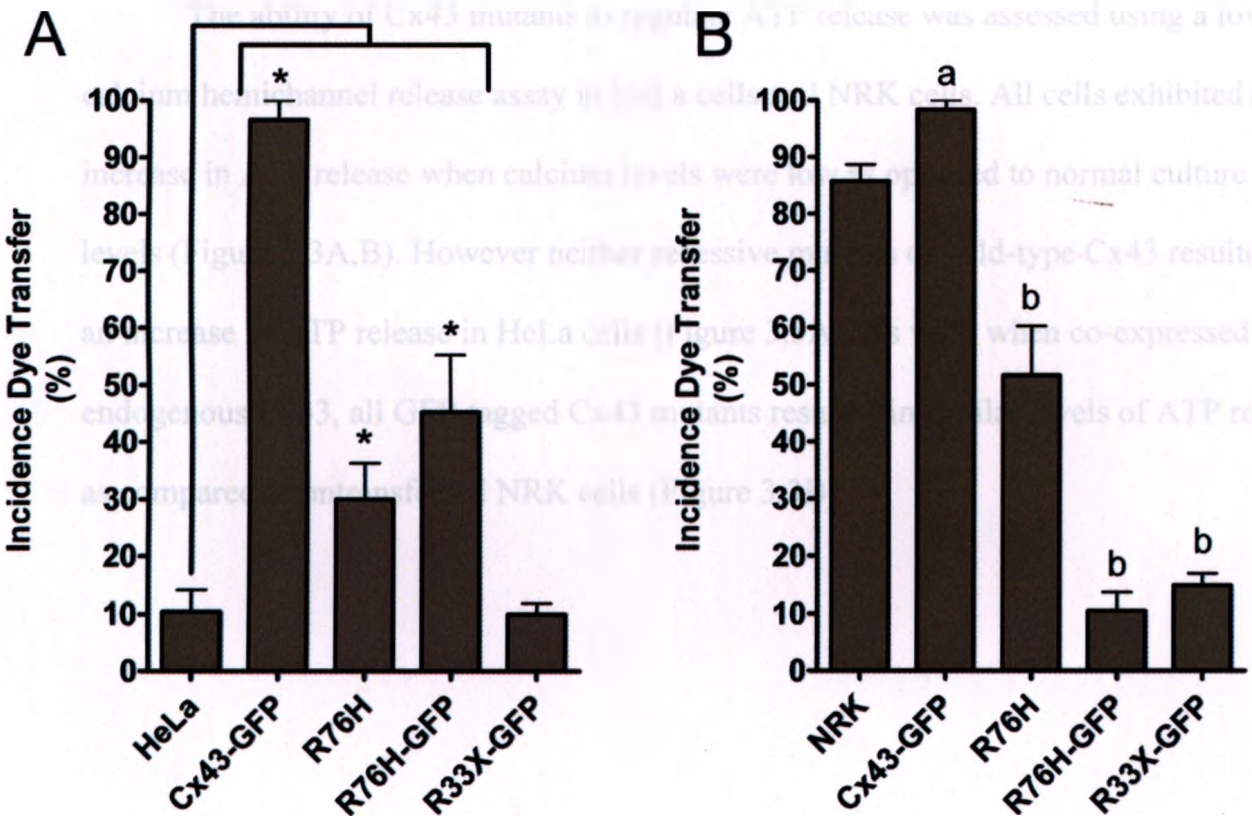
A
B
Incidence Dye Transfer (%)
100
90
80
70
60
50
40
30
20
10
0
*
*
*
a
b
b
b
HeLa
Cx43-GFP
R76H
R76H-GFP
R33X-GFP
NRK
Cx43-GFP
R76H
R76H-GFP
R33X-GFP

Figure 3.2

channels, although maybe not to the same efficiency as wild-type Cx43. The R33X mutant did not form functional channels. Furthermore, both mutants reduced the incidence of dye transfer by in NRK cells ~34-76% (Figure 2B), suggesting that these mutants can act as dominant-negatives to the function of co-expressed wild-type Cx43.

3.3.3 The recessive mutants produced similar levels of ATP release as wild-type HeLa and NRK cells

The ability of Cx43 mutants to regulate ATP release was assessed using a low calcium hemichannel release assay in HeLa cells and NRK cells. All cells exhibited an increase in ATP release when calcium levels were low as opposed to normal culture levels (Figure 3.3A,B). However neither recessive mutants or wild-type Cx43 resulted in an increase in ATP release in HeLa cells (Figure 3.3A). As well, when co-expressed with endogenous Cx43, all GFP-tagged Cx43 mutants resulted in similar levels of ATP release as compared to untransfected NRK cells (Figure 3.3B).

Figure 3.3 Recessive mutants R76H and R33X did not alter ATP release in HeLa or NRK cells. HeLa and NRK cells transfected with cDNA encoding Cx43-GFP, R76H-GFP, R33X-GFP were treated using HBSS with calcium (+Ca^{2+}) or without calcium (-Ca^{2+}) and assessed for extracellular ATP concentrations. HeLa cells expressing GFP-tagged Cx43 mutants resulted in similar extracellular ATP concentrations as untransfected HeLa and wild-type Cx43 expressing cells (A). As well, when co-expressed with wild-type Cx43 the all GFP-tagged Cx43 mutants resulted in similar extracellular ATP concentrations as untransfected NRK cells (B). Controls HeLa, HeLa (Cx43-GFP), NRK and NRK (Cx43-GFP) are the same data set used in sections 2.3. Bars represent means ± s.e.m. * = significant difference from Cx43. N = 3 replicate transfections ($P<0.05$)

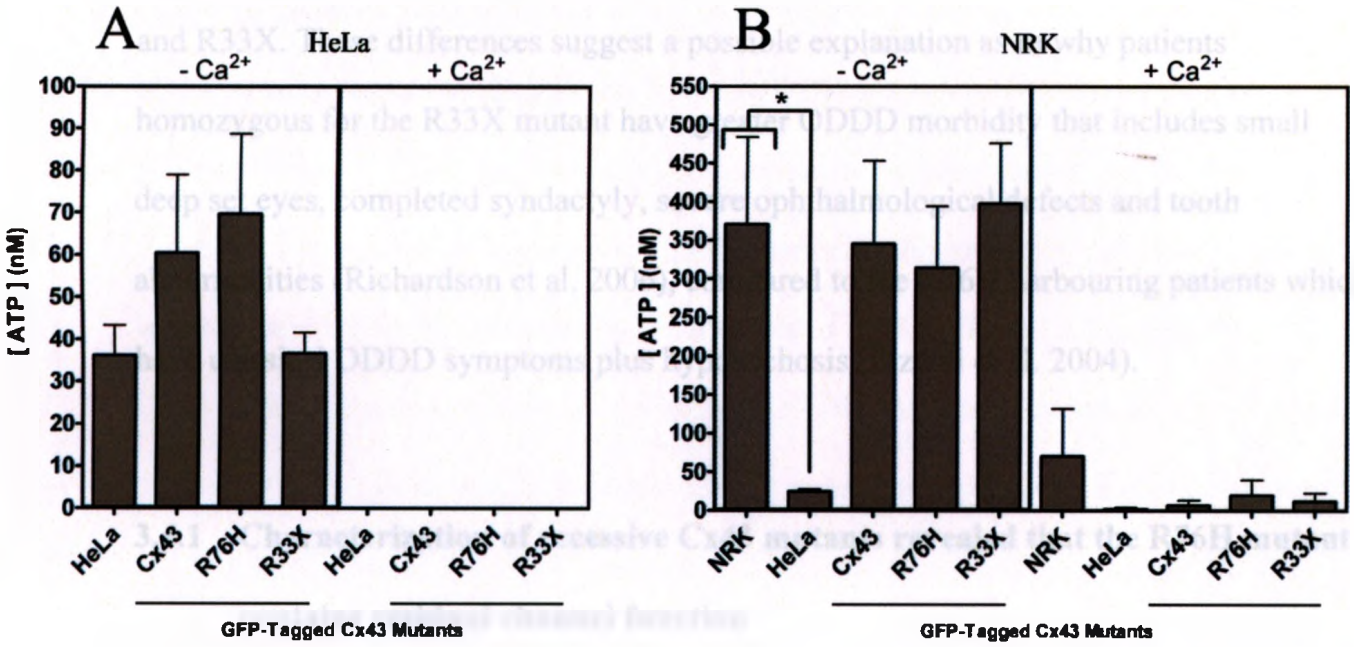
A
HeLa
- Ca2+
+ Ca2+
[ATP] (nM)
100
90
80
70
60
50
40
30
20
10
0
HeLa
Cx43
R76H
R33X
HeLa
Cx43
R76H
R33X
GFP-Tagged Cx43 Mutants
B
NRK
- Ca2+
+ Ca2+
*
[ATP] (nM)
550
500
450
400
350
300
250
200
150
100
50
0
NRK
HeLa
Cx43
R76H
R33X
NRK
HeLa
Cx43
R76H
R33X
GFP-Tagged Cx43 Mutants

Figure 3.3

3.4 Discussion

To date, all autosomal dominant Cx43 mutants examined exhibited a loss of gap junction channel function and acted as dominate-negatives on the channel function of endogenous Cx43 (Dobrowolski et al. 2007, Gong et al. 2007, McLachlan et al. 2005, Roscoe et al. 2005, Shibayama et al. 2005). However, recent clinical presentation and genetic studies have uncovered two recessive Cx43 mutants, R76H and R33X (Paznekas et al. 2003, Pizzuti et al. 2004, Richardson et al. 2006). In the present study, we examined the localization and GJIC differences between the two recessive Cx43 mutants, R76H and R33X. These differences suggest a possible explanation as to why patients homozygous for the R33X mutant have greater ODDD morbidity that includes small deep set eyes, completed syndactyly, severe ophthalmological defects and tooth abnormalities (Richardson et al. 2006), compared to the R76H harbouring patients which have classical ODDD symptoms plus hypotrichosis (Pizzuti et al. 2004).

3.4.1 Characterization of recessive Cx43 mutants revealed that the R76H mutant contains residual channel function

Our study revealed that the GFP-tagged and untagged recessive R76H mutant, but not the R33X mutant, possesses the ability to form gap junction-like plaques at sites of cell to cell apposition in both Cx43 –positive (NRK) and –negative (HeLa) cells. The R33X mutant, consisting of only the amino terminus and part of the first transmembrane domain of Cx43, exhibited a diffuse intracellular expression profile. The fact that the R33X mutant did not assemble into gap junctions is not surprising as less than 10% of the entire Cx43 protein is encoded by this mutant. As predicted from it diffused intracellular

localization, the R33X mutant also failed to form functional gap junction channels. Thus the R33X mutant might be considered a Cx43 knockout. Interestingly, our study revealed that the R76H mutant formed functional gap junction-like plaques at sites of cell-cell apposition. This mutation is localized to the second transmembrane domain of Cx43 where an arginine (R) residue is substituted by a histidine (H) residue thus maintaining the positive charge at this amino acid site. Thus, the conservative nature of this missense mutation may serve to retain the channel function. The functional and non-functional channels produced by the R76H and R33X mutants, respectively, provide an explanation as to why patients harbouring the R76H mutants, as opposed to those harbouring the R33X mutant, appear to display less severe ODDD symptoms (Pizzuti et al. 2004, Richardson et al. 2006). Interestingly, both recessive mutants caused a reduction in the function of co-expressed endogenous Cx43 channels, although it is important to note that we are likely highly overexpressing the mutants in NRK cells and exceeding the 1:1 Cx43 to mutant ratio expected to be found in ODDD patients. Nevertheless, the parents of the ODDD patients would both be expected to have reduced overall Cx43 function but the degree of channel function appears not to be sufficiently severe to cause ODDD (Richardson et al. 2006). Interestingly, in the heterozygous state these are the first Cx43 mutants identified that appear to not cause disease and only cause disease when both alleles encode the mutation. In all cases of autosomal dominant disease, when the mutant is expressed in reference cells in the absence of endogenous Cx43, no functional channels form.

The residual gap junction channel function exhibited by the R76H mutant may suggest that this mutant is also capable of forming hemichannels. It is well documented

that wild-type Cx43 hemichannels open and release ATP under reduced extracellular calcium (Dobrowolski et al. 2007, Stout et al. 2002, Tong et al. 2007). As well, one research group showed that the G143S, I31M and G138R autosomal dominant Cx43 mutants have increased hemichannel activity suggesting that these mutants have a gain-of-hemichannel function (Dobrowolski et al. 2007). However, neither the R33X or R76H recessive mutants exhibited gain-of-hemichannel function or acted as dominant-negatives to the hemichannel function of co-expressed endogenous Cx43.

3.4.2 Conclusion

Collectively, we found the recessive R76H mutant contains residual gap junction channel function which is unique from any other Cx43 mutant studied to date. We also found that the R33X mutant does not form gap junction-like structures or functional gap junction channels. Interestingly, parents of these ODDD patients do not display any ODDD symptoms despite harbouring a single copy of the Cx43 mutation, similar to the autosomal dominant patient population. Therefore, it appears that the residual function of the R76H mutant, and possibly the inability of the R33X mutants to oligomerize with wild-type Cx43 may enable heterozygotes of recessive mutants to be disease-free. The findings presented here also provide plausible explanations as to why patients harbouring the R76H mutant have less morbidity than patients harbouring R33X mutants. Both mutants represent unique opportunities to study the importance of Cx43 in development, as well as normal tissue function.

3.5 References

Alexander DB, Goldberg GS (2003). Transfer of biologically important molecules between cells through gap junction channels. *Curr Med Chem* 10: 2045-2058.

Brink PR, Cronin K, Banach K, Peterson E, Westphale EM, Seul KH *et al.* (1997). Evidence for heteromeric gap junction channels formed from rat connexin43 and human connexin37. *Am J Physiol* 273: C1386-1396.

Cronier L, Crespin S, Strale PO, Defamie N, Mesnil M (2009). Gap junctions and cancer: new functions for an old story. *Antioxid Redox Signal* 11: 323-338.

Dobrowolski R, Sommershof A, Willecke K (2007). Some oculodentodigital dysplasia-associated Cx43 mutations cause increased hemichannel activity in addition to deficient gap junction channels. *J Membr Biol* 219: 9-17.

Falk MM (2000). Biosynthesis and structural composition of gap junction intercellular membrane channels. *Eur J Cell Biol* 79: 564-574.

Gong XQ, Shao Q, Langlois S, Bai D, Laird DW (2007). Differential potency of dominant negative connexin43 mutants in oculodentodigital dysplasia. *J Biol Chem* 282: 19190-19202.

Goodenough DA, Goliger JA, Paul DL (1996). Connexins, connexons, and intercellular communication. *Annu Rev Biochem* 65: 475-502.

Jordan K, Solan JL, Dominguez M, Sia M, Hand A, Lampe PD *et al.* (1999). Trafficking, assembly, and function of a connexin43-green fluorescent protein chimera in live mammalian cells. *Mol Biol Cell* 10: 2033-2050.

Li H, Liu TF, Lazrak A, Peracchia C, Goldberg GS, Lampe PD *et al.* (1996). Properties and regulation of gap junctional hemichannels in the plasma membranes of cultured cells. *J Cell Biol* 134: 1019-1030.

McLachlan E, Manias JL, Gong XQ, Lounsbury CS, Shao Q, Bernier SM *et al.* (2005). Functional characterization of oculodentodigital dysplasia-associated Cx43 mutants. *Cell Commun Adhes* 12: 279-292.

Musil LS, Goodenough DA (1990). Gap junctional intercellular communication and the regulation of connexin expression and function. *Curr Opin Cell Biol* 2: 875-880.

Paznekas WA, Boyadjiev SA, Shapiro RE, Daniels O, Wollnik B, Keegan CE *et al.* (2003). Connexin 43 (GJA1) mutations cause the pleiotropic phenotype of oculodentodigital dysplasia. *Am J Hum Genet* 72: 408-418.

Paznekas WA, Karczeski B, Vermeer S, Lowry RB, Delatycki M, Laurence F *et al.* (2009). GJA1 mutations, variants, and connexin 43 dysfunction as it relates to the oculodentodigital dysplasia phenotype. *Hum Mutat* 30: 724-733.

Pizzuti A, Flex E, Mingarelli R, Salpietro C, Zelante L, Dallapiccola B (2004). A homozygous GJA1 gene mutation causes a Hallermann-Streiff/ODDD spectrum phenotype. *Hum Mutat* 23: 286.

Rackauskas M, Neverauskas V, Skeberdis VA (2010). Diversity and properties of connexin gap junction channels. *Medicina (Kaunas)* 46: 1-12.

Reaume AG, de Sousa PA, Kulkarni S, Langille BL, Zhu D, Davies TC *et al.* (1995). Cardiac malformation in neonatal mice lacking connexin43. *Science* 267: *1831-1834.

Richardson RJ, Joss S, Tomkin S, Ahmed M, Sheridan E, Dixon MJ (2006). A nonsense mutation in the first transmembrane domain of connexin 43 underlies autosomal recessive oculodentodigital syndrome. *J Med Genet* 43: e37.

Roscoe W, Veitch GI, Gong XQ, Pellegrino E, Bai D, McLachlan E *et al.* (2005). Oculodentodigital dysplasia-causing connexin43 mutants are non-functional and exhibit dominant effects on wild-type connexin43. *J Biol Chem* 280: 11458-11466.

Shibayama J, Paznekas W, Seki A, Taffet S, Jabs EW, Delmar M *et al.* (2005). Functional characterization of connexin43 mutations found in patients with oculodentodigital dysplasia. *Circ Res* 96: e83-91.

Sohl G, Willecke K (2004). Gap junctions and the connexin protein family. *Cardiovasc Res* 62: 228-232.

Stout CE, Costantin JL, Naus CC, Charles AC (2002). Intercellular calcium signaling in astrocytes via ATP release through connexin hemichannels. *J Biol Chem* 277: 10482-10488.

Thomas T, Jordan K, Simek J, Shao Q, Jedeszko C, Walton P *et al.* (2005). Mechanisms of Cx43 and Cx26 transport to the plasma membrane and gap junction regeneration. *J Cell Sci* 118: 4451-4462.

Tong D, Li TY, Naus KE, Bai DL, Kidder GM (2007). In vivo analysis of undocked connexin43 gap junction hemichannels in ovarian granulosa cells. *Journal of Cell Science* 120: 4016-4024.

CHAPTER 4

General Discussion

4.1 Summary

4.1.1 Chapter 1 - Objective #1: Characterize the localization and function of bladder disease- associated (D3N and G143S) and unassociated (G2V and I130T) Cx43 mutants when expressed in reference cell models.

Previous studies have investigated the localization and functional status of the Cx43 mutants I130T and G143S (Dobrowolski et al. 2007, Lai et al. 2006, Shibayama et al. 2005); however the Cx43 mutants G2V and D3N were not previously studied. Our study was the first to compare and contrast the localization of function of bladder disease –associated (D3N and G143S) and –unassociated (G2V and I130T) Cx43 mutants linked to ODDD. All mutants formed non-functional gap junction plaque-like structures at sites of cell to cell apposition and acted as dominant-negatives to the function of co-expressed endogenous Cx43. Interestingly, the G2V and D3N mutants did exhibit a gain-of-hemichannel function, as assessed by ATP release, but no change in ATP release was found when cells expressed either the G143S or I130T mutants. Therefore, we found no discernable difference in the localization, gap junction channel function as assessed by dye transfer or hemichannel ATP release between bladder disease –associated and –unassociated Cx43 mutants.

4.1.2 Chapter 1 – Objective #2: Determine the expression and distribution of Cx43 and Cx26 in the bladder of G60S and I130T Cx43 mutant mice and their control littermates.

To our knowledge this study is the first to localize Cx43 and Cx26 proteins in the mouse bladder, as previous studies have focused on bladders from either rats or humans

(Imamura et al. 2009, Kuhn et al. 2008b, Miyazato et al. 2009, Neuhaus et al. 2002a, Zhang et al. 2008). Our study employed two genetically different heterozygous mutant mouse models harbouring Cx43 mutants G60S and I130T, which phenotypically resemble ODDD patients (Flenniken et al. 2005, Kalcheva et al. 2007). *In vitro* studies have previously shown that the G60S and I130T mutants form non-functional gap junction plaques at sites of cell to cell apposition, therefore disrupting various organ development and normal function (Flenniken et al. 2005, Kalcheva et al. 2007, McLachlan et al. 2005, Shibayama et al. 2005, Toth et al. 2010). We found that Cx43 is expressed in both the lamina propria and detrusor layers of all wild-type and mutant mice. While in both mutant mouse lines Cx43 was localized to punctate structures indicative of gap junctions, in G60S mice, Cx43 also appeared more diffuse and intracellular. Nevertheless, both mutant mouse lines exhibited a reduction in the most highly phosphorylated Cx43 species which is known to be important in plaque formation and GJIC (Koo et al. 1997, Solan and Lampe 2009). As well, Cx26, which was localized to the basal urothelial layer in a unique intracellular distribution, was similar in all wild-type and mutant mice, suggesting that Cx26 expression did not act to compensate for perturbed Cx43 function. Therefore, if Cx43 is required for normal bladder function, and if the G60S and I130T mutants are non-functional (McLachlan et al. 2005, Shibayama et al. 2005), the mutant mice may possess a bladder phenotype similar to that reported by ODDD patients.

4.1.3 Chapter 2 – Objective #1: Characterize the localization and function of two recessive Cx43 mutants (R33X and R76H) when expressed in reference cell models.

The current knowledge regarding Cx43 recessive mutants R76H and R33X is restricted to their original identification and documentation of the clinical symptoms expressed by the patients harbouring these mutants. Our study is the first to assess the localization, gap junction channel and hemichannel function of these mutants. While the R76H mutant formed plaques at sites of cell to cell appositions similar to wild-type Cx43, the R33X mutant displayed a more diffuse intracellular expression pattern. Interestingly, the R76H mutant contained residual gap junction channel function not seen by the R33X mutant or other autosomal dominant Cx43 mutants (Dobrowolski et al. 2007, Gong et al. 2007, McLachlan et al. 2005, Roscoe et al. 2005, Shibayama et al. 2005). Interestingly, both recessive mutants acted as dominate-negatives to the function of co-expressed endogenous Cx43, a situation which partially mimics the genotype of the heterozygous parents of these patients. This latter result was unexpected as heterozygous carriers of these recessive mutants are disease-free. This discrepancy may be attributed to the extensive overexpression of the mutants in relationship to the endogenous Cx43. Importantly, both mutants did not affect ATP release in Cx43 –negative and –positive cells. These results provide clues as to why patients homozygous for the R76H mutant display classical ODDD symptoms similar to patients homozygous for autosomal-dominant mutants such as hypotrichosis and dental abnormalities (Pizzuti et al. 2004), while patients harbouring the R33X mutants have more extensive morbidities including:

small deep set eyes, complete syndactyly, ophthalmological defects and dental abnormalities (Richardson et al. 2006).

4.2 Contribution of Research

4.2.1 ODDD Patients and Bladder Abnormalities

Our study showed no molecular or Cx43 channel differences between disease –associated (D3N and G143S) and –unassociated (G2V and I130T) Cx43 mutants. Thus, we propose that patients harbouring any Cx43 mutant could exhibit a bladder defect depending upon the genetic background of the patient, age, or presence of additional disease burden. Similar to the normal population, bladder abnormalities generally do not manifest in ODDD patients until the second half of the patient's life (Loddenkemper et al. 2002). However, as Cx43 seems to play a significant role in bladder function (Fry et al. 2007, Hashitani et al. 2004, Neuhaus et al. 2007, Sui et al. 2002), it may be possible that ODDD patients are more susceptible to developing bladder abnormalities and/or may develop them earlier in life compared to the general population. Interestingly, a small population of ODDD patient that have been diagnosed with bladder abnormality are in their teen and early twenties (Paznekas et al. 2009), which is extremely rare for the normal population. Currently, the percentage of bladder defects that are linked to ODDD patients is lower than the percentage of the general population documented with bladder defects (Abrams et al. 2003, Paznekas et al. 2009). However, we propose that the number of ODDD patients suffering from bladder defects may be larger than reported, as early diagnosis of bladder defects may be impossible in younger ODDD patients. Similar to the general population ODDD patients are dependent on diapers during their first several

years. Therefore, bladder defects experienced by ODDD patients may not be linked back to the original diagnosis of ODDD, which generally occurs early in a patients life.

4.2.2 Recessive R76H Mutant Retains Residual Gap Junction Channel Function

Our results revealed differences in the localization and functional characteristics of the two autosomal-recessive Cx43 mutants, R76H and R33X. Interestingly, the R76H mutant forms gap junction channels that retain residual function, a characteristic not seen in the R33X mutant or any autosomal-dominant Cx43 mutant (Gong et al. 2007, McLachlan et al. 2005, Roscoe et al. 2005). The fact that the homozygous autosomal recessive R76H mutant retain residual channel function similar to heterozygous autosomal dominant mutants (Gong et al. 2007, McLachlan et al. 2005, Roscoe et al. 2005) suggests a possible explanation as to why patients harbouring the R76H mutant display ODDD symptoms similar to patients harbouring autosomal dominant mutants. As well, it appears that the R76H mutant may disrupt the function of endogenous Cx43; however, the residual function of the R76H mutant may allow for heterozygous carriers of this mutation to maintain sufficient GJIC and remain disease free. Similarly, heterozygous carriers of the recessive R33X mutant are also disease free. The R33X mutant, which consists of only the amino terminus and a part of the first transmembrane domain may not be able to oligomerize with wild-type Cx43, thus not affected the channel. Interestingly, previous studies on Cx43 knock-out mice revealed that loss of Cx43 is lethal at birth while heterozygous mice are relatively normal (Reaume et al. 1995). Thus, the homozygous R33X mutant patients are essentially human Cx43-knock-

outs. The reason why R33X patients survive remains unknown but may be rooted in a compensatory action of other connexin proteins.

4.3 Future Studies

4.3.1 Determine if Cx43 mutant mice possess a bladder phenotype.

The bladders of G60S and I130T mice exhibit Cx43 intracellular distribution and reduction in highly phosphorylated Cx43 species. This information along with the *in vitro* loss-of-channel function assays of the same Cx43 mutants may suggest that the mutant mice may possess a bladder phenotype similar to that reported by ODDD patients. Therefore, future research should be directed toward determining if the Cx43 mutant mice possess a bladder phenotype by analyzing bladder cystometry and smooth muscle strip contractions (Imamura et al. 2009, Kim et al. 2010, Lee et al. 2011, Vahabi et al. 2011, Zhang et al. 2011). Information obtained from these assays would provide insight into urinary frequency, pressure, urine output and smooth muscle contraction strength and shed insights into whether the bladders from mutant mice have any organ-based functional defects.

Interestingly, even though Cx43 appears to be important in the normal function of the bladder (Fry et al. 2007, Neuhaus et al. 2005, Sui et al. 2002, Sui et al. 2003, Wang et al. 2006), not all ODDD patients have been reported to carry bladder abnormalities. It remains possible that the specific mutations harbored by an ODDD patients is not critical for the onset of incontinence, but rather the issue relates extensively to the age of the patient and their specific genetic background. To assess this question further it would be beneficial to generate a mutant mouse line that harbors one of the bladder associated

mutants (D3N or G143S) used in the current study. As well as studying the mouse at difference age points, including infant, middle aged and elderly mice.

4.3.2 Use primary cultured smooth muscle and urothelial cells to assess the channel activity in mutant and wild-type mice

Analyzing Cx43 mutants in reference cell lines is an excellent start to understanding how mutants function. However, to truly understand how a protein functions, it is important to look at its endogenous expression in primary cell types that are known to express the protein. The urothelium, lamina propria and detrusor layers of the bladder are known to express Cx43 (Grossman et al. 1994, Haefliger et al. 2002, Hashitani et al. 2004, John et al. 2003b, Sui et al. 2003, Wang et al. 2006), and therefore represent excellent models for studying endogenous Cx43 function. It is possible to produce primary cultures of the urothelium and detrusor layers of the bladder from Cx43 mutants mice (Boselli et al. 2002, Galvin et al. 2004, Neuhaus et al. 2002b, Tyagi et al. 2006), which can subsequently be use to study the gap junction channel or hemichannel activity via dye transfer, ATP release, dual-whole cell patch clamp and Ca^{2+} wave propagations (Neuhaus et al. 2002b). The information obtained about endogenous connexin status and function may reveal more pertinent information pertaining to the function of Cx43 within these layers; as well as the effects that mutant Cx43 proteins produce.

4.4 Conclusion

Collectively, this study has revealed several unique findings, previously unknown in the field of gap junctions. First, with respect to the bladder, it appears that the specific mutation harbored by an ODDD patient on its own may not be important in the manifestation of a bladder defect. Bladder abnormalities may be the result of multiple genetic factors associated with ODDD or the aging process itself. Although the current information suggests that ODDD patients are less susceptible as the general population to develop bladder defects (Abrams et al. 2003, Paznekas et al. 2009), more thorough record keeping of ODDD patients is required to determine this definitively. Second, as seen with the recessive R76H mutant, it appears as though some mutants retain residual gap junction channel function. Therefore, the varied functional status of different Cx43 mutants may be an indicator of why ODDD patients do not all display the same symptoms. Further inquiry into both bladder-associated and recessive mutants is necessary to identify the molecular mechanisms that subsequently result in the symptoms displayed by patients harboring the associated mutants.

4.5 References

Abrams P, Cardozo L, Fall M, Griffiths D, Rosier P, Ulmsten U *et al.* (2003). The standardisation of terminology in lower urinary tract function: report from the standardisation sub-committee of the International Continence Society. *Urology* 61: 37-49.

Boselli C, Govoni S, Vicini D, Lanni C, Racchi M, D'Agostino G (2002). Presence and passage dependent loss of biochemical M3 muscarinic receptor function in human detrusor cultured smooth muscle cells. *J Urol* 168: 2672-2676.

Dobrowolski R, Sommershof A, Willecke K (2007). Some oculodentodigital dysplasia-associated Cx43 mutations cause increased hemichannel activity in addition to deficient gap junction channels. *J Membr Biol* 219: 9-17.

Flenniken AM, Osborne LR, Anderson N, Ciliberti N, Fleming C, Gittens JE *et al.* (2005). A Gja1 missense mutation in a mouse model of oculodentodigital dysplasia. *Development* 132: 4375-4386.

Fry CH, Sui GP, Kanai AJ, Wu C (2007). The function of suburothelial myofibroblasts in the bladder. *Neurourol Urodyn* 26: 914-919.

Galvin DJ, Watson RW, O'Neill A, Coffey RN, Taylor C, Gillespie JI *et al.* (2004). Hypoxia inhibits human bladder smooth muscle cell proliferation: a potential mechanism of bladder dysfunction. *Neurourol Urodyn* 23: 342-348.

Gong XQ, Shao Q, Langlois S, Bai D, Laird DW (2007). Differential potency of dominant negative connexin43 mutants in oculodentodigital dysplasia. *J Biol Chem* 282: 19190-19202.

Grossman HB, Liebert M, Lee IW, Lee SW (1994). Decreased connexin expression and intercellular communication in human bladder cancer cells. *Cancer Res* 54: 3062-3065.

Haefliger JA, Tissieres P, Tawadros T, Formenton A, Beny JL, Nicod P *et al.* (2002). Connexins 43 and 26 are differentially increased after rat bladder outlet obstruction. *Exp Cell Res* 274: 216-225.

Hashitani H, Yanai Y, Suzuki H (2004). Role of interstitial cells and gap junctions in the transmission of spontaneous Ca2+ signals in detrusor smooth muscles of the guinea-pig urinary bladder. *J Physiol* 559: 567-581.

Imamura M, Negoro H, Kanematsu A, Yamamoto S, Kimura Y, Nagane K *et al.* (2009). Basic fibroblast growth factor causes urinary bladder overactivity through gap junction generation in the smooth muscle. *Am J Physiol Renal Physiol* 297: F46-54.

John H, Wang X, Wehrli E, Hauri D, Maake C (2003). Evidence of gap junctions in the stable nonobstructed human bladder. *J Urol* 169: 745-749.

Kalcheva N, Qu J, Sandeep N, Garcia L, Zhang J, Wang Z *et al.* (2007). Gap junction remodeling and cardiac arrhythmogenesis in a murine model of oculodentodigital dysplasia. *Proc Natl Acad Sci U S A* 104: 20512-20516.

Kim JH, Lee SR, Hong CH, Choi YD (2010). Increased Urinary Nerve Growth Factor as a Predictor of Persistent Detrusor Overactivity After Bladder Outlet Obstruction Relief in a Rat Model. *J Urology* 183: 2440-2444.

Koo SK, Kim DY, Park SD, Kang KW, Joe CO (1997). PKC phosphorylation disrupts gap junctional communication at G0/S phase in clone 9 cells. *Mol Cell Biochem* 167: 41-49.

Kuhn A, Stadlmayr W, Monga A, Cameron I, Anthony F (2008). A pilot study of Connexin 43 (Cx43) in human bladder tissue in patients with idiopathic detrusor overactivity. *Eur J Obstet Gyn R B* 141: 83-86.

Lai A, Le DN, Paznekas WA, Gifford WD, Jabs EW, Charles AC (2006). Oculodentodigital dysplasia connexin43 mutations result in non-functional connexin hemichannels and gap junctions in C6 glioma cells. *J Cell Sci* 119: 532-541.

Lee WC, Chuang YC, Chiang PH, Chien CT, Yu HJ, Wu CC (2011). Pathophysiological studies of overactive bladder and bladder motor dysfunction in a rat model of metabolic syndrome. *J Urol* 186: 318-325.

Loddenkemper T, Grote K, Evers S, Oelerich M, Stogbauer F (2002). Neurological manifestations of the oculodentodigital dysplasia syndrome. *J Neurol* 249: 584-595.

McLachlan E, Manias JL, Gong XQ, Lounsbury CS, Shao Q, Bernier SM *et al.* (2005). Functional characterization of oculodentodigital dysplasia-associated Cx43 mutants. *Cell Commun Adhes* 12: 279-292.

Miyazato M, Sugaya K, Nishijima S, Kadekawa K, Machida N, Oshiro Y *et al.* (2009). Changes of bladder activity and connexin 43-derived gap junctions after partial bladder-outlet obstruction in rats. *Int Urol Nephrol* 41: 815-821.

Neuhaus J, Weimann A, Stolzenburg JU, Wolburg H, Horn LC, Dorschner W (2002a). Smooth muscle cells from human urinary bladder express connexin 43 in vivo and in vitro. *World J Urol* 20: 250-254.

Neuhaus J, Wolburg H, Hermsdorf T, Stolzenburg JU, Dorschner W (2002b). Detrusor smooth muscle cells of the guinea-pig are functionally coupled via gap junctions in situ and in cell culture. *Cell Tissue Res* 309: 301-311.

Neuhaus J, Pfeiffer F, Wolburg H, Horn LC, Dorschner W (2005). Alterations in connexin expression in the bladder of patients with urge symptoms. *BJU Int* 96: 670-676.

Neuhaus J, Heinrich M, Schlichting N, Oberbach A, Fitzl G, Schwalenberg T *et al.* (2007). [Structure and function of suburothelial myofibroblasts in the human urinary bladder under normal and pathological conditions]. *Urologe A* 46: 1197-1202.

Reaume AG, de Sousa PA, Kulkarni S, Langille BL, Zhu D, Davies TC *et al.* (1995). Cardiac malformation in neonatal mice lacking connexin43. *Science* 267: 1831-1834.

Roscoe W, Veitch GI, Gong XQ, Pellegrino E, Bai D, McLachlan E *et al.* (2005). Oculodentodigital dysplasia-causing connexin43 mutants are non-functional and exhibit dominant effects on wild-type connexin43. *J Biol Chem* 280: 11458-11466.

Shibayama J, Paznekas W, Seki A, Taffet S, Jabs EW, Delmar M *et al.* (2005). Functional characterization of connexin43 mutations found in patients with oculodentodigital dysplasia. *Circ Res* 96: e83-91.

Solan JL, Lampe PD (2009). Connexin43 phosphorylation: structural changes and biological effects. *Biochem J* 419: 261-272.

Sui GP, Rothery S, Dupont E, Fry CH, Severs NJ (2002). Gap junctions and connexin expression in human suburothelial interstitial cells. *BJU Int* 90: 118-129.

Sui GP, Coppen SR, Dupont E, Rothery S, Gillespie J, Newgreen D *et al.* (2003). Impedance measurements and connexin expression in human detrusor muscle from stable and unstable bladders. *BJU Int* 92: 297-305.

Toth K, Shao Q, Lorentz R, Laird DW (2010). Decreased levels of Cx43 gap junctions result in ameloblast dysregulation and enamel hypoplasia in Gja1Jrt/+ mice. *J Cell Physiol* 223: 601-609.

Tyagi S, Tyagi P, Van-le S, Yoshimura N, Chancellor MB, de Miguel F (2006). Qualitative and quantitative expression profile of muscarinic receptors in human urothelium and detrusor. *J Urol* 176: 1673-1678.

Vahabi B, Lawson K, McKay NG, Sellers DJ (2011). Phasic activity of urinary bladder smooth muscle in the streptozotocin-induced diabetic rat: effect of potassium channel modulators. *Eur J Pharmacol* 660: 431-437.

Wang HZ, Brink PR, Christ GJ (2006). Gap junction channel activity in short-term cultured human detrusor myocyte cell pairs: gating and unitary conductances. *Am J Physiol Cell Physiol* 291: C1366-1376.

Zhang X, Kuppam DS, Melman A, DiSanto ME (2011). In vitro and in vivo relaxation of urinary bladder smooth muscle by the selective myosin II inhibitor, blebbistatin. *BJU Int* 107: 310-317.

Zhang ZB, Zhou DR, Song B (2008). Gap junctional protein connexin 43 in rat detrusor muscle with unstable bladder. *Chin Med J (Engl)* 121: 1698-1701.

APPENDIX

Date: 10.15.09

Dear Dr. Laird

A **MAJOR MODIFICATION** to your "Animal Use Protocol" entitled:

The role of gap junctions in disease has been approved.

The protocol **2006-101** and yearly expiry date of **10.31.10** remain unchanged.

Please ensure that individual(s) performing procedures, as described in this protocol, are familiar with the contents of this document.

The holder of this *Animal Use Protocol* is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

c.c. W Lagerwerf

The University *of* Western Ontario
Animal Use Subcommittee / University Council on Animal Care
Health Sciences Centre, • London, Ontario • CANADA N6A 5C1
PH: 519-661-2111 ext. 86770 • FL 519 661-2028 • www.uwo.ca / animal