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Myogenic bladder defects in mouse models of human oculodentodigital dysplasia

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Abstract

To date, over 65 mutations in the gene encoding Cx43 (connexin43) have been linked to the autosomal-dominant disease ODDD (oculodentodigital dysplasia). A subset of these patients experience bladder incontinence which could be due to underlying neurogenic deterioration or aberrant myogenic regulation. BSMCs (bladder smooth muscle cells) from wild-type and two Cx43 mutant lines (Cx43^{G60S} and Cx43^{I130T}) that mimic ODDD exhibit a significant reduction in total Cx43. Dye transfer studies revealed that the G60S mutant was a potent dominant-negative inhibitor of co-expressed Cx43, a property not equally shared by the I130T mutant. BSMCs from both mutant mouse strains were defective in their ability to contract, which is indicative of phenotype changes due to harbouring the Cx43 mutants. Upon stretching, Cx43 levels were significantly elevated in controls and mutants containing BSMCs, but the non-muscle myosin heavy chain A levels were only reduced in cells from control mice. Although the Cx43^{G60S} mutant mice showed no difference in voided urine volume or frequency, the Cx43^{I130T} mice voided less frequently. Thus, similar to the diversity of morbidities seen in ODDD patients, genetically modified mice also display mutation-specific changes in bladder function. Furthermore, although mutant mice have compromised smooth muscle contraction and response to stretch, overriding bladder defects in Cx43^{I130T} mice are likely to be complemented by neurogenic changes.

Keywords

bladder; connexin43 (Cx43); disease; gap junction; mutation; oculodentodigital dysplasia

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AUTHOR CONTRIBUTION

The majority of experiments were carried out by Tao Huang under the immediate supervision and assistance of Qing Shao. Jamie Simek assisted in image acquisition and analysis. Kevin Barr did all of the mouse breeding and genotyping and assisted with the *in vivo* experiments. The I130T mutant mice were generously provided by Glen Fishman who also reviewed the paper before submission. Dale Laird oversaw the design and writing of the paper.

INTRODUCTION

Gap junctions are composed of tightly packed intercellular channels assembled from members of the connexin family that allow for the intercellular exchange of small molecules [e.g. cAMP, IP₃ (inositol trisphosphate) and ATP] in a process known as GJIC (gap junctional intercellular communication) [1]. After cotranslational import into the endoplasmic reticulum, connexins oligomerize into hexamers referred to as either 'connexons' or 'hemichannels' [2]. Once delivered to the cell surface, two adjacent connexons from opposing plasma membranes dock to form a complete gap junction channel that proceeds to tightly cluster with other channels into a gap junction plaque [3]. Even though connexins undergo multiple steps of assembly, they generally have a short half-life of less than 4 h both in cultured cells and tissues [4]. The most ubiquitously found connexin is Cx43 (connexin43) which is a phosphoprotein found in the vast majority of human tissues [4].

Since Cx43 is expressed in many human organs it was not surprising when mutations in the gene encoding Cx43 result in a primarily autosomal-dominant development disease known as ODDD (oculodentodigital dysplasia) [5,6]. Patients harbouring Cx43 mutants share common clinical characteristics, such as syndactyly and camptodactyly of the digits, microdontia, craniofacial bone deformities, and enamel loss [5,6]. For reasons that remain unclear, in addition to these morbidities some ODDD patients have additional disease burdens and suffer from glaucoma, skin diseases, central nervous system disorders or incontinence [5,6]. Although some of these disorders manifest with aging, others present at a relatively young age. Bladder disturbances are one condition that can manifest in the young, but it is not clear whether this is linked to a specific mutation site in the Cx43 gene, or if neurogenic or myogenic changes in the bladder are responsible. Since Cx43 is abundantly expressed in BSMCs (bladder smooth muscle cells) [7–10] and interstitial cells [11], but not found in neurons or the skeletal muscle cells of the sphincter, we propose that the bladder defect would have a myogenic origin. In order to address this question, two genetically modified mouse lines that mimic ODDD (Cx43^{G60S} and Cx43^{I130T}) were acquired [12,13]. Cx43^{G60S} mice have been studied extensively and used to assess the role of Cx43 in enamel formation, cardiac function, bone formation, skin diseases, oogenesis, breast cancer and vision [14–21]. In all of these scenarios the expression of the G60S mutant was found to be dominant to the co-expressed endogenous Cx43, resulting in a greater than 50% reduction in Cx43-based gap junction coupling. Remarkably, this finding did not always reflect developmental disorders or the onset of disease during aging. For example, Cx43 is widely and abundantly expressed within the intercalated discs of the myocardium, but rarely do ODDD patients ever exhibit cardiopathologies [5,19]. This observation was supported further by the genetically modified mice harbouring the I130T mutant where total Cx43 function was reduced to approximately 50% and only mild cardiopathologies were observed [13]. On the basis of these mutant mouse models of ODDD, it is difficult to determine where developmental defects will be seen and secondly, what organs will be affected.

From the recent findings demonstrating that Cx43 levels in the bladder are subject to circadian oscillations as defined by clock genes [22], and further evidence linking increased Cx43 and micturition [23–25], it is clear that Cx43 plays a critical role in bladder function in

health and disease. Interestingly, four generations of a Hispanic family harbouring the Cx43 I130T mutant exhibited multiple neurological abnormalities including spastic paraparesis and abnormal brain and spinal cords and, furthermore, three out of four affected family members exhibited bladder dysfunction [26]. Given the strong correlation between the I130T mutation in humans and bladder dysfunction we hypothesized that Cx43^{I130T} mice would have compromised bladder function, whereas the Cx43^{G60S} mice might be unaffected. Furthermore, we proposed that through a comparative study between these two genetically modified mouse models of ODDD we would be able to determine whether bladder dysfunction was linked to myogenic or neurogenic disorders. To that end, we initially showed that there were no overt changes in the histological architecture of the bladder or detrusor thickness in mutant compared with control mice [7], suggesting that if a defect existed it would likely be linked to functional changes in the smooth muscle or have neurogenic origins.

In the present study, we show that indeed both mutant mouse lines have altered BSMC contraction and stretch response properties, but this only manifested as a defective bladder in Cx43^{I130T} mice. These divergent findings in genetically modified mice recapitulated the human ODDD condition where only some patients suffer from incontinence and we further suggest that when bladder defects are found in patients they may be at least partially rooted in myogenic changes in the bladder smooth muscle, but also require neurogenic changes.

MATERIALS AND METHODS

Animals

Animal studies were conducted on two mutant mouse lines harbouring heterozygous mutations, G60S or I130T, in the gene *Gja1*, which encodes Cx43. Heterozygote mice expressing the I130T mutant (*Gja1*^{tm3GFi}) were originally created as described previously [13] and were bred on a mixed background of CD1 and C57BL/6 mice. All Cx43^{I130T/+} mice (referred to as Cx43^{I130T} or simply I130T mice) used were at generation three to five of backcrossing to C57BL/6 and compared with WT (wild-type) littermate controls. Heterozygote *Gja1*^{Jrt/+} (*Gja1*^{m1Jrt}; also known as Cx43^{G60S/+}) mice [12] (referred to as Cx43^{G60S} or simply G60S mice) carrying the G60S mutant on a background of C3H/HeJ and C57BL/6J mice were used for our experiments. Cx43^{G60S/+} mice used in the experiments were compared with littermate controls. The mice are expected to translate a 1:1 ratio of mutant to endogenous Cx43 protein which would genetically match human ODDD patients. All experiments were approved by the Animal Care Committee at Western University and conducted according to the guidelines of the Canadian Council on Animal Care. After confirming 100% penetrance of the syndactyly feature in all mutant mice, as well as determination by PCR genotyping [12], genotype determination was completed by visual inspection of the pups. A 100% incidence rate of syndactyly ($n = 203$) in I130T mice was confirmed by PCR genotyping. However, although syndactyly was found on the front limbs of all mice, the back limbs did not always exhibit this phenotype.

Isolation and primary culture of mouse BSMCs

BSMCs were isolated from the bladders of 1-year-old male G60S or I130T mice and littermate controls as described previously [27]. Briefly, the bladders were isolated and physically separated from the outer tunica serosa and tunica adventitia layers. The stripped bladder tissue was then digested in 0.25% trypsin-EDTA (Life Technologies) for 30 min at 37°C with gentle agitation. Cells released by this digestion were discarded and the remaining tissue minced and suspended in RPMI 1640 medium containing 0.1% collagenase A (Roche Diagnostics). Digestion was carried out for 30 min at 37°C with shaking. The resulting cell suspension was pelleted at 250 g for 5 min. The cell pellet was suspended in RPMI 1640 medium/10% FBS followed by centrifugation at 150 g for 2 min. The supernatant containing BSMCs was collected while the pellet was again re-suspended in RPMI 1640 medium/FBS, followed by centrifugation at 125 g for 2 min. Finally, both cell supernatant fractions of primarily BSMCs were combined and 5×10^5 cells cultured on collagen I (BD Biosciences, catalogue number 354231)-coated plastic dishes with RPMI 1640 medium/FBS. The culture medium was changed every 3 days and grown to 80–90% confluence. The purity of the BSMCs was assessed by staining for α -SMA (α -smooth muscle actin). Primary or secondary cultured BSMCs were used for all the experiments in the present study.

Immunocytochemistry

Isolated BSMCs were immunolabelled as described previously [19,28,29]. Briefly, cells were grown on glass coverslips and fixed with 80% methanol/20% acetone at 4°C for 15 min. After fixation, cells were blocked with 3% BSA and 0.02% Triton X-100 in PBS for 45 min at room temperature (25°C). WT and Cx43 mutant BSMCs were labelled with a 1:500 dilution of anti-Cx43 antibody (Sigma–Aldrich, C6219) and 1:200 dilution of anti- α -SMA antibody (Sigma–Aldrich, A2547). Primary antibody binding was detected using goat anti-mouse or donkey anti-rabbit antibodies conjugated to Alexa Fluor® 555 or Alexa Fluor® 488 (Life Technologies). Nuclei were stained with Hoechst 33342 (10 μ g/ml; Life Technologies). Coverslips were rinsed in distilled water, mounted and analysed on a Zeiss LSM 510 Meta confocal microscope as described previously [30,31].

Dye transfer studies

Mouse BSMCs were pressure microinjected with a glass pipette containing 10 mM Alexa Fluor® 350 (Molecular Probes) using an Eppendorf FemtoJet automated pressure microinjector attached to a Leica DM IRE2 inverted epifluorescence microscope. Images were collected 1–2 min after microinjection using OpenLab software (Improvision). Data were collected from at least three independent primary cultures from different mouse preparations. The percentage of cells that passed dye to at least one neighbour as well as the degree of dye spread from the microinjected cell (first degree, cells that contact the injected cell; second degree, cells not in direct contact with the injected cell) were recorded and analysed as described previously [32,33].

Collagen gel contraction assay

Optimal gel formation was determined by varying rat tail collagen type I concentrations and NaOH titration for correct pH buffering. A final concentration of 1.2 mg/ml collagen medium mixture supplemented with $\sim 2 \mu\text{l}$ of 1 M NaOH per gel (300 μl) was determined to be optimal. Fully confluent BSMCs were detached by 0.1% trypsin and suspended in 5 ml of RPMI 1640 medium. To form cell-populated gels, two parts cell suspension was mixed with one part prepared rat tail collagen type I solution (3.6 mg/ml), yielding a final concentration of 0.75×10^5 cells/ml and 1.2 mg/ml collagen I. The mixture was triturated briefly, and transferred on to a 48-well plate (300 μl /well). Gels were incubated at 37 °C to allow cells to distribute uniformly throughout the gel. Once the gels had set, 300 μl of fresh medium was added to each well. Gels were incubated at 37°C in a 5 % CO₂ humidified incubator, and imaged by using the Bio-Rad Laboratories ChemiDoc Imaging System every 24 h for 5 days. Gel surface areas were measured using ImageJ software (NIH). Contraction was quantified by calculating the gel area as a percentage of the initial surface area. Data are presented as means $\pm$ S.E.M. ($\#P < 0.01$).

BSMCs stretch assay

Mechanical stretch of mouse BSMCs was performed by using a tension system as described previously [34]. Freshly isolated mouse BSMCs from WT, G60S or I130T mice were plated on to six-well silicone elastomer-bottomed culture plates (Flexcell) coated with collagen I. Cells were cultured in RPMI 1640 medium/10 % FBS for 3–4 days, achieving ~ 80 % confluence. In fresh medium, cells were subjected to mechanical deformation with the Flexcell FX-5000 tension system. The stress unit consists of a computer-controlled vacuum unit and a base plate to hold the culture dishes. Vacuum (≈ 15 –20 kPa) is repeatedly applied to the rubber-bottomed dishes via the base plate, which is placed in a humidified incubator with 5% CO₂ at 37 °C. Cells were subjected to a 10 % cyclic biaxial strain at a frequency of 1.0 Hz. For controls, cells were cultured under the same conditions, but were not subjected to mechanical strain. After 48 h of stretching, cells were harvested and processed for Western blotting.

Western blotting

Cultured BSMCs were lysed in detergent-based buffer (150 mM NaCl, 10 mM Tris/HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.5% Nonidet P-40, 1% Triton X-100, 1 mM NaF, 1mM Na₃VO₄ and 1 Complete™ Mini protease inhibitor tablet, Roche). For each lane, 40 μg of protein was resolved by SDS/PAGE (10% gel). Resolved proteins were transferred on to nitrocellulose membranes, blocked in 3% BSA at room temperature and incubated with primary antibodies overnight at 4°C. Cx43 and NM-MHC-IIA (non-muscle myosin heavy chain II-A) were detected using polyclonal anti-Cx43 (1:5000 dilution; Sigma–Aldrich, C6219) and polyclonal anti-NM-MHC-IIA (1:1000 dilution; Covance PRB-440P) antibodies. The membranes were also probed for GAPDH (glyceraldehyde-3-phosphate dehydrogenase; anti-GAPDH antibody, 1:10000 dilution; Chemicon MAB374) as a protein loading control. Immunoreactive bands were revealed following a 1 h incubation with Alexa Fluor® 680 (red; 1:10000 dilution; Life Technologies) or IRDye 800-conjugated secondary antibodies (green; 1:10000 dilution; Rockland) and visualized using the Odyssey Infrared

Imaging System (Li-Cor Biosciences). Band intensity was quantified using the Odyssey Infrared Imaging System.

Micturition analysis in mice

The 24-h urine volume from WT, G60S or I130T mice were determined by the VSOP (voided stains on paper) method as described previously [35]. Normal saline ranging from 50 to 800 μ l was poured on to a piece of filter paper by a micropipette. The recorded stain areas were imaged using a Bio-Rad Laboratories Chemidoc Imaging System in conjunction with Adobe Photoshop and recorded as total pixels. The correlation between the amount of saline and stained area was calculated to generate a standard formula for further mouse studies. Urine volume over 24 h from WT and mutant mice were analysed by placing filter paper overlaid with soft plastic mesh on the bottom of the cages. After 24 h, the stain on the filter paper was imaged, and the amount of urine voided per gram over 24 h was determined using the standard curve calculated above.

To evaluate the 24-h urine frequency of control and mutant mice, we set up a similar device to the VSOP device described previously [36] where the filter paper was on rolling device so that fresh paper was provided to collect the urine. The number of urine stains on the underlying filter paper was recorded every 2 h for a 24-h period. Water was provided to the mice during urine collection.

Statistics

All values are presented as means $\pm$ S.E.M. unless otherwise indicated. All results were analysed using the Student's two-tailed independent sample *t* test or a one-way ANOVA test in which a *P* < 0.05 was considered to be a statistically significant difference. All analyses were carried out by using the SigmaStat statistical software.

RESULTS

Cx43 levels and GJIC are greatly reduced in BMSCs from mutant mice

Immunolabelling for α -SMA in primary cultures of BMSCs isolated from control and mutant mice revealed that all cells exhibited a uniform spindle-shape and expressed α -SMA, indicating that the cultures were highly enriched populations of smooth muscle cells (Figures 1A and 1C). When BMSCs from G60S mice were immunolabelled for Cx43, there was a noticeable reduction in Cx43 at cell–cell interfaces, whereas the perinuclear staining for Cx43 was more evident (Figure 1A). This change in Cx43 distribution was not found in BMSCs from I130T mice when compared with littermate control cells (Figure 1C). As assessed by Western blotting, the total level of Cx43 was significantly reduced with the G60S mutant causing a $\sim$ 65% reduction (Figure 1B), whereas the I130T mutant caused a less dramatic $\sim$ 35% reduction in total Cx43 (Figure 1D). Not surprisingly these changes in total Cx43 levels reflected a dramatic reduction of up to $\sim$ 75% in dye coupling seen in BMSCs obtain from G60S mice (Figures 2A, 2B and 2E) and a less dramatic reduction of $\sim$ 40–65% in dye coupling seen in BMSCs isolated from I130T mice (Figures 2C, 2D and 2F). Taken together, these studies would suggest that the G60S mutant destabilizes the entire Cx43 population in BMSCs and acts as a dominant on co-expressed endogenous Cx43.

Meanwhile, the I130T mutant appears to traffic and assemble into gap junction plaques with little negative effects on co-expressed endogenous Cx43. These genetically modified mice represent two distinct manifestations of ODDD when considering molecular and functional changes to Cx43.

Defective smooth muscle contraction and lack of response to stretch in BMSCs from mutant mice

Since Cx43 and coupling status was dramatically altered in BMSCs from mutant mice, we examined whether this would manifest in phenotypic changes to the BMSCs. BMSCs from control mice underwent substantial contraction over a 5-day period (Figure 3). BMSCs from G60S mice exhibited only modest levels of cell contraction in collagen; this level was only slightly greater in BMSCs from I130T mice. These findings would suggest that BMSCs from mutant mice have compromised contraction which could, in principle, result in changes in bladder physiology.

Since the bladder undergoes routine and repetitive physical deformation with changeable levels of stretch reflecting urine accumulation and voiding cycles, we investigated the response of BMSCs to mechanical stretch. In control cells, we found that stretch caused a ~50–75% increase in Cx43 and this same stretch-induced increase in Cx43 was observed in BMSCs from both mutant mouse lines (Figures 4A–4C and 4E). Mechanical strain also caused a significant decrease in NM-MHC-IIA in WT BMSCs, consistent with the predicted decrease in protein expression levels expected to occur upon stretch [34] (Figures 4A, 4B, 4D and 4F). However, the NM-MHC-IIA levels in BMSCs from both mutant mice were unchanged before and after stretched (Figures 4D and 4F). Collectively, these studies would suggest that BMSCs from mutant mice not only have reduced ability to contract, but are defective in reprogramming following the stretch stimulus.

Bladder function in mutant mice

With the evidence that BMSCs are defective in their ability to contract and fully respond to stretch we predicted that these defects may manifest as changes in bladder function in one or both of our ODDD mouse models. To first establish whether urine production and urine voiding was altered, we assessed the total urine voided over 24 h and found that there was no difference between the mutant mice and their control counterparts (Figure 5). However, when the number of voids over a 24-h period was assessed, we found that I130T mice voided only half as frequently as their littermate counterparts, whereas there was no difference detected between the G60S mice and their control counterparts (Figure 6). Taken together, these experiments would indicate that in mice harbouring a systemic Cx43 mutant the overall production of urine is unchanged which would suggest that kidney function is unaltered. Yet, the fact that the I130T mice urinate less often, but void the same amount of total urine, would suggest that the bladders distend more than controls to accommodate more urine before receiving sufficient stimulus to void.

DISCUSSION

It has been known for some time that smooth muscle cells of the detrusor muscle are coupled by Cx43-based gap junctions [37]. Furthermore, an increase in Cx43 status in the bladder has been associated with overactive bladder and incontinence [25]. Thus it was somewhat surprising to find that 12% of ODDD patients who would be expected to have reduced Cx43 function in the bladder and elsewhere would suffer from bladder abnormalities that often manifested in the second decade of life [5]. This raised the question as to whether Cx43 function was truly compromised in the bladder and if this resulted in changes in smooth muscle contraction or bladder function. To that end, we examined the coupling status in BMSCs from control and genetically modified (G60S and I130T) mice that mimic the clinical characteristics of ODDD. The G60S mutant has no documented linkage to bladder abnormalities, whereas up to half of the patients harbouring the I130T mutant suffer from incontinence [26]. BMSCs from both mutant mouse lines exhibited severely compromised GJIC, a condition that resulted in their reduced ability to contract and a reduction in their NM-MHC-IIA levels suggesting a phenotype change. Behavioural studies revealed that both mutant mouse strains produced normal levels of urine suggesting that their bladders and kidneys could function properly, but I130T mutant mice required greater urine volume accumulation in the bladder before receiving the stimulus to urinate. Collectively, these studies suggest that mutant mice have defective smooth muscle cell properties, but this alone may not be sufficient to result in bladder defects, rather I130T mice may have compromised neurogenic responses to stretch requiring the bladder to overfill before urination.

Cx43 and coupling status of BMSCs from mutant mice

In the bladder, although the detrusor muscle in G60S or I130T mutant mice was found previously to be of similar size and architecture [7], it was unclear whether Cx43 expression and coupling status was altered in BSMCs. In the present study we show that Cx43 levels in the G60S mutant mice are reduced by ~65%, whereas coupling status is even further reduced to ~25% of control littermates. Thus the channel-dead [38] G60S mutant was clearly acting as a dominant-negative to co-expressed WT Cx43 and this dominance appeared to be due to the retention of Cx43 within the Golgi apparatus. In the case of BMSCs from I130T mutant mice, the levels of total Cx43 were reduced by only ~35% with dye coupling being reduced by ~40–65%. The reduced severity of the I130T mutant may be explained by the fact that it traffics and assembles into gap junction plaques and exhibits limited negative properties on co-expressed WT Cx43. Furthermore, we and others have shown previously that the I130T mutant, when expressed in GJIC-deficient cells, does retain ~25% residual coupling, indicating that the mutation does not cause a complete loss of Cx43 channel function [38,39]. Thus although both these genetically modified mouse models of ODDD result in overriding phenotypic presentations commonly found in humans, the molecular changes to Cx43 trafficking, assembly and function are variable which could result in bladder defect differences.

BMSCs from mutant mice are defective

Not surprisingly, BMSCs from both mutant mouse models exhibited defective contraction properties in collagen gels, a condition that was more pronounced in BMSCs harbouring the G60S mutation. This difference in contraction between the two strains of mutant mice could be explained by the fact that Cx43 function in the G60S mice is more severely compromised than what is observed in I130 mutant mice. The question then was raised as to whether BMSCs from both mutant mouse strains would respond to stretch, a process encountered by BMSCs during the normal cycles of bladder filling and voiding. First, we found that Cx43 levels increased by ~30–75% in all control and mutant mice, indicating that Cx43 gene expression was stimulated upon stretch and/or that the life cycle of Cx43 was prolonged in cells following stretching. These findings support previous studies at the organ level where Cx43 levels were elevated in obstructed bladders [40], which may further have therapeutic implications [41]. Secondly, to assess whether stretch promoted a phenotypic change in BMSCs, we assessed the changes in myosin heavy chain isoforms where NM-MHC-IIA has been shown to be down-regulated in vascular smooth muscle cells following mechanical strain [34]. Consistent with these past findings, in both our control groups, NM-MHC-IIA was down-regulated by at least 50%, indicating that stretch was reprogramming the cells and altering the phenotypic state of the cell. However, in the case of the G60S mice, these cells exhibited levels of NM-MHC-IIA consistent with already being under strain and, furthermore, the addition of stretch failed to cause further changes in gene regulation. Taken together, these findings suggest that BMSCs from mutant mice can up-regulate their Cx43 following mechanical stretch, but fail to change their resting differentiation status.

I130T mutant mice have altered bladder function

Since BMSCs in both mutant mouse lines have altered molecular and cellular properties the question remained as to whether this would manifest as changes in bladder function. We first established that neither genetically modified mutant mouse line had altered production of urine or urine voiding. Since Cx43 is expressed in glomerular epithelial and proximal tubular cells of the kidney [42], and the Cx43 mutants would be co-expressed in these cells, it was important to rule-out changes in urine production that might reflect kidney defects. Although urine production was unchanged, we were interested to find that G60S mutant mice voided at the same frequency of littermate control mice, whereas the I130T mutant mice voided half as frequently. This was somewhat unpredicted given that the molecular and cellular properties assessed in BMSCs were more severe in G60S mice, as compared with I130T mice, raising the likelihood that the myogenic changes in the BMSCs are not the sole determinant in whether a physiological bladder defect will be observed.

Bridging findings in genetically modified mice to the ODDD condition

Previously, it has been clearly shown that Cx43 sensitizes the bladder for cyclic and circadian oscillations in bladder storage of urine and micturition [22,25,43,44]. Thus, on one hand, it is not surprising that rodent models of a hyperactive bladder suggest that this condition is correlated with increased levels of Cx43 and GJIC in the bladder [25,45]. On the other hand, it is interesting to find that at least some ODDD patients with compromised Cx43 function have early-onset incontinence [5]. To the best of our knowledge, the I130T

mutation in ODDD patients has the highest correlation with early onset of bladder abnormalities occurring in approximately 40–50% of the cases reported [26], although one extended Pakistani family has been reported to have no evidence of neurological or bladder disorders [46]. Since only the I130T mutant mice exhibited reduced, but significant, myogenic changes, and further resulted in a physiological change in micturition, we propose that there may be a neurogenic component upstream of cholinergic neural stimuli in these mice that additively result in bladder dysregulation. This position is supported by MRI scans of ODDD patients harbouring the I130T mutant where significant changes in the brain and spinal cord were observed [26]. Moreover, the fact that bladder incontinence is not fully penetrant in all I130T harbouring patients suggest that other genes, epigenetic factors or comorbidities may also contribute to the clinical presentation of the disease.

In summary, our studies support a critical role for Cx43 in the bladder that is rooted, in part, in the function of BMSCs. Comparative analysis using unique genetically modified mouse lines that mimic ODDD, suggest that the I130T mice are more prone to bladder dysfunction even though the BMSCs are less severely compromised than those found in G60S mice. Finally, our studies point to a neurogenic defect that must accompany compromised myogenic properties in the bladder to instil organ dysfunction.

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Abbreviations

BSMC	bladder smooth muscle cell
Cx43	connexin43
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GJIC	gap junctional intercellular communication
NM-MHC-IIA	non-muscle myosin heavy chain II-A
ODDD	oculodentodigital dysplasia
α-SMA	α -smooth muscle actin
VSOP	voided stains on paper
WT	wild-type

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Figure 1. Localization and expression of Cx43 in primary cultures of mouse BSMCs
 Primary cultured BSMCs from WT or Cx43 mutant mice were immunolabelled for Cx43 (green) and α -SMA (red) before staining of the nuclei with Hoechst (blue) (A and C). Arrowheads denote Cx43 gap junction plaques (A and C), and arrows revealed the perinuclear distribution of Cx43 in BSMCs obtained from G60S mice (A). Scale bars, 20 μ m. Western blots revealed that Cx43 was expressed at lower levels in BSMCs from G60S and I130T mice compared with littermate control mice (B and D). GAPDH was used as a loading control (B and D). ** $P < 0.01$.

Figure 2. BSMCs from mutant mice have greatly reduced GJIC

Cultured BSMCs from WT or Cx43 mutant mice were microinjected with Alexa Fluor® 350 (A and C). Fluorescent images collected before dye injection and 1–2 min after dye injection were correlated with phase-contrast images of the same field. Microinjected cells are denoted by the asterisks (A and C). Scale bars, 20 μ m. The incidence of dye transfer to surrounding cells was quantified (B and D). Brackets denote the number of dye injected cells (B and D). The degree of dye transfer (see the Materials and methods section) was

quantified (**E** and **F**). Note that the BSMCs from Cx43 mutant mice exhibited significantly reduced dye transfer. $**P < 0.01$, $***P < 0.001$.

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Figure 3. BMSCs from mutant mice have reduced contractility

BSMCs from mutant and littermate controls mice were mixed with collagen I into gels and plated on to 48-well plates. Gels containing cells were imaged every 24 h for 5 days (**A** and **B**). Contraction was quantified by calculating the area occupied by the cells as a percentage of the initial surface area (**C** and **D**). Note that BSMCs from Cx43 mutant mice exhibited less contraction than cells from littermate control mice. # $P < 0.01$ ($n = 3$).

Figure 4. Effect of cyclic mechanical stretch on Cx43 and NM-MHC-IIA expression in BSMCs from control and mutant mice

BSMCs from control and mutant mice were exposed to 1.0 Hz, 10 % cyclic biaxial strain for 48 h before immunoblotting for Cx43 and NM-MHC-IIA (**A** and **B**). GAPDH was used as a loading control (**A** and **B**). Quantification revealed that Cx43 was significantly elevated in BSMCs for mutant and control mice (**C** and **E**). However, although NM-MHC-IIA was reduced in WT mice, it did not significantly change following mechanical stretching in BSMCs from mutant mice (**D** and **F**). ** $P < 0.01$, *** $P < 0.001$ ($n = 3$).

Figure 5. WT and mutant mice produce the same amount of urine

In order to establish a standard curve, the stained area generated from 50 to 800 µl of saline was imaged (**A**) and plotted against the volume of saline (**B**). Urine voided over a 24-h period was determined for mutant and control mice and normalized to mouse weight (**C** and **D**). For both mutant mice, the amount of urine voided was not significantly different from littermate controls. NS, not significant (n 4).

Figure 6. Frequency of urination in mutant and control mice

By using a VSOP-like device, urine frequency over a 24-h period for mutant and control mice was determined. The number of voids on filter paper was recorded every 2 h for a 24-h period and quantified (**A** and **B**). Note that the I130T mutant mice displayed less than half the voids than its littermate control mice (**B**). NS, not significant, *** $P < 0.001$ ($n = 5$).