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CELLULAR PATHWAYS CONTRIBUTING TO FIBROSIS IN THE BLADDER WALL OF CHILDREN WITH EXSTROPHY

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HYPOTHESIS / AIMS OF STUDY

Bladder exstrophy, as part of the exstrophy-epispadias complex, is characterised by a pathologically open and protruding organ. Despite improvements in surgical repair, many patients continue to have poorly contractile, low-capacity bladders. These pathophysiological features are associated with significant fibrosis and greater biomechanical stiffness of the bladder wall (1). The current investigation was designed to yield insight into changes to Wnt-signalling pathways, important in organ and tissue development, but also in the regulation of connective tissue deposition (2). The study used multiplex immunolabelling of target proteins and single mRNA molecules, followed by imaging and unbiased, quantitative image analysis. Data were obtained from children undergoing exstrophy repair and compared to those from children with functionally normal bladders (controls). We hypothesise that exstrophy bladders show excessive connective tissue deposition associated with altered regulation of Wnt-signalling targets.

STUDY DESIGN, MATERIALS AND METHODS

Anterior bladder wall samples were obtained from N = 9 control patients with normally-functioning bladders (age; 26 [18,48] months; 2 female, 7 male; for ureteric re-implantation) or N = 18 children with exstrophy (age; 62 [63,86] months; 6 female, 12 male; for bladder closure). Samples were immediately placed in 10% formaldehyde at 4°C, dehydrated and fixed in paraffin (FFPE samples). The study design was double-blind, all tissue blocks and antibodies used were blinded to the experimenter; the code was broken only when all analyses were completed. Previous functional data obtained with these tissue samples indicated a sample size of N = 7 was adequate for $p < 0.05$ at 80% power.

Tissue arrays were made from 0.8 mm diameter cores of FFPE samples and sectioned at 6-8 μm with an HM355S automatic microtome (Thermo Scientific). Sections were stained using a Leica Automated Bond System RXTM IHC/ISH for H&E/van Gieson staining (Figure 1A) and also probed with four different primary antibodies against Pygo1, Cx43, FRA1 and TCF7L1(Figure 1B) and single molecule RNAscope detection for CCND2, JUN, SOX-9 and TCF7L2(Figure 1C). These markers were chosen as they are known transcriptional downstream targets for Wnt-signalling pathways. Protein expression and gene transcription were detected by specific fluorophore labels for each antibody and chromogens for RNAscope labelling and were adjusted for variation in total amount of tissue in each core. Data are medians [25,75% interquartiles], differences between data sets were tested by two-way ANOVA with post-hoc non-parametric tests.

RESULTS

Exstrophy tissue had a lower proportion of smooth muscle in tissue cross sections, compared to samples from normally functioning bladders (30.6% [21.7,56.9] vs 65.6% [57.0,75.8]; p = 0.0150: Figure 1A), as measured by van Gieson staining. Protein expression was assessed by measuring the appropriate fluorophore label intensity (pixel counts) for each section and collected from ten regions of interest (RoI; each 60 μm diameter); five from areas dominated by smooth muscle and five from non-muscle areas. Antibody or RNAscope label intensity and total amount of tissue in each core was calculated using modified ImageJ plugins. Data from smooth muscle areas show increased expression in exstrophy sections for Pygo1 (p = 0.044) and Cx43 (p = 0.001) compared to normal tissue, but no differences for FRA1 and TCF7L1: Figure 2A). For non-muscle regions only Cx43 counts were different between exstrophy and normally-functioning bladder (p = 0.006: Figure 2A). Single molecule RNA counts for CCND2 were significantly fewer in exstrophy tissue (p = 0.013) but similar for JUN, SOX-9 probes. Counts for TCF7L2 were on the borderline for significance (p = 0.051: Figure 2B).

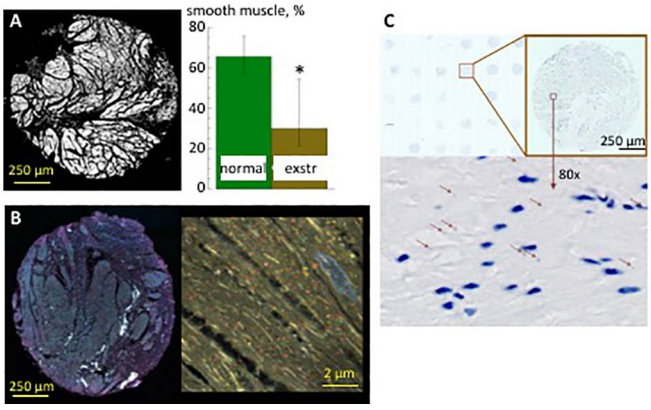
INTERPRETATION OF RESULTS

The data confirm previous findings that the smooth muscle content of exstrophy bladder wall is less than that of normally functioning bladders. However, changes to pathways associated with Wnt-signalling were not uniformly altered. Particular reasons for choosing these targets were: they may have a role in development of the congenital anomaly itself, and they are potentially associated with fibrosis deposition. Pygo1 is an essential transcription factor of beta-catenin canonical Wnt signalling which enhances and upregulates this pathway. In the bladder wall Cx43 expression is found in the interstitial/fibroblast cell population (3) consistent with greater deposition of connective tissue. However, FRA1 another transcription factor, known to be regulated by Wnt signal activation showed no change in expression. Previous work suggests that there was actually a decrease in the key transducer of Wnt signaling, namely, beta-catenin whose expression is reduced in exstrophy (1) and is consistent with unchanged expression of TCF7L1, another protein involved in Wnt signal mediated gene transcription. This is the first study to demonstrate the feasibility of high throughput, single molecule mRNA imaging in formalin-fixed bladder wall specimens. Although counts were low it has been possible to demonstrate dysregulation of transcription of Wnt target genes; in particular CCND2, critical in G1/S transition in the cell cycle.

CONCLUDING MESSAGE

These experiments emphasise the increased deposition of extracellular matrix at the expense of smooth muscle in exstrophy human bladder. Whilst an enhanced activity of Wnt-signalling pathways is associated with such fibrosis the particular sub-pathway requires further investigations and assessment of a larger number of target genes. A further possibility will be that in some bladder diseases downregulation of Wnt signal activity induces fibrosis.

FIGURE 1



A: van Gieson stained normal bladder bit map; muscle, white. Percentage smooth muscle:normal & exstrophy sections; *p < 0.05. B: four-protein label image, exstrophy section & high-power image. C: single molecule mRNA labels (brown arrows); normal bladder

FIGURE 2

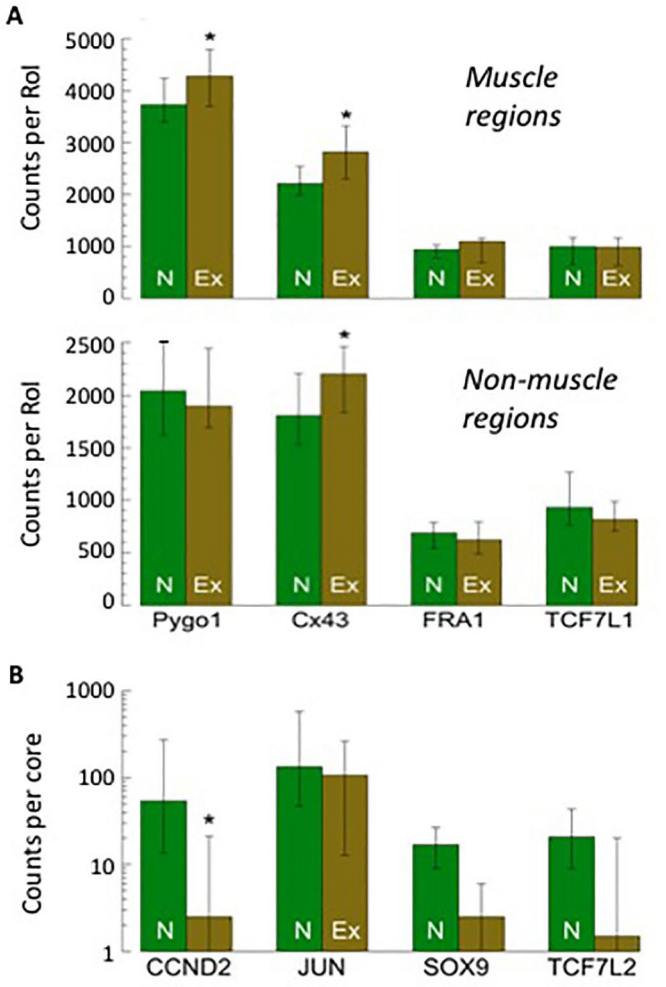


Figure 2. Protein, mRNA counts. A: protein counts in muscle and non-muscle areas in sections from and exstrophy bladder sections. B: single molecule mRNA counts in normal and exstrophy bladder sections. N - normal; Ex - exstrophy *p < 0.05

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