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Ectodermal Wnt signaling regulates abdominal myogenesis during ventral body wall development

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ABSTRACT

Defects of the ventral body wall are prevalent birth anomalies marked by deficiencies in body wall closure, hypoplasia of the abdominal musculature and multiple malformations across a gamut of organs. However, the mechanisms underlying ventral body wall defects remain elusive. Here, we investigated the role of Wnt signaling in ventral body wall development by inactivating *Wls* or *β-catenin* in murine abdominal ectoderm. The loss of *Wls* in the ventral epithelium, which blocks the secretion of Wnt proteins, resulted in dysgenesis of ventral musculature and genito-urinary tract during embryonic development. Molecular analyses revealed that the dermis and myogenic differentiation in the underlying mesenchymal progenitor cells was perturbed by the loss of ectodermal *Wls*. The activity of the Wnt-Pitx2 axis was impaired in the ventral mesenchyme of the mutant body wall, which partially accounted for the defects in ventral musculature formation. In contrast, epithelial depletion of *β-catenin* or *Wnt5a* did not resemble the body wall defects in the ectodermal *Wls* mutant. These findings indicate that ectodermal Wnt signaling instructs the underlying mesodermal specification and abdominal musculature formation during ventral body wall development, adding evidence to the theory that ectoderm-mesenchyme signaling is a potential unifying mechanism for the origin of ventral body wall defects.

Introduction

Ventral body wall closure defects include highly variable phenotypes, which can be categorized as gastroschisis, amniocentesis, omphalocele, umbilical hernia, limb-body wall defect and prune belly syndrome. For example, prune belly syndrome (PBS, OMIM 100100) is a specific body wall defect with characteristics of ventral body wall musculature deficiency or hypoplasia, various urinary tract abnormalities and bilateral cryptorchidism in males (Hassett et al., 2012). According to the accumulated literature on ventral body wall defects, most of these body wall closure defects are thought to be of embryonic origin (Sadler and Feldkamp, 2008). Before E12.0 during mouse embryogenesis, the parietal layer of the lateral plate mesoderm, together with the surface ectoderm, forms the somatopleure and migrates ventrally to give rise to the primary body wall (Eng et al., 2012; Sadler, 2010). Then, abdominal myogenic progenitor cells move into the somatopleure and progressively differentiate into secondary body wall, including the three layers of abdominal musculature, dermis and epithelium. The bilateral ventral body walls join at the midline, and the body wall is fully closed at E16.5 (Brewer and Williams, 2004a; Sadler, 2010; Sadler and Feldkamp, 2008).

In contrast to the long list of clinical reports and experimental studies, molecular hypotheses for ventral body wall defects are controversial. Four types of theories have been presented as the etiology of these deficiencies: embryonic dysplasia, vascular disruption, mechanical disruption and malfunction of the ectodermal placodes (Brewer and Williams, 2004a). Recently, a novel hypothesis has been proposed that crosstalk between the epithelium and mesenchyme is a vital signaling process essential for normal development and closure of the ventral body wall (Brewer and Williams, 2004a). This theory is based on the observation that inactivation of the transcription factor AP-2α leads to one of the most severe ventral body wall closure defects, thoracoabdominoschisis. Histological and molecular analyses suggest that AP-2α is required for signaling from the surface ectoderm to the underlying mesoderm for proper development and closure of ventral body wall (Brewer and Williams, 2004b).

Wnt signaling has a pivotal role in embryonic myogenesis and myoblast differentiation of skeletal muscles. In mouse paraxial mesoderm, Wnt1 from the embryonic dorsal neural tube and

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Wnt7a in the dorsal ectoderm induce somite myogenesis (Tajbakhsh et al., 1998). Ectodermal Wnt6 is required for myogenic induction in the limb bud (Geetha-Loganathan et al., 2006; Geetha-Loganathan et al., 2005). In concert with Shh signaling, canonical Wnt/β-catenin signaling regulates myogenic differentiation and proliferation through direct induction of *Myf5* expression within the epaxial dermomyotome (Borello et al., 2006). Non-canonical Wnt11, together with Wnt6 secreted from the surface ectoderm, maintains the essential epithelial nature of the dorsomedial and ventrolateral lips of the dermomyotome and acts as a directional cue for the elongation of easy muscle fibers (Gros et al., 2009). Furthermore, the Wnt/Lef1-Pitx2 pathway has been suggested to regulate somite myogenesis (Abu-Elmagd et al., 2010). The loss of *Pitx2* resulted in a deficiency in ventral body wall closure and agenesis of abdominal musculatures (Eng et al., 2012). However, the role of the Wnt-Pitx2 pathway in abdominal muscle formation remains unclear.

Here, we inactivated *Wntless* (*Wls*) gene expression in the mouse abdominal ectoderm through the *Msx2-cre* transgene (Lewandoski et al., 2000). Wls is required for the secretion of various Wnt proteins (Banziger et al., 2006; Bartscherer et al., 2006; Franch-Marro et al., 2008; Fu et al., 2011; Gasnereau et al., 2012), and depletion of the *Wls* allele is sufficient to block the paracrine effect of Wnt signaling during multiple developmental processes (Augustin et al., 2012; Fu et al., 2009; Huang et al., 2012; Maruyama et al., 2012; Myung et al., 2012; Zhong et al., 2012; Zhu et al., 2012). The deficiency of epithelial *Wls* led to deformities in abdominal musculature in the secondary ventral body wall, which are typical characteristics of prune belly syndrome. Our findings provide novel insight into the role of Wnt-mediated epithelium–mesenchyme signaling during ventral body wall development.

Materials and methods

Mice

Wls$^{c/c}$ and *Wnt5a*$^{+/-}$ mice were generated as previously described (Zhu et al., 2012). *Wls*$^{c/c}$ (C57BL/6) mice were crossed with a *Msx2-cre* transgenic mouse. *Msx2-cre*; *Wls*$^{c/+}$ offspring were backcrossed to *Wls*$^{c/c}$ mice to generate the *Msx2-cre*; *Wls*$^{c/c}$ knockout mice. The floxed status of the *Wls* conditional allele was genotyped with the following primers: P1: 5′-ATACTTTTTCT-GATCTGTTGT-3′ and P2: 5′-AAGTTTTAATAGGTCTGTGTT-3′. The presence of *Msx2-cre* was identified by PCR using the following primers: F: 5′-CAAAAGTTGGAGTCTTCGCT-3′ and R: 5′-CAGAAG-CATTTTCCAGGTAT-3′. *Catnb*$^{c/c}$ mice were generated and genotyped as previously described (Guo et al., 2004). The *Wnt5a* knockout mice were genotyped with the primers: P1: 5′-TTCCAAGTTCTTCC-TAATGGC-3′ and P2: 5′-TTGGGTGGAGAGGCTATTCG-3′. The mice were maintained in a specific pathogen-free environment with free access to food and water and a 12 h/12 h light–dark cycle. Animal welfare and experimental procedures were conducted strictly in accordance with the care and use of laboratory animals and the related ethical regulations of Shanghai Jiao Tong University.

LacZ staining, histology and immunohistochemistry

Whole mount X-gal staining was performed as described previously (Day et al., 2005). After re-fixing the stained samples, the samples were either photographed or sectioned to observe Cre enzyme activity. Wild-type and mutant embryos were harvested at specific stages and fixed with 4% PFA in PBS. The fixed samples were dehydrated, embedded in paraffin, and cut into 10 μm thick sections. H&E staining was performed on rehydrated samples. For immunohistochemistry (IHC), sections were rehydrated, blocked with 5% goat serum, and incubated overnight at 4 °C with anti-Wls (Santa Cruz, USA), anti-Lef1 (CST, USA), anti-Myosin (Epitomics, CA, USA), anti-keratin5 (Covance, NJ, USA), anti-BrdU (CST, USA), and anti-MyoD (BD, USA) primary antibodies according to the recommended dilution. Alexa Fluor 488 (Invitrogen, USA) was used as the secondary antibody. A TUNEL assay kit (Promega, WI, USA) was used to label apoptotic cells. The labeled samples were then counterstained with DAPI, and a Leica SP5 confocal microscope was used to observe and document the results.

qRT-PCR and in situ hybridization

Total RNA isolated from E12.5 and E13.5 mouse abdominal skin was extracted using TRIzol reagent (Invitrogen, CA, USA). cDNA was reverse transcribed using SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, CA, USA). qRT-PCR was performed on an Applied Biosystems 7500 using SYBR reagents. β-actin was used as a control. Whole-mount RNA *in situ* hybridizations and section RNA *in situ* hybridizations were performed as described previously using probes for *MyoD*, *Myogenin*, *Wls*, *Dermo1* and *Pitx2* (Zhu et al., 2012). Pitx2 cDNA was amplified with the primers: F: 5′-AGAAACCGCTACCCAGAC-3′ and R: 5′-TCAGAAACAAGGCATCCA-3′.

Results

A variety of Wnt genes are expressed in the ventral body wall

To identify the nature of Wnt proteins that contribute to ventral body wall development, we examined the expression levels of all 19 Wnt genes in the developing ventral body wall by qRT-PCR. At E13.5, the expression of *Wnt2*, *Wnt3*, *Wnt5a*, *Wnt6*, *Wnt7b* and *Wnt11* were relatively higher in the abdominal body wall (Fig. 1A). *Wnt6* expression in the ventral body wall ectoderm from E11.5 to E13.5 was validated by *in situ* hybridization (arrow in Fig. 1C–I). In contrast, *Wnt5a* expression existed in somites and the dorsal mesenchyme (Fig. 1D), but was not strongly detected in the ventral most ectoderm at E13.5 (Fig. 1H), consistent with a previous report (Witte et al., 2009). *Wls* expression has been detected in a variety of tissues and cell types during organogenesis (Yu et al., 2010). We also detected that *Wls* was expressed in the ventral body wall, especially in the abdominal ectoderm (arrows in Fig. 2I and K). These results showed that *Wls* and several *Wnt* genes, such as *Wnt6*, are expressed in the developing ventral body wall.

Ectodermal inactivation of Wls impairs ventral body wall development

To investigate the role of Wnt proteins in ventral body wall development, *Msx2-cre* was used to inactivate *Wls* to block Wnt secretion in the ectoderm of the ventral body wall. X-gal staining of *Msx2-cre*; *R26R* reporter mice showed that the Cre activity first appeared in the ventral body wall between the hindlimbs at E11.5 (Fig. 2A and B). Thereafter, its expression progressively expanded to the entire ventral body wall between the forelimbs and hindlimbs from E12.5 to E13.5 (Fig. 2C–F). Consistent with its expression pattern in the limb ectoderm (Barrow et al., 2003), Cre activity was also detected in the ectoderm of the ventral body wall in the *Msx2-cre*; *R26R* mice (Fig. 2G and H).

The *Msx2-cre* mice were crossed to *Wls*$^{c/c}$ mice to generate *Msx2-cre*; *Wls*$^{c/+}$ mice. The heterozygous mice did not display defects in growth and development. By backcrossing *Wls*$^{c/c}$ mice to *Msx2-cre*; *Wls*$^{c/+}$ mice, *Msx2-cre*; *Wls*$^{c/c}$ homozygous mutants were generated and identified to be perinatal lethal. In the

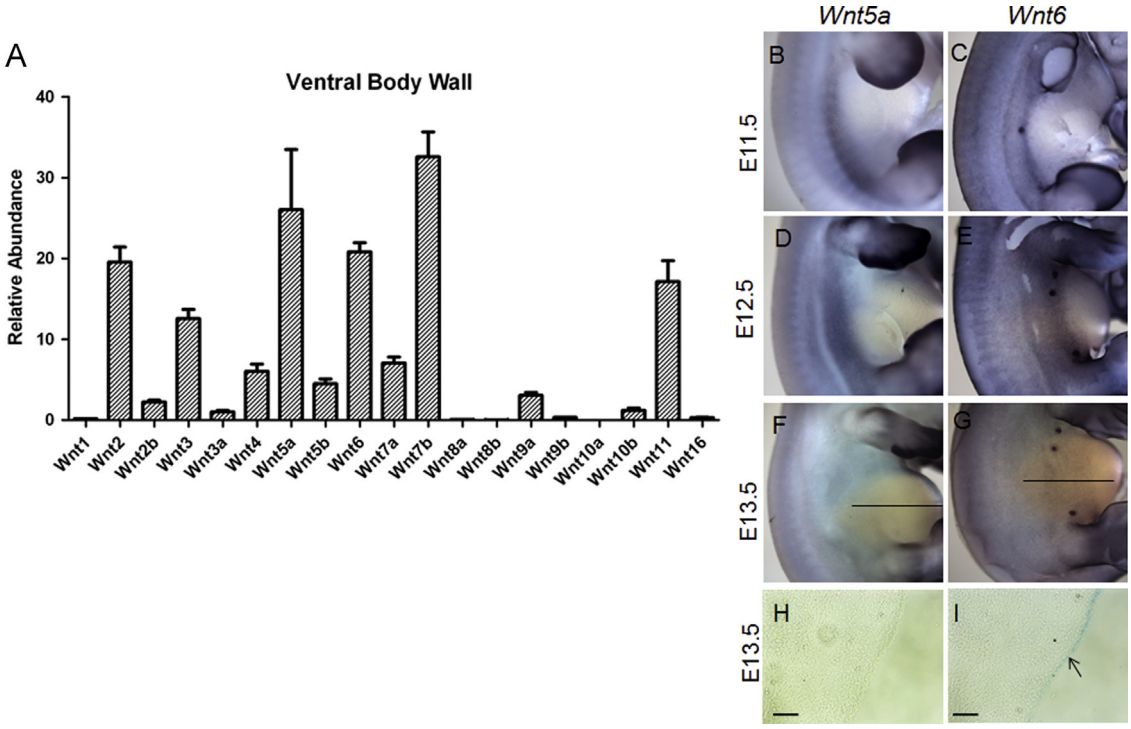

Fig. 1. Relative abundance of Wnt expression in the ventral body wall ectoderm. (A) qRT-PCR revealed that a variety of Wnts was expressed in the ventral body wall at E13.5. (B–I) *Wnt6* (C, E, G, I) but not *Wnt5a* (B, D, F, H) was remarkably expressed in the distal ventral ectoderm of the developing ventral body wall from E11.5 to E13.5. Panels H–I correspond to cross-sections along the lines in F–G.

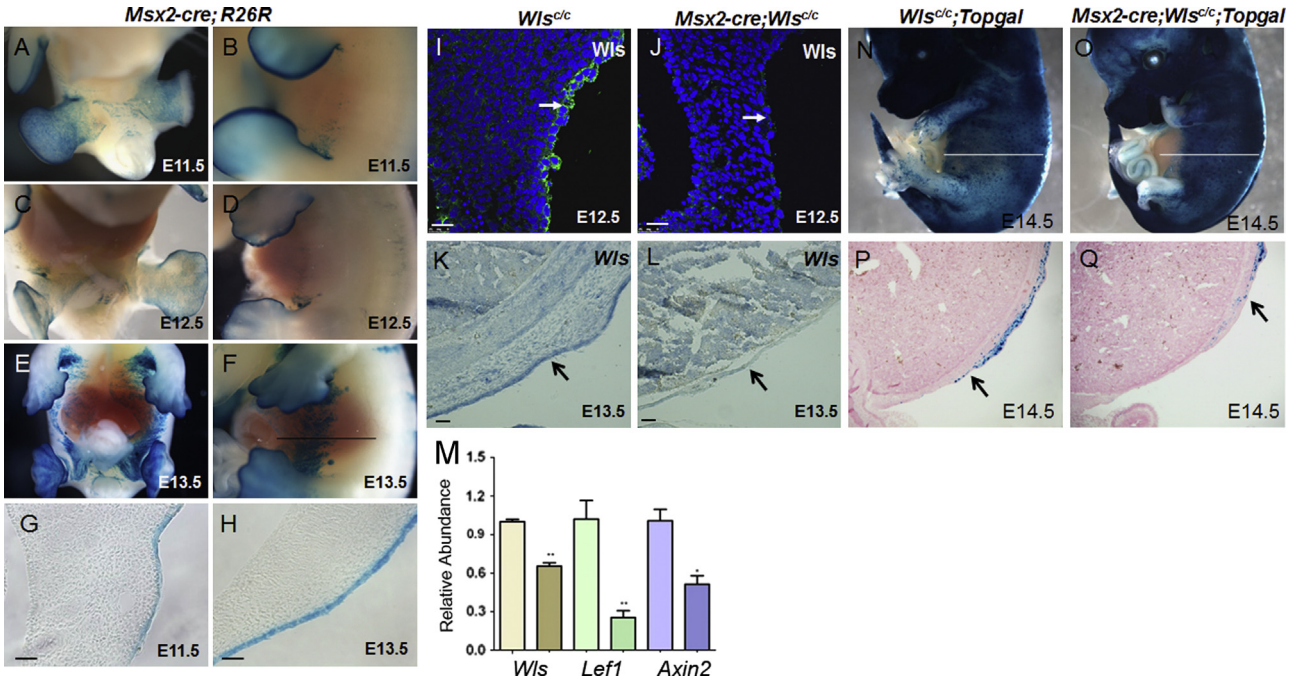

Fig. 2. Cre activity in the ventral body wall ectoderm in the *Msx2-Cre* mice. (A–F) X-gal staining of *Msx2-cre*; *R26R* embryos at E11.5 (A, B), E12.5 (C, D) and E13.5 (E, F) showing Cre activity in the abdominal body wall ectoderm. A–E, ventral view; B–F, lateral view. (G–H) Transverse sections of the embryo trunk at E11.5 (G) and E13.5 (H) corresponding to panels B and F. (I–L) The expression of the Wls protein (I, J) and mRNA (K, L) was significantly reduced in the ventral body wall ectoderm in the *Msx2-cre*; $Wls^{c/c}$ mutant embryo at E12.5, indicating effective deletion of *Wls* by *Msx2-cre*. (M) qRT-PCR analysis indicated that the expression of *Wls*, *Lef1* and *Axin2* was downregulated in the ventral body wall in the control and *Msx2-cre*; $Wls^{c/c}$ mutant E12.5 embryos. $N=3$. (N–O) LacZ staining for $Wls^{c/c}$; *Topgal* and *Msx2-cre*; $Wls^{c/c}$; *Topgal* embryos at E14.5. (P–Q) Section of LacZ staining in N–O. White lines indicated the position of sections.

knockout embryos, *Wls* expression was efficiently depleted in the ectoderm of the ventral body wall, indicated by IHC with a Wls antibody (arrows in Fig. 2I and J) and by *in situ* hybridization with a *Wls* probe (arrows in Fig. 2K and L). qRT-PCR validated the decrease in expression levels of *Wls*, *Lef1* and *Axin2* in the mutant ventral body wall compared to the controls (Fig. 2M, $p<0.05$, $n=3$). Furthermore, diminished LacZ staining was observed in ventral epithelium of *Msx2-cre*; $Wls^{c/c}$; *Topgal* relative to $Wls^{c/c}$; *Topgal* embryos at E14.5 (arrows in Fig. 2P and Q), indicating the downregulation of canonical Wnt signaling activity.

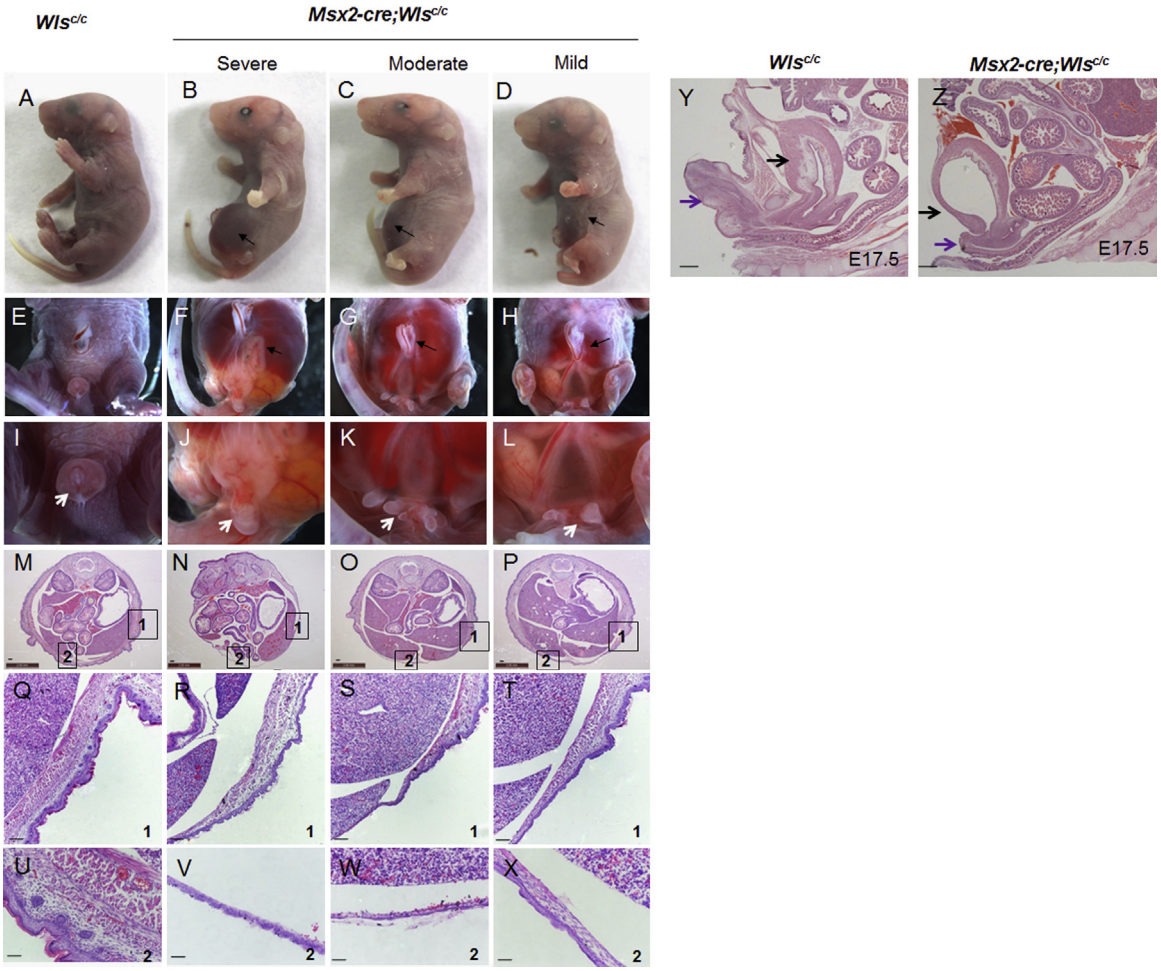

Fig. 3. Abnormal abdominal body wall phenotypes in the *Msx2-cre*; *Wls*$^{c/c}$ embryos. (A–D) Lateral view of the wild-type and *Msx2-cre*; *Wls*$^{c/c}$ mutant embryos at E18.5. Arrows indicate abdominal body walls with severe, moderate, and mild phenotypes. (E–H) Ventral high-magnification view of the mouse body wall corresponding to panels A–D. The abdominal body wall in *Msx2-cre*; *Wls*$^{c/c}$ mutant embryos is nearly transparent. (I–L) Defects in the genito-urinary tracts in the *Msx2-cre*; *Wls*$^{c/c}$ mutant embryos. (M–P) HE staining of transverse sections of the trunk (50 × magnification). (Q–T) High-magnification view of the box 1 lateral region in M–P (200 × magnification). (U–X) High-magnification view the of box 2 ventral region in M–P (200 × magnification). Indiscernible muscle and ventral dermis were formed in the mutant ventral body wall compared to the controls. (Y–Z) HE staining of sections of the genito-urinary system in the mutants. Black arrows mark the enlarged bladder, and purple arrows show the defective urinary tracts.

The homozygous *Msx2-cre*; *Wls*$^{c/c}$ embryos exhibited varied deformities in ventral body wall development, categorized as severe, moderate and mild phenotypes according to the width of secondary ventral body wall and the extent of lateral muscle migration at E18.5 (Fig. 3). More than 30 mutant mice were analyzed and the percentage of each type are as following: severe (40%) , moderate (40%), mild(20%). The severe and moderate mutants had outward extension of the visceral organs in the belly (Fig. 3B–D), which was covered by a nearly transparent abdominal body wall (Fig. 3F–H). The body wall in the mutants was composed of a thin layer of epithelium at the most ventral part, whereas the body wall was relatively normal in the lateral part. Meanwhile, the most ventral part of the body wall in the mild mutant consisted of a thin layer of epithelium and underlying mesenchyme (Fig. 3M–P and U–X). In addition, genitalia formation was disrupted in the *Msx2-cre*; *Wls*$^{c/c}$ embryos (arrows in Fig. 3I–L). According to HE staining of abdominal sections at E17.5, the bladder was dilated and the bladder mesenchyme was diminished in the mutants compared to the controls (arrows in Fig. 3Y and Z). These deficiencies in *Msx2-cre*; *Wls*$^{c/c}$ mice were 100% penetrant. The range of severity was concordant between ventral body wall and genito-urinary tracts. The structure of the ventral body wall in the severe mutants resembled the primary abdominal body wall in wild-type embryos before E12.5, indicating agenesis of the secondary ventral body wall. Together, these data indicated that the loss of *Wls* in epithelium impairs secondary body wall and genito-urinary system development.

Ectodermal loss of Wls disrupts abdominal myogenesis

The secondary ventral body wall is formed by mesenchyme derived from the lateral plate mesoderm and myogenic progenitor cells originating from the dermomyotome. To dissect the developmental defects in the *Msx2-cre*; *Wls*$^{c/c}$ embryos, myogenic differentiation and migration were evaluated in cross-sections by IHC or *in situ* hybridization. Epidermal development appeared normal in the mutant ventral body wall, as indicated by the normal expression of K5 (Fig. 4A–D), a keratinocyte marker in the skin. However, muscle formation was remarkably diminished in the ventral most part of the body wall, as revealed by an anti-MLC antibody (Fig. 4E–H).

Myogenic progenitor cells migrate ventrally along the musculature formation in secondary ventral body wall. To dissect the molecular pathways involved in the myogenic defects in the mutant ventral body wall, several myoblast markers were employed in IHC or *in situ* hybridization. MyoD is an early myoblast marker while Myogenin is expressed in differentiated myoblasts. Defects in myogenic development in the mutants were

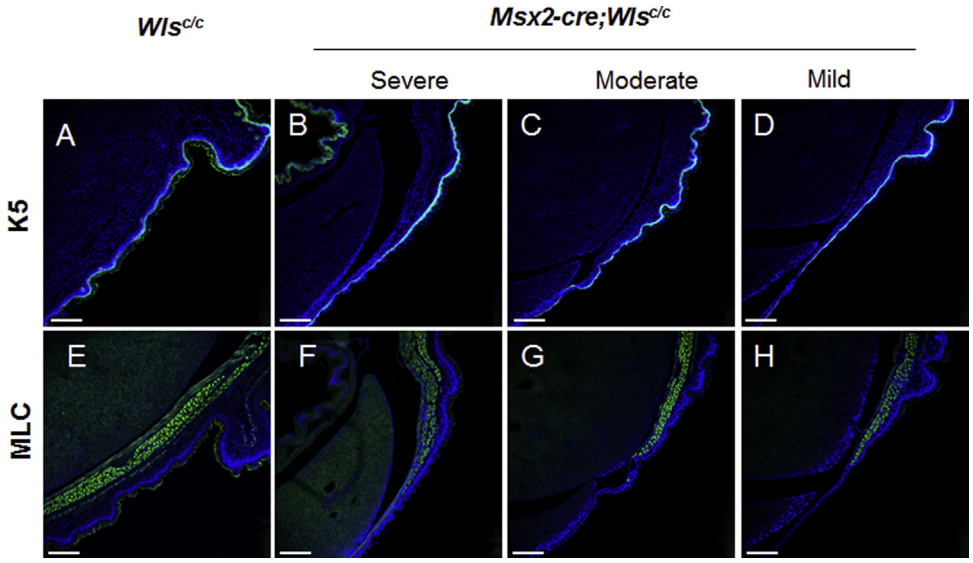

Fig. 4. Impaired muscle development in the ventral body wall of *Msx2-cre*; *Wls*$^{c/c}$ mutants. (A–D) Immunofluorescence staining for K5, a marker of the basal layer, showed relatively normal epithelium formation in the *Msx2-cre*; *Wls*$^{c/c}$ mutant. (E–H) Immunostaining with a myosin antibody indicated that the development of the ventral body wall muscle was perturbed in the mutant. DAPI, blue; K5 or myosin, green.

found at E13.5 and E14.5 and were marked by the excluded expression of *Myogenin* in the furthermost ventral body wall (Fig. 5H′–I′). The distance of the *Myogenin* positive cells from the ventral front to the dorsal midline was shorter in the mutants at E13.5 compared to the wild-type controls (dual arrows in Fig. 5H–I′). In contrast, *MyoD* expression in the lateral ventral body wall was not remarkably different between wild-type and mutant, as evidenced by *in situ* hybridization in embryos from E11.5 to E13.5 (arrows in Fig. 5A–C′) and IHC analysis in E12.5 sections (Fig. 6A and B). However, mesenchymal cell migration and differentiation underlying the ventral ectoderm was disrupted in the furthermost ventral part of the body wall in the *Msx2-cre*; *Wls*$^{c/c}$ embryos, as indicated by the significant exclusion of *Dermo1* and *Pitx2* expression from the ventral most mesenchyme (arrows in Fig. 6L–M′). *Pitx2* has been reported to be expressed in somite-derived myogenic progenitor cells and act as an early inducer of myogenic differentiation (Eng et al., 2012). In line with the start of ventral musculature deficiency, *Pitx2* expression level was comparable in lateral body wall between the wild type and mutant embryos at E11.5 and E12.5 (arrows in Supplemental Fig. S1). But it was excluded from ventral body wall of E13.5 mutant (double head arrows in Supplemental Fig. S1). These findings suggested that the loss of ectodermal *Wls* disrupted late abdominal myogenic differentiation during secondary ventral body wall development.

Depletion of epithelial Wls does not affect the proliferation and survival of abdominal myogenic progenitor cells

To investigate the cellular mechanisms underlying the abdominal myogenesis defects, cell proliferation and death were examined by BrdU labeling and a TUNEL assay. In E12.5 sections, the MyoD expression domain in the ventral body wall was outlined, and BrdU positive cells were counted in a similar region in consecutive sections (Fig. 6A–D). Meanwhile, BrdU positive cells were also counted in the underlying mesenchyme of the ventral body wall (Fig. 6E and F). Statistical analysis showed that cell proliferation was not altered in the *Msx2-cre*; *Wls*$^{c/c}$ embryos compared to the controls (Fig. 6G, $p > 0.05$, $n=3$). Moreover, no differences were detected in cell death in the ventral body wall, as revealed by the TUNEL assay (Fig. 6H–K, $p > 0.05$, $n=3$). These data indicated that cell proliferation and survival were not primary contributors to the abdominal musculature defects in the mutant.

Ablation of epithelial β-catenin or Wnt5a has no effect on ventral body wall development

The loss of *Wls* decreased the activity of canonical Wnt signaling in the ventral body wall (Figs. 2M, 5K and K′). To test whether the musculature defects in the mutant ventral body wall resulted from the cell autonomous effect of Wnt signaling ablation in the ectoderm, we revisited *Msx2-cre*; *Catnb*$^{c/c}$ mice with β-catenin deficiency in ventral body wall ectoderm. The *Msx2-cre*; *Catnb*$^{c/c}$ mutant mice had no limb formation (Fig. 7A and B), which was in line with a previous report (Barrow et al., 2003). However, the mice displayed no obvious defects in ventral body wall development compared to the controls, as evidenced by HE staining and IHC with anti-MLC on transverse sections (Fig. 7C–H). Therefore, the ventral body wall defects caused by the loss of epithelial *Wls* may not have resulted from the intrinsically defective Wnt signaling in the ectoderm, but may have been the consequence of blocking ectodermal Wnt secretion.

We also investigated the ventral body wall structure in *Wnt5a*$^{-/-}$ mice by HE staining and IHC analysis on transverse sections at E16.5. Similarly, no remarkable defect in ventral body wall was detected in *Wnt5a* null mice (Fig. 7I–N). These results also indicated that Wnt5a, the non-canonical Wnt, may not be the major Wnt protein that signals from epithelium to mesenchyme during ventral body wall development.

Discussion

In this study, we investigated *Msx2-cre*; *Wls*$^{c/c}$, *Msx2-cre*; *Catnb*$^{c/c}$ and *Wnt5a*$^{-/-}$ embryos and compared their defects in ventral body wall development. Based on these genetic and molecular analyses, we conclude that ectodermal Wnt signaling instructs the differentiation and specification of the underlying mesenchyme in a paracrine manner during ventral body wall formation. These data shed new insight into our understanding of the molecular mechanisms of myogenesis. The Wnt/Pitx2 pathway might be commonly employed in myogenesis through ectoderm-

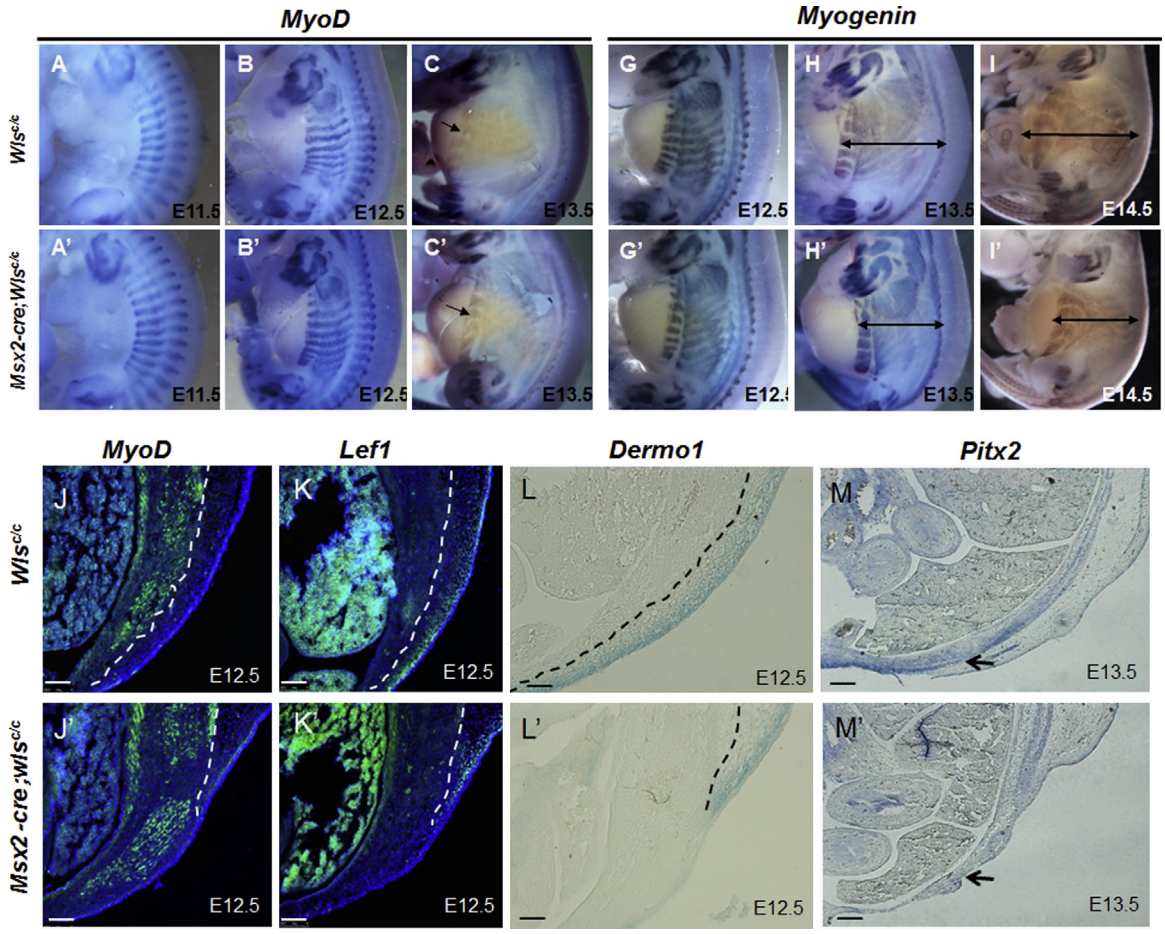

Fig. 5. Myoblast migration and differentiation is impaired in the ventral body wall of *Msx2*$^{cre/+}$; *Wls*$^{c/c}$ mutants. (A–C′) *MyoD* expression, which represented myoblast progenitor cells, was relatively normal in the ventral body wall from E11.5 to E13.5 in the *Msx2-cre*; *Wls*$^{c/c}$ mutant compared to the controls. (G–I′) *Myogenin* expression, which marked differentiated muscle fibers, was excluded from the far most ventral body wall in the *Msx2-cre*; *Wls*$^{c/c}$ mutant at E13.5 and E14.5 (double arrows). However, its expression was comparatively normal at E12.5. (J–J′) MyoD expression was comparable in the ventral body wall between sections from the control and *Msx2-cre*; *Wls*$^{c/c}$ mutant at E12.5. (K–K′) Immunofluorescence staining showed that the expression of Lef1 was significantly reduced in the mutant. (L–L′) *Dermo1* mRNA was absent from the ventral mesenchyme at E12.5 in a *Msx2-cre*; *Wls*$^{c/c}$ mutant (dashed lines). (M–M′) *Pitx2* expression was not detected in the far most ventral mesenchyme in a E12.5 mutant (arrows).

mesoderm crosstalk across multiple systems, including somites, limbs and the ventral body wall. Furthermore, these results add new evidence for the potential unifying theory that claims that most of the ventral body wall deficiencies arise from defective epithelial–mesenchymal signaling.

Ectodermal Wnt signaling regulates abdominal myogenesis during secondary ventral body wall development

The developmental processes of limb myogenesis and abdominal myogenesis share many aspects, which involve the coordinated migration of lateral plate mesoderm and myogenic differentiation from the dermomyotome. Wnt signaling is an important regulator of myogenic differentiation and muscle fiber elongation in somitogenesis and limb formation (Borello et al., 2006; Brand-Saberi and Christ, 1999; Francis-West et al., 2003; Gros et al., 2009; van der Velden et al., 2008). Ectodermal Wnt6 is essential for limb myogenesis (Geetha-Loganathan et al., 2006; Geetha-Loganathan et al., 2005). Our previous study reported that the *Msx2-cre*; *Wls*$^{c/c}$ mice display deficiencies in limb myogenesis (Zhu et al., 2012). *Msx2-cre* was originally detected to be expressed in the limb ectoderm (Barrow et al., 2003). Here, we identified that *Msx2-cre* also had Cre activity in the abdominal ectoderm. Our further investigations revealed defects in abdominal musculature formation and dysplasias in the ventral body wall in *Msx2-cre*; *Wls*$^{c/c}$ embryos. Therefore, *Wls*-mediated ectodermal Wnt signaling regulates myogenesis during both limb and ventral body wall development.

According to our qRT-PCR examinations, several Wnts, including *Wnt2*, *Wnt3*, *Wnt5a*, *Wnt6*, *Wn7b* and *Wnt11*, were comparatively enriched in the developing ventral body wall (Fig. 1A). The expression of *Wnt6* in the ectoderm was validated by *in situ* hybridization (Fig. 1B). In the *Msx2-cre*; *Wls*$^{c/c}$ embryos, canonical Wnt signaling activity was significantly decreased in the underlying mesenchyme of the ventral body wall (Fig. 2M), and abnormalities in the dermis specification and myogenic differentiation were identified (Fig. 5K–M′). In contrast, the loss of β-catenin in the ectoderm of *Msx2-cre*; *Catnb*$^{c/c}$ embryos caused no comparable defects in the ventral body wall as that in the epithelium-specific *Wls* null embryos (Fig. 7A–H). However, Wnt5a is a typical non-canonical Wnt that is involved in the regulation of distal mesenchyme extension or the outgrowth of multiple organs (Gao, 2012; Yamaguchi et al., 1999). However, the *Wnt5a* null embryos also had no remarkable defects in ventral body wall myogenesis (Fig. 7I–N). *Wnt5a* expression was also absent from the ventral ectoderm (Fig. 1F and H). Therefore, non-canonical Wnt5a signaling might not contribute to ventral body wall development. These analyses indicate that ectodermal canonical Wnt ligands, most likely Wnt6, regulate ventral body wall development through epithelia-mesenchymal signaling. Of course, to validate this hypothesis, ventral body wall development could be investigated in embryos that were implanted with beads soaked with Wnt6 protein or anti-Wnt6 antibody.

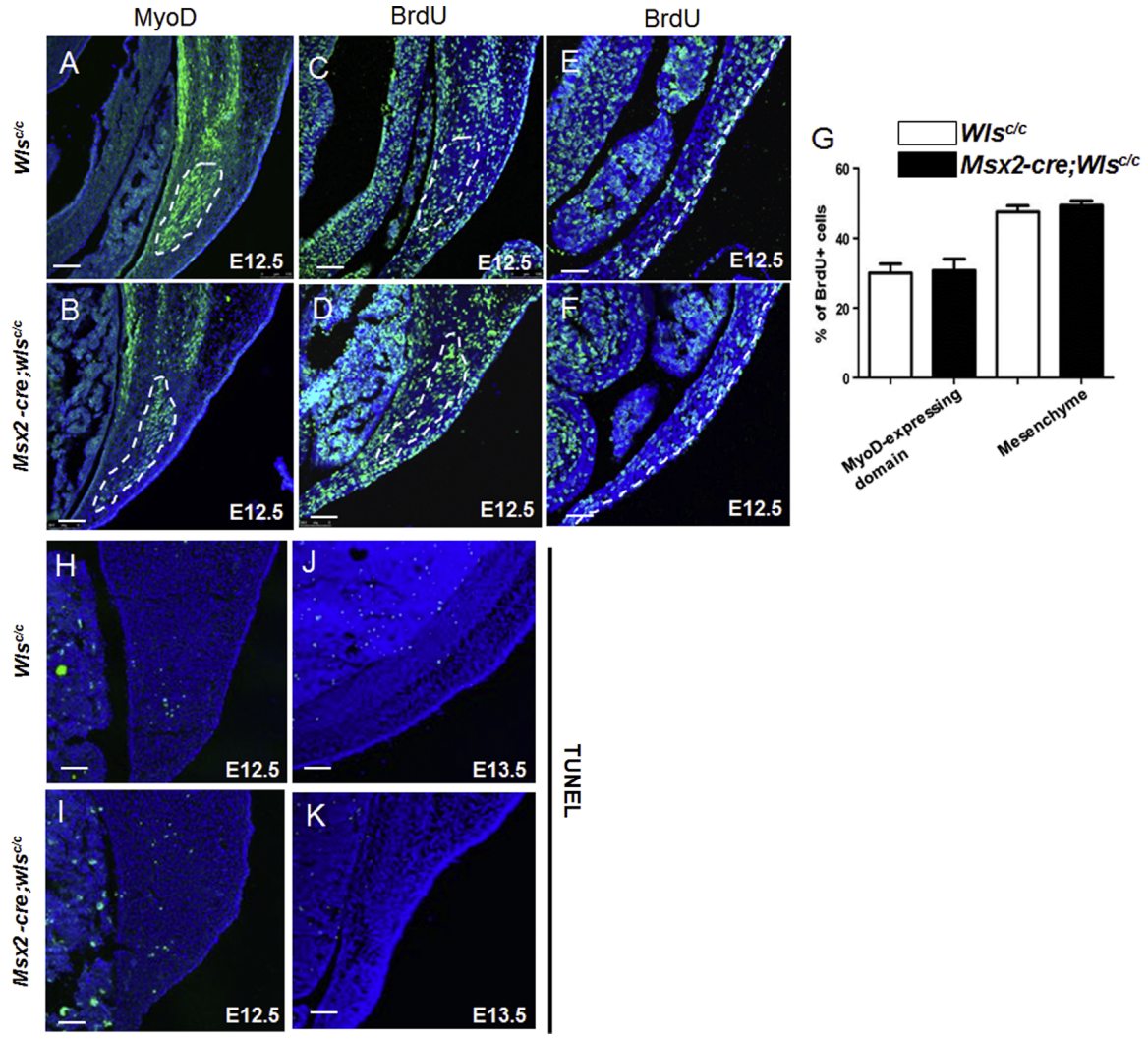

Fig. 6. Loss of ectodermal *Wls* has no impact on mesodermal cell proliferation. (A–B) The MyoD-expressing domain in the ventral body wall at E12.5. (C–D) BrdU labeling in the presumptive MyoD expression domain at E12.5 (dashed line). (E–F) BrdU labeling in mesenchymal cells in the ventral body wall at E12.5. (G) Cell proliferation in MyoD-expressing and mesenchymal cells was not significantly different in the mutants compared to the controls at E12.5. (H–K) TUNEL staining showed that cell apoptosis was not significantly altered in the ventral body wall at E13.5.

The Wnt-Pitx2 signaling pathway has been reported to regulate cell proliferation in limb myogenesis and somite myogenesis (Abu-Elmagd et al., 2010; Briata et al., 2003; Kioussi et al., 2002). *Pitx2* has been identified as one cause of Reiger syndrome, which includes defects in the ventral body wall in addition to eye, craniofacial and cardiac abnormalities (Gage and Camper, 1997; Semina et al., 1996). Furthermore, the loss of *Pitx2* in mice leads to deficiencies in abdominal musculature formation and closure of the ventral body wall (Eng et al., 2012; Kitamura et al., 1999). In our results, the deficiency of ectodermal *Wls* led to a decrease in *Pitx2* and *Lef1* expression in the ventral mesenchyme and myogenic progenitor cells (Fig. 5L–M′). Taken together, our findings suggest that the Wnt/β-catenin-Pitx2 pathway, at least in part, mediated the signaling from the overlaying ectoderm to instruct abdominal myogenesis. The Wnt-Pitx2 pathway might commonly regulate myogenesis in multiple tissues, including limbs, somites and the ventral body wall.

Ectodermal Wnt signaling is a potential etiological factor for PBS

Animal mutants displaying typical PBS-like deficiencies are rarely reported. The transcriptional factor HNF1β, which regulates embryogenesis and visceral endoderm specification (Coffinier et al., 1999; Mefford et al., 2007), contributes to the etiology of some clinical cases of PBS, especially in patients with renal cystic dysplasia and prostatic hypoplasia (Granberg et al., 2012; Haeri et al., 2010). The most common cause of prune belly syndrome (PBS) was previously thought to be urethral obstruction malformation, which results in an enormous bladder and ureteral dilation that distends the fetal abdomen (King and Prescott, 1978; Pagon et al., 1979). However, our observations did not support this hypothesis. In our study, specific inactivation of *Wls* in the mouse abdominal ectoderm led to typical PBS-like phenotypes. The loss of epithelial *Wls* affected the differentiation of myogenic progenitor cells in the underlying mesenchyme, as well as the formation of the genito-urinary system. Previous report also reveals that epithelial Wnt signaling directs external genitalia formation (Miyagawa et al., 2009). Therefore, the defective epithelial–mesenchymal signaling seems to be a more important cause for PBS etiology. Wnt/Pitx2 axis might be a potential genetic factor contributing to the pathology of PBS.

Epithelial-mesenchymal signaling may be a unifying mechanism for the origin of ventral body wall defects

Several animal models of ventral body wall closure defects have been established. For example, TGF-β2/TGF-β3 double knockout mice

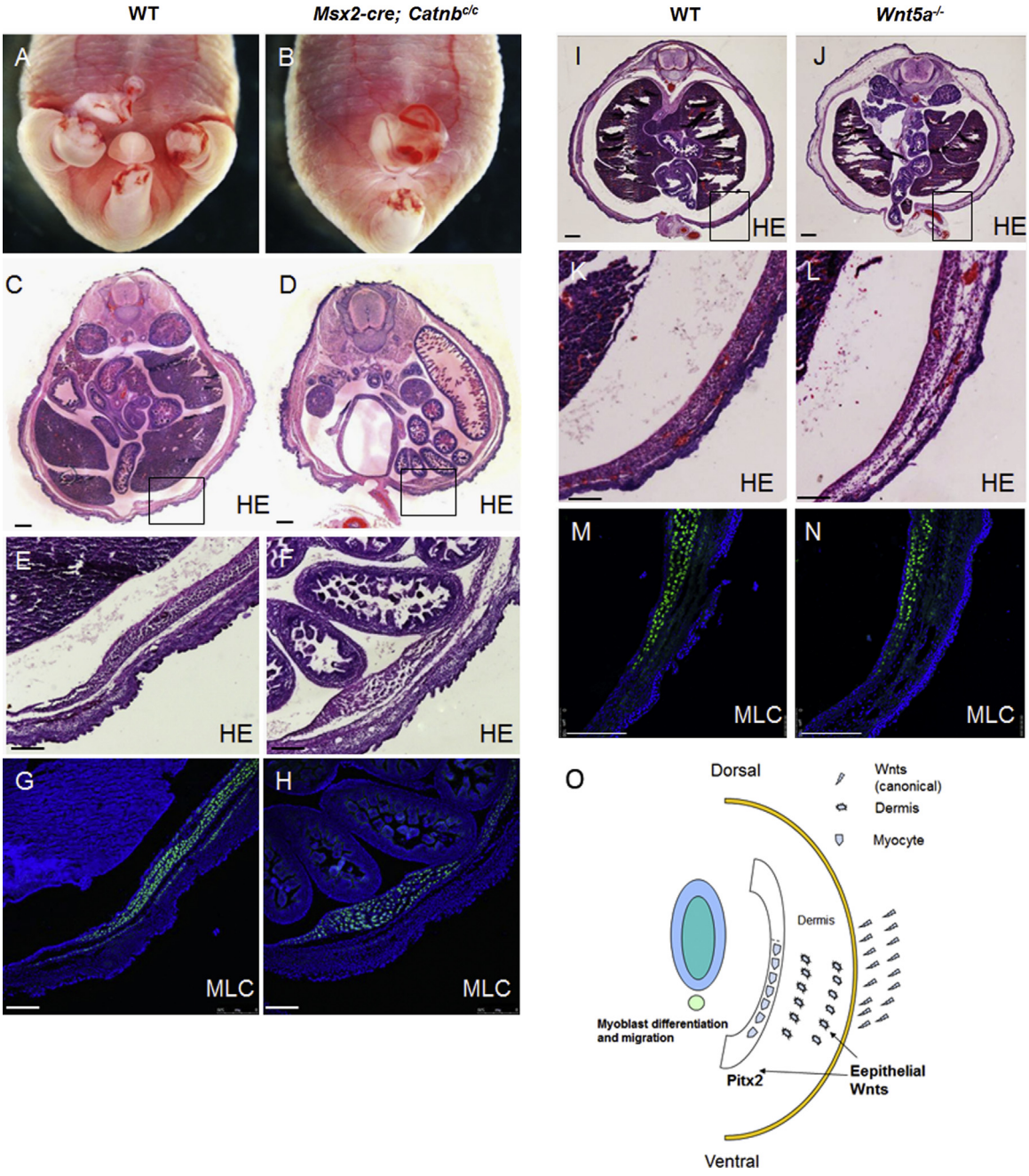

Fig. 7. Loss of ectodermal *β-catenin* or *Wnt5a* has no obvious impact on the ventral body wall. (A–B) Ventral view of a *Msx2-cre*; *Catnb*$^{c/c}$ embryo at E17.5 showed the loss of hindlimbs and a relatively normal ventral body wall. (C–D) HE staining of transverse sections of *Msx2-cre*; *Catnb*$^{c/c}$ embryos. (E–F) Enlarged view of boxed region in (C–D). (G–H) Immunostaining for myoblasts by a MLC antibody at E17.5. (I–J) HE staining of transverse sections of the *Wnt5a*$^{-/-}$ embryos at E16.5. (K–L) Enlarged view of the boxed region in I–J. (M–N) Immunostaining of myoblasts by a MLC antibody at E16.5. These analyses indicated normal muscle development or migration in the ventral body walls of *Wnt5a* or epithelial *Wls* mutants. (O) Diagram showed that epithelial Wnt signaling regulated underlying myogenesis and dermis specification during ventral body wall development through β-catenin-Pitx2 axis.

and AP2-α knockout mice both display the most severe defects in ventral body wall development (Brewer and Williams, 2004b; Dunker and Krieglstein, 2002). *IGF-II receptor*/*IGF-II* mutants (Eggenschwiler et al., 1997) and *Bmp1* null mice (Suzuki et al., 1996) exhibit abnormalities across different organs in the ventral body wall. This evidence suggests that multiple signaling pathways, including TGF-β, IGF and Bmp signaling, are involved in regulating ventral body wall development. Further investigations employing conditional knockout mice of these signaling pathways would provide better understanding of their contributions to the development of the ventral body wall. And more, studies have showed that multiple signaling pathways, including Hh and Wnt signaling regulate development of external genitalia through epithelial–mesenchymal interaction (Miyagawa et al., 2009; Suzuki et al., 2009). On the other hand, several other mutants, including *Shroom* (Hildebrand and Soriano, 1999), *PS1* (Hartmann et al., 1999), *Hic1* (Carter et al., 2000), *Zic3* (Klootwijk et al., 2000), *Alx4* (Qu et al., 1997), *Hoxb2*/*b4* (Manley et al., 2001) and *p57*^{KIP2} (Zhang et al., 1997), present partially penetrant body wall phenotypes. These genetic factors are also possible mesenchymal factors that are responsive to ectodermal signaling in regulating ventral body wall formation. Together with these reports, our findings support the theory that disrupted epithelial–mesenchymal signaling may be a unifying mechanism that accounts for most ventral body wall defects.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2013.12.027.

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