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SPECIAL ISSUE RESEARCH ARTICLE

Identification of Responsive Cells in the Developing Somite Supports a Role for β-Catenin-Dependent Wnt Signaling in Maintaining the DML Myogenic Progenitor Pool

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Somitic β-catenin is involved in both maintaining a stem cell population and controlling myogenic differentiation. It is unclear how β-catenin-dependent Wnt signaling accomplishes these disparate roles. The present study shows that only dorsal cells in the early somite respond to β-catenin-dependent Wnt signaling and as the somites compartmentalize to form the dermomyotome and myotome, responding cells are detected primarily in the dorsomedial lip (DML). Forced activation of Wnt target genes in DML cells prevents their progeny from entering the myotome, while blocking activation allows myotomal entry. This suggests a role for β-catenin-dependent/Wnt signaling in maintaining progenitor cells in the DML and that if β-catenin-dependent/Wnt signaling is required to induce myogenesis, the response is transitory and rapidly down-regulated. *Developmental Dynamics 239:222–236, 2010.* © 2009 Wiley-Liss, Inc.

Key words: somite; myotome formation; Wnt signaling; nuclear beta-catenin; dorsomedial lip

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INTRODUCTION

All skeletal muscle in the vertebrate trunk is derived from somites, the segmental structures that form from the paraxial mesoderm in a rostral to caudal progression during development. As new somites form caudally, the more rostral somites undergo a process of compartmentalization. Newly formed somites are epithelial balls, and as development proceeds, cells migrate away from the ventral region to form the sclerotome; the remaining dorsal cells form an epithelial layer, the dermomyotome. Skeletal muscle precursors arise primarily from the dermomyotome to form the back as well as the body wall and limb muscles (for a recent review, see Bryson-Richardson and Currie, 2008).

Skeletal myogenesis is initiated in somitic cells as they delaminate from the lips of the dermomyotome. The dorsomedial lip (DML) represents the initial site of primary myotome induction and is the site of involution of myotomal precursors of the epaxial muscles. During primary myotome formation, the DML is an important source of epaxial muscle precursors (Denetclaw et al., 1997; Ordahl et al., 2001); later, mitotically competent muscle precursors invade the myotomal compartment of the somite from the central domain of the dermomyotome and contribute cells to the myotome (Gros et al., 2004, 2005; Ben-Yair and Kalcheim, 2005; Kassar-Duchossoy et al., 2005; Relaix et al., 2005).

A complex array of signals emanating from surrounding tissues has been shown to influence primary myogenesis and somite patterning. For example, ablation of dorsal tissues, (dorsal neural tube and ectoderm) results in defective dermomyotome development, whereas ablation of ventral tissues (notochord and floor plate) negatively affects sclerotome formation (Brand-Saberi et al., 1993; Dietrich et al., 1997). When epithelial somites are cultured without neighboring tissues, somite patterning and

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differentiation of muscle and cartilage does not occur (Kenny-Mobbs and Thorogood, 1987; Buffinger and Stockdale, 1994; Munsterberg and Lassar, 1995; Stern and Hauschka, 1995). Notochord and neural tube–derived signals are thought to guide myogenic differentiation in the first myotomal cells and induce epaxial myogenesis (Ordahl and Le Douarin, 1992; Buffinger and Stockdale, 1995; Munsterberg and Lassar, 1995; Stern and Hauschka 1995; Pownall et al., 1996; Spence et al., 1996; Dietrich et al., 1997). Wnt3a, Wnt1, and Wnt6 all mimic the effect of the dorsal neural tube and surface ectoderm, suggesting that these Wnt ligands regulate myogenesis in the primary myotome (reviewed in Geetha-Loganathan et al., 2008).

Numerous studies have implicated signaling through the canonical Wnt pathway in the activation and maintenance of myogenesis both in vivo and in vitro. Wnts initially were shown to be required for induction of myogenesis in somite explants in coculture experiments (Stern and Hauschka, 1995; Munsterberg et al., 1995; Fan et al., 1997; Reshef et al., 1998). Similarly, Wnt signaling, acting via β-catenin, was shown to be necessary and sufficient for skeletal myogenesis in P19 cells (Ridgeway et al., 2000; Petropoulus and Skerjenic, 2002) and to activate myogenesis in adult myogenic stem cells (Polesskaya et al., 2003). Furthermore, mice null for the neural tube–derived Wnts 1 and 3a (Ikeya and Takada, 1998) and mice treated with the Wnt antagonist Frzb1 display myotomal defects (Borello et al., 1999). These data all suggest a crucial role for signaling through the canonical Wnt pathway in myogenic induction. This is further supported by the localized expression of transcripts of components of the canonical Wnt pathway in the dorsomedial-most region of the somite where the first myogenic cells are specified (Schmidt et al., 2000; Cauthen et al., 2001).

Wnts are thought to induce myogenesis in the primary myotome through the upregulation of β-catenin (Schmidt et al., 2000), the key mediator of signaling through the canonical Wnt pathway (Logan and Nusse, 2004). Transcripts of the Wnt receptor Frizzled 1 (Fz1), β-catenin, and the nuclear partner of β-catenin, Lef1, are all expressed during somitogenesis with Lef1 and β-catenin transcripts restricted to the developing myotome at later stages (Schmidt et al., 2000). Fz2 and Fz7 are also expressed in epithelial somites and in the dermomyotome (Linker et al., 2003).

Although signaling through the canonical Wnt pathway participates in the patterning of dorsal structures and induction of myogenesis in the somite, the cellular processes that are regulated by β-catenin-dependent Wnt signaling are not well understood. This is compounded by conflicting reports concerning the activation of myogenic genes and the role of Wnts in the somite. For example, it has been proposed that induction of myogenesis in the somite is through a β-catenin-independent Wnt pathway requiring PKA and CREB (Chen et al., 2005). Similarly, a proliferative role for signaling through the canonical Wnt-pathway via Wnt3a in the dermomyotome has been demonstrated (Galli et al., 2004), but it is unclear precisely which cells are responding and how they respond. While Wnt1 and Wnt3a have been shown to preferentially activate Myf5, and Wnts6 and 7a preferentially activate MyoD (Tajbakhsh et al., 1998; Cossu and Borello, 1999), Wnt signaling directly regulates Myf5 expression through direct binding of TCF/Lefs and β-catenin to its enhancer (Borello et al., 2006) but MyoD is thought to be activated through a β-catenin-independent mechanism (Brunelli et al., 2007). This implies that signaling through the non-canonical, β-catenin-independent, Wnt pathway also has a role in the induction of myogenesis. Further complicating the role of Wnts in myogenesis is the recent finding that β-catenin, but not TCF/Lefs, is required for muscle differentiation and that MyoD interacts directly with β-catenin and that β-catenin/MyoD complexes, instead of the β-catenin/TCF complex, are involved in Wnt-mediated regulation of muscle differentiation (Kim et al., 2008).

Since β-catenin is a multifunctional protein, with a role in cell adhesion as well as Wnt signaling, transcript expression is not indicative of where β-catenin/TCF functions to activate Wnt target genes. However, β-catenin observed in the nucleus (Batlle et al., 2002) and the activation of a Wnt reporter (Dorsky et al., 2002) can be used to identify and define cells that are responding to signaling through the canonical Wnt pathway in the developing somite. To begin to determine which cells of the somite respond to signaling through the canonical Wnt pathway, we used both immunohistochemistry to determine where β-catenin is nuclearized and in ovo electroporation of the reporter, TOPGFP, to identify where in the developing somite β-catenin/TCF/Lefs activate Wnt target gene transcription.

We found that as the somite progresses through compartmentalization, the cells responding to β-catenin/Wnt signaling are present in specific regions. In both the early somite, prior to myotome formation (somite IV–VI), and late somite, after myotome formation (somite X and older), Wnt-responding cells are restricted to the dorsal Pax7-positive domain of the somite. Wnt-responsive cells were not found in either the myotome or the sclerotome. Furthermore, cells expressing an activated form of TCF were restricted from entering the myotome, suggesting that β-catenin/Wnt signaling maintains the myogenic progenitor population in the DML, but must be "turned off" for myogenic differentiation to proceed.

RESULTS

Nuclear β-Catenin Localization in Somitic Cells

We first examined somites at different developmental stages for nuclear localization of β-catenin to determine where and when in the somite cells are responding to signaling through the canonical Wnt pathway. Because β-catenin degradation is blocked upon the receipt of a Wnt signal and β-catenin subsequently enters the nucleus, nuclear localization of β-catenin can be used to identify cells that are likely to be responding to signaling through the canonical Wnt pathway. We examined 5-μm transverse sections of chicken embryos using immunocytochemistry and antibody against β-catenin, coupled with propidium iodide

nuclear staining. Since propidium iodide stains nuclei red, antigen detection employed a secondary antibody that fluoresced green, and in this way, co-labeled nuclei would appear yellow. Somites were analyzed at two stages: early somites (stage IV–VI; Fig. 1 A) prior to myotome formation and later somites (stages VII–X; Fig.1 G) that have formed a myotome. These are the same somite stages as those examined in later electroporation experiments.

In early somites, there was a distinct difference in Wnt signaling between dorsal and ventral cells. Intense staining for nuclear β-catenin, indicated by yellow nuclei due to co-localization of β-catenin (green) and the nuclear signal (red), was observed in the dorsal epithelial cells of the presumptive dermomyotome (Fig.1B, C, E). Strong membrane-associated β-catenin staining was also observed along the apical surfaces of these cells. In contrast, no appreciable β-catenin staining was observed in the nuclei of ventral cells in the presumptive sclerotome, which appear red (Fig. 1D).

In older somites, high levels of nuclear β-catenin were observed in cells restricted to the dorsomedial lip (DML, Fig. 1H). Fewer cells with β-catenin nuclear staining were observed in other regions of the dermomyotome (Fig. 1I). Most importantly, there was no nuclear β-catenin staining of cells in the myotome. Unlike the cells of the DML, which have yellow nuclei (Fig. 1H), the nuclei of the myotomal cells appear red despite high levels of membrane-bound β-catenin (Fig. 1J) and close proximity to the dorsal neural tube, a known source of Wnt signals. This suggests a lack of response to β-catenin-mediated Wnt signaling in the myotomal cells. That nuclear β-catenin was most clearly observed in the cells of the DML is consistent with data showing that Wnt 1 and Wnt 3a double knockout mice lack a DML (Ikeya and Takada, 1998).

Expression of TOPGFP Is Restricted to Dorsal Pax7-Expressing Cells in Young Somites

To further investigate which cells in the somite respond to β(-catenin/Wnt signaling, we used the Wnt reporter TOPGFP (Dorsky et al., 2002), where the expression of a destabilized GFP protein with a half-life of approximately 12 hr is dependent upon reiterated TCF/LEF1 binding elements. Detection of GFP expression, to a first approximation, therefore reflects current activation of the promoter by β-catenin-mediated Wnt signaling or activation in the cell's recent history. In ovo electroporation was used to deliver either TOPGFP or the control plasmid pCAX/GFP, which constitutively expresses GFP, to cells in somites II–IV of HH stage-13–14 embryos. Initially, we examined differences in the β-catenin-mediated Wnt responsiveness of dorsal versus ventral cells, and examined somites 6 hr post-electroporation. Pax7 expression was used to identify dorsal somitic cells. These early somites had compartmentalized into dorsal and ventral halves but had not yet formed a myotome.

When TOPGFP was directed towards cells in the dorsal half of the somite, GFP-positive cells were detected in all electroporations (Fig. 2B–D) and GFP expression was detected only in cells that co-labeled for Pax7. In contrast, when TOPGFP was directed to cells in the ventral half of the somite, GFP expression was not observed in the Pax7-negative, ventral cells (Fig. 2E, F). Occasionally, however, GFP-expressing cells were observed when ventral cells were electroporated, but these cells always co-labeled with Pax7. These results are consistent with our data demonstrating that nuclear β-catenin staining is observed in the dorsal but not in the ventral half of the early somite. As observed here and by

Fig. 1. Nuclear β-catenin is restricted to cells in the dorsal half of early somites and is found in cells in the dorsomedial lip and dermomytome, but not in the myotome, of later (compartmentalized) somites. β-catenin nuclear localization was determined by co-labeling sections with the nuclear dye propidium iodide (red) and anti-β-catenin antibody (green). Wherever β-catenin and the nuclear stain co-localize, cells show distinct yellow nuclei (arrow in B). **A**: In the early somite (stage IV–VI), distinct membrane-associated β-catenin staining was observed along the apical surfaces of dorsal cells, which will form the dermomyotome (DMT; asterisks in **B, C, E**). Co-localization of the nuclear stain and anti-β-catenin is observed in the dorsal cells (B, C, E). In contrast, sclerotomal cells (SC; **D**) do not show pronounced β-catenin staining of any kind. In later somites that have formed a DMT and a myotome (MYO; **G–I**), nuclear β-catenin is detected in cells of the dorsomedial lip (DML) and to a lesser extent in the cells of the DMT. β-catenin staining is also pronounced in the membranes of the myotomal cells but is not detected in myotomal cell nuclei (**J**; arrowhead). This indicates that the nuclear β-catenin we observe is not a consequence of high levels of membrane bound β-catenin in the somitic cells. Sections of comparable somites (early somite, **F**; later somite, **K**) stained with nuclear stain and secondary antibody alone never demonstrate yellow nuclei.

Fig. 2. Detection of canonical Wnt pathway responsive cells in early somites using a TOPGFP reporter. DNA encoding TOPGFP (**A–F**) or GFP under the direction of the chicken β-actin promoter (pCAX/GFP) was electroporated into early somites (stage IV–VI) and somites examined later for GFP positive cells (**G, H**). Sections were also labeled with anti-Pax7 (red), which labels dorsal somitic cells and cells of the dorsal neural tube (NT). The position of the positive electrode for each electroporation is summarized in the diagram in A. When TOPGFP is directed to the dorsal region of the somite (B–D), GFP is detected at a later time in the cells of the dermomyotome (DMT). When TOPGFP is directed ventrally (E, F), GFP expression is not detected in ventral cells that form the presumptive sclerotome (SC). Non-Pax7-expressing cells rarely express TOPGFP. To demonstrate that lack of sclerotomal expression of TOPGFP is not due to an inability to electroporate the ventral somitic cells, epithelial somites were electroporated and the control construct, pCAX/GFP, directed to ventral cells. In these experiments, sclerotomal cells readily express GFP under the direction of the β-actin promoter (G, H). As a negative control, co-electroporations were performed into the dorsal somite using the constitutively active construct pDsRED and FOPGFP, which contains mutated TCF binding sites (**Ia–Ic**). Somitic cells fail to express GFP (Ic) at appreciable levels when electroporated with FOPGFP despite the expression of DsRED throughout the DMT (**Ia**, **Ib**). All sections are immunostained with anti-Pax7 antibody (blue).

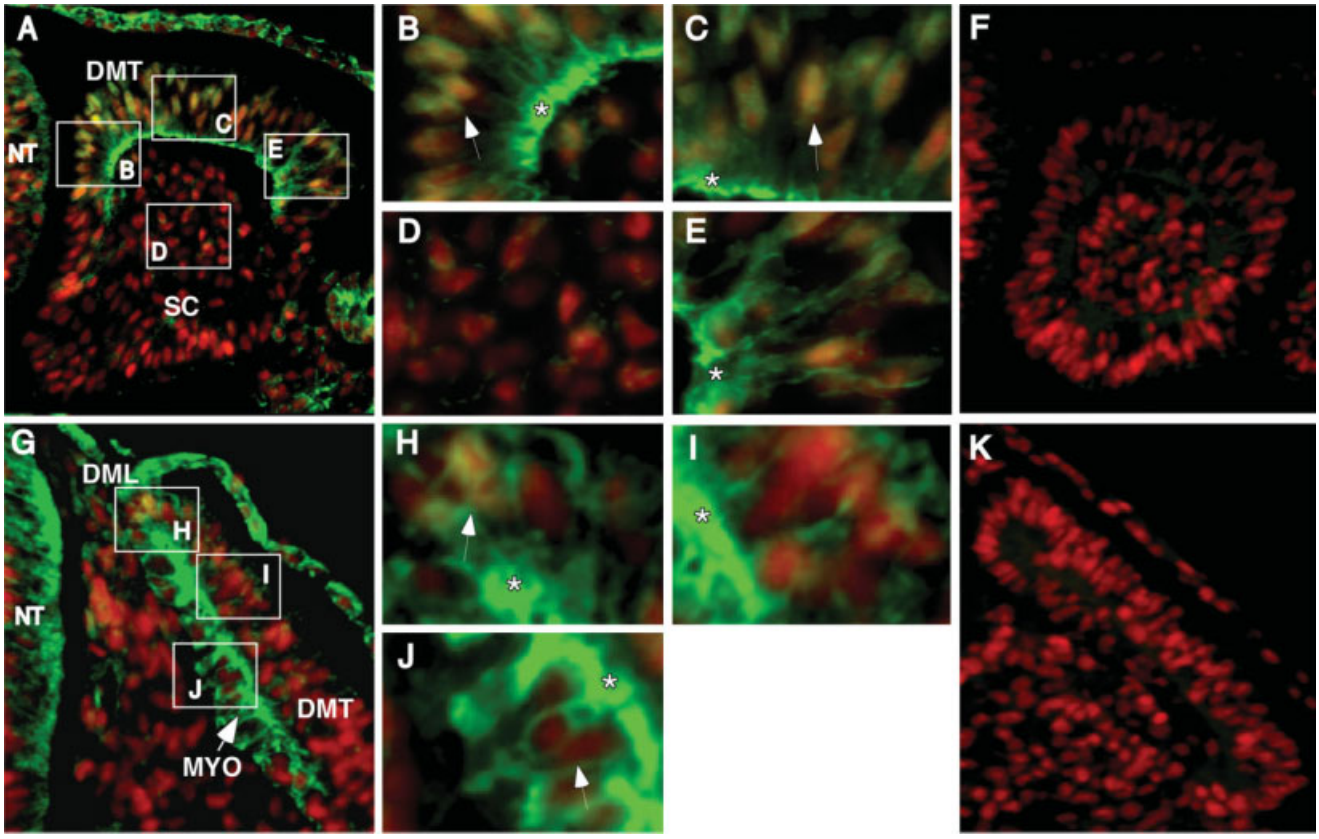
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Fig. 1.

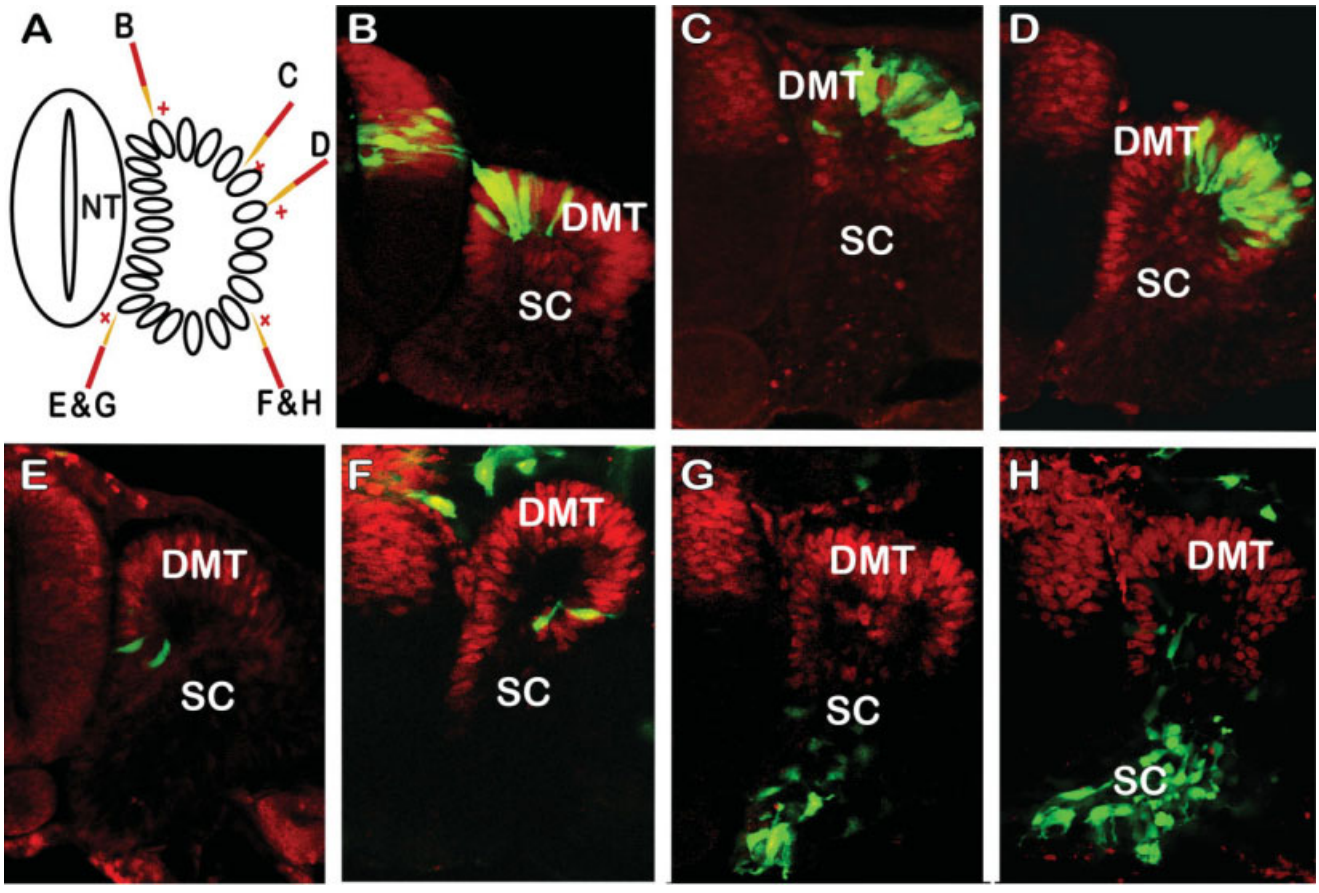
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Fig. 2.

others (Scaal et al., 2004), plasmid DNA leaks out of the somitocoel immediately after injection. If leaked DNA floats to the dorsal side of the somite, transfection of dorsal cells can occur during a ventral electroporation as the electric field includes all cells between the anode and cathode (Krull, 2004). Therefore, electroporation of plasmids into the ventral somite sometimes resulted in GFP expression not only in the cells targeted by electroporation but also some dorsal cells that were within the electric field between the anode and cathode.

To demonstrate that the lack of TOPGFP expression in ventral somitic cells was not due to an inability to electroporate these cells, we electroporated the control plasmid pCAX/GFP, which constitutively expresses GFP, under the same conditions as TOPGFP. In these electroporations, GFP expression was observed in the ventral cells (Fig. 2G, H). These data combined with the nuclear β-catenin staining indicate that all the cells in the dorsal half of the early somites are responding to β-catenin-dependent Wnt signaling. At this early stage, Wnt-responsive cells in the somite correspond to Pax7-expressing cells.

To further demonstrate that dorsal, Pax7-positive cells, but not ventral Pax7-negative cells, of the early somite respond to signaling through the canonical Wnt pathway, we co-electroporated TOPGFP and pDsRED, or pCAX/GFP and pDsRED, into cells in the ventral half of the somite. When TOPGFP was co-electroporated with pDsRED, GFP expression was only detected in a few dorsal dermomyotomal cells that expressed Pax7, while DsRED-expressing cells were detected throughout the ventral sclerotome (Fig. 3B,a–c). However, when both control plasmids (pDsRED and pCAX/GFP) were co-electroporated into ventral cells, both DsRED and GFP were observed (Fig. 3C, a–c).

As a negative control for TOPGFP electroporations, FOPGFP and pDsRED were co-electroporated into the dorsal somite. FOPGFP contains mutated TCF binding sites and is expressed at significantly reduced levels compared to TOPGFP. FOPGFP and pDsRED co-electroporations resulted in DsRED-expressing cells throughout the dermomyotome but no significant GFP was detected (Fig. 3D, a–c).

Wnt Responsiveness Is Restricted to Dermomyotmal Cells in Older Somites

We further investigated the differences in the Wnt responsiveness of cells in the dermomyotome and myotome that we observed in later somites based on β-catenin nuclear localization. TOPGFP and pDsRED or pCAX/GFP and pDsRED were co-electoporated into the dorsomedial quadrant of somites and the embryos were examined 24 hr post-electroporation for GFP expression. To identify the different compartments of the somite, sections of the electroporated embryos were immunostained with either Pax7 or MF20 antibodies to identify the dermomyotome and myotome, respectively.

Co-electroporation of TOPGFP and pDsRED resulted in strong co-expression of both GFP and DsRED in the DML of the dermomyotome (Fig. 4B, a–c and 4C, a–c). However, GFP expression was reduced in dermomyotomal cells outside the DML while DsRED expression was qualitatively equal in all dermomyotomal cells (Fig. 4B, a–c and 4C, a–c). When pCAX/GFP and pDsRED were co-electroporated (Fig. 4), we rarely observed cells that only expressed one of the plasmids without the other; therefore, while it cannot be completely eliminated as a possibility, it is unlikely that the difference in expression is the result of the plasmids segregating during co-electroporation. This argues that cells that express only DsRED after co-electroporation of pDsRED and TOPGFP are not Wnt responsive. This is supported by the co-electroporation of the control plasmids pCAX/GFP and pDsRED that resulted in equivalent intensities of both GFP and DsRED in the DML and dermomyotome (Fig. 4Ea–f).

Our earlier data indicated that myotomal cells do not contain nuclear β-catenin and suggested that cells in the myotome do not respond to signaling through the canonical Wnt pathway. To further examine this unexpected result, pDsRED and TOPGFP DNA was co-electroporated into the dorsal half of the somite and embryos were examined 24 hr later. Embryo sections were immunolabeled with MF20, an anti-myosin antibody, to differentiate myotomal cells from the overlying dermomyotome. We detected DsRED-expressing, but not GFP-expressing, cells in the myotome (Fig. 4C, a–c, arrows). In contrast, an approximately equivalent number of GFP-positive cells was observed in the myotome after electroporation of pCAX or DNTCF-pMES (see Fig. 8E).

When the negative control plasmid FOPGFP was tested in co-electroporations of FOPGFP and pDsRED at this later stage, DsRED was strongly expressed, whereas only low levels of GFP were detected in the somite (Fig. 4F, a–c). The lack of significant GFP expression in the dorsal somite after electroporation of FOPGFP indicates that the expression of TOPGFP in somitic cells is dependent on a functional TCF binding site as well as nuclear β-catenin. In addition, in these experiments examining older somites as in earlier experiments with younger somites, only cells that expressed Pax7 appeared to be responding to signals through the canonical Wnt pathway.

Wnt Responsiveness Is Highest in the DML

To ascertain if there were, indeed, regional differences in the number of cells that demonstrate Wnt responsiveness in the somite, the number of cells expressing TOPGFP was compared to the number of DsRED-expressing cells after co-electroporation. The dorsal half of somites II–IV at HH stages 13–14 was electroporated with TOPGFP and pDsRED and embryos examined 24 hr later. To count the percentage of GFP-expressing electroporated cells in different regions of the somite, embryos were embedded and vibratome sectioned and the dermomyotome was sub-divided into regions 1–3 and the myotome was designated region 4 (Fig. 5). Cells that expressed GFP and/or DsRED were counted in each region. The percentage of Wnt-responsive

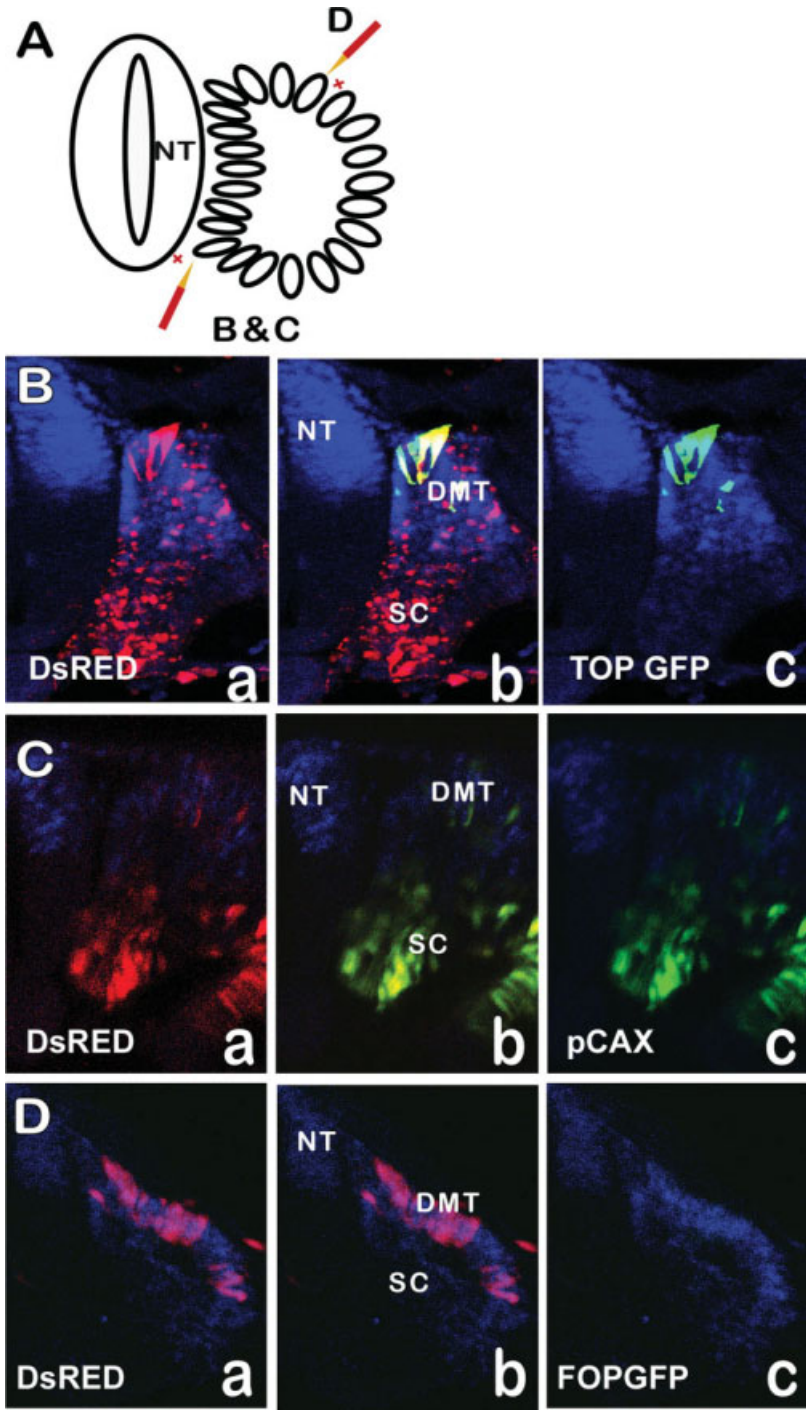

Fig. 3. Sclerotomal cells do not respond to signals through the canonical-Wnt pathway. To demonstrate that the inability of sclerotomal cells (SC) to express the reporter TOPGFP is not due to inefficient electroporation, early somites (stage IV–VI) were co-electroporated with TOPGFP and the control construct, pDsRED (which should be expressed in all cells), and examined later for GFP and DsRED expression. Electrodes were positioned as illustrated in the diagram in **A**. In co-electroporations of TOPGFP with pDsRED (**Ba–c**) into the ventral half of the somite, DsRED is expressed in both the ventral (SC) and dorsal somitic cells (DMT), but GFP is only expressed in dorsal cells (DMT). In contrast, when the GFP expression construct, pCAX/GFP, was co-electroporated with pDsRED into the ventral domain, both GFP and DsRED are expressed in ventral sclerotomal cells (**Ca–c**). As a negative control, co-electroporations were performed into the dorsal somite (shown as position D in the cartoon in A) using FOPGFP, which contains mutated TCF-binding sites, together with the constitutively expressed construct pDsRED (**Da–c**). Somitic cells fail to express GFP (Dc) at appreciable levels when electroporated with FOPGFP but express DsRED throughout the DMT (Da,b). All sections are immunostained with anti-Pax7 antibody (blue).

cells is the percentage of DsRed-positive cells that also expressed GFP. As previously indicated (Fig. 4B, a–c and 4C, a–c), nearly 100% of the DsRed-expressing cells in the dorsal DML (region 2) of the dermomyotome also expressed GFP, while approximately half the cells that expressed DsRED also expressed GFP in regions outside the dorsal DML (regions 1 and 3; Fig. 5). In contrast to the dermomyotome where GFP expression was observed in cells of all three regions, no GFP-positive cells were detected in the myotome (region 4; Fig. 5). That nearly 100% of the cells that express DsRED also expressed GFP in the DML of the dermomyotome (region 2; Fig. 5) suggests that this region represents a population of highly responsive cells to β-catenin-mediated Wnt signaling. The cells in the DML represent a pool of progenitor cells for both the myotome and dermomyotome

Fig. 4. Wnt-responsive cells are concentrated in the DML as the primary myotome forms. To identify Wnt-responsive cells in older somites (stages X and older), TOPGFP and control constructs were co-electroporated into epithelial somites (the placement of electrodes for each set of electroporations is summarized in the cartoon in **A**), and examined after somites had compartmentalized into a dermomyotome (DMT) and myotome (MYO). Sections were immunolabeled for either Pax7 (blue) to identify the DMT (**B, D, F**) or MF20 (blue) to identify cells in the MYO (**C, E**). When pDsRED and TOPGFP were targeted to the dorsomedial aspect of the early somite, both DsRED and GFP are detected later in the DMT and dorsomedial lip (DML; B), but cells expressing GFP were not detected in the MYO. The arrow in Bb points to a presumptive myotomal cell outside the Pax7-positive region that expresses only DsRED. The arrow in Cb points to an MF20-positive myotomal cell that co-expresses DsRED but not GFP. When somites are co-electroporated with the control constructs, pCAX/GFP and pDsRED, cells throughout the later somite co-express both GFP and DsRED (Da–c, arrows, Ea–f). When co-electroporated ventrally (Da–c), sclerotomal cells (SC) as well as cells in the DMT and MYO (arrow in Db) co-express DsRED and GFP. When pCAX/GFP and pDsRED are co-electroporated dorsally, cells in both the DMT and MYO co-express GFP and DsRED (arrows in Ee; Ed–f are enlargements of the boxed areas in Ea–c, respectively). When FOPGFP and pDsRED were co-electroporated as a negative control (electrode position F in the cartoon in A), DsRED expression is strong throughout the DMT, but no appreciable GFP expression is detected.

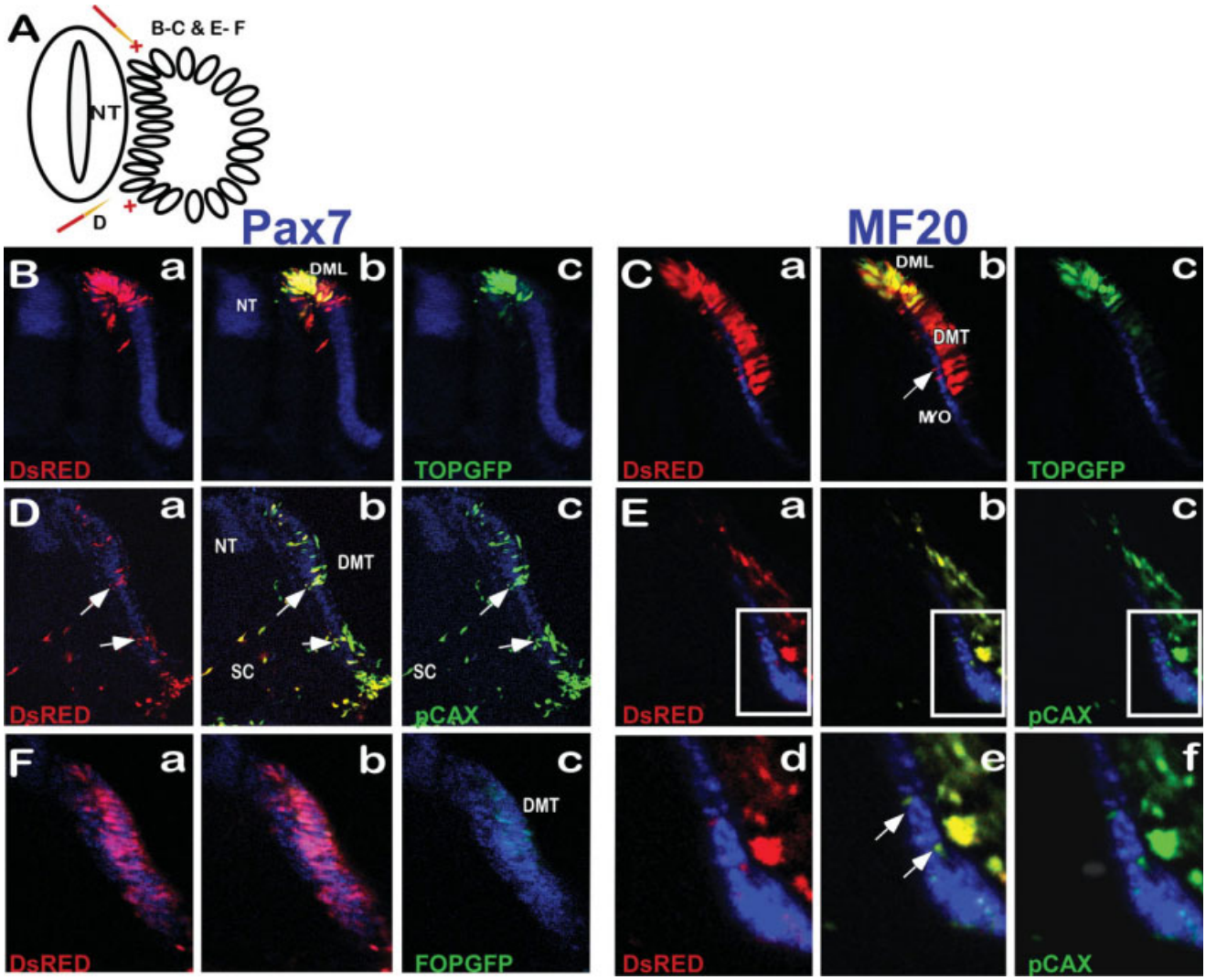

Fig. 4.

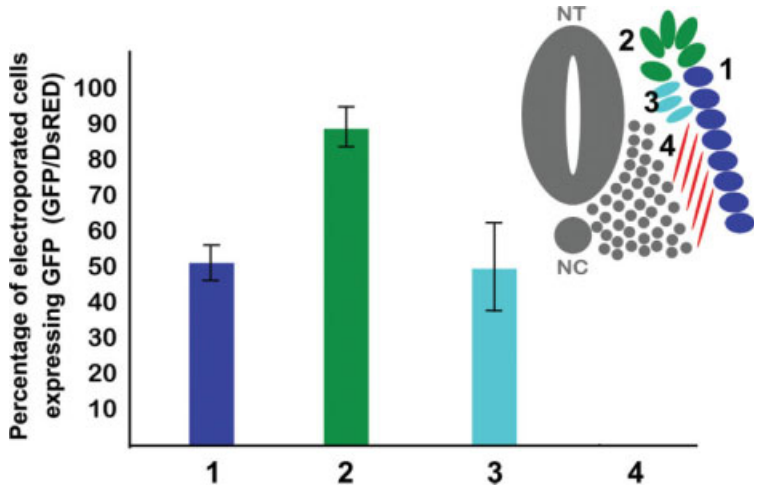

Fig. 5.

Fig. 5. β-catenin-dependent Wnt-responsive cells are enriched in the DML and are not detected in the myotome. After co-electroporation of TOPGFP and pDsRED, the number of GFP- and DsRED-positive cells were counted in transverse sections of somites. Different regions of the somite were designated as shown in the cartoon. DsRED-positive cells represent all cells electroporated and GFP-positive cells represent the subset of electroporated cells in which the β-catenin/Wnt-dependent reporter TOPGFP was activated. Nearly all cells in the DML (region 2) express both DsRED and GFP. In other regions of the dermomyotome (regions 1 and 3), approximately 50% of cells that express DsRED also express GFP. In the myotome (region 4), however, no GFP expression was observed. The number of cells counted in each region was 265 (region 1), 120 (region 2), 63 (region 3), and 66 (region 4), respectively.

(Venters and Ordahl, 2002; Venters and Ordahl, 2005). The reduced number of cells expressing GFP in regions 1 and 3 of the dermomyotome suggests that the progeny of the DML cells have a reduced response to β-catenin/Wnt signaling. Of particular note is that once cells are in the myotome and begin to differentiate, they appear to be non-responsive to β-catenin-mediated Wnt signaling.

Ectopic Activation and Repression of β-catenin/Wnt Signaling

Because our data suggest that cells in the myotome do not respond to signaling through the canonical Wnt pathway, we reasoned that activation of β-catenin/Wnt signaling in dermomyotomal cells might prevent cells from entering the myotome. Our β-catenin immunostaining and TOPGFP data suggest that there is a reduced response in cells derived from the DML. We, therefore, examined the effect of constitutively activating the downstream targets of Wnt signaling in DML cells and determine how this affected their progeny. To determine the effect of constitutively activating or repressing β-catenin/Wnt target genes, we electroporated constructs into the dorsal medial quadrant that expressed GFP constitutively with either a dominant-negative or a dominant-positive form of TCF1 (DNTCF-pMES and DPTCF-pMES, respectively), or with the control pCAX/GFP construct.

Before electroporating DNTCF-pMES and DPTCF-pMES into somitic cells, we tested their efficacy in cell culture and in somite electroporations. We transfected either DNTCF-pMES or a construct encoding Wnt1, pWnt1, together with the LEF1/TCF1-responsive luciferase reporter, TOPflash, into HEK 293 cells. Transfection of HEK 293 cells with either pWnt1 or DPTCF-pMES resulted in comparably high levels of luciferase activity (Fig. 6). Although pWnt1 and DPTCF-pMES each activated TOPflash significantly, neither activated the control reporter, FOPFlash (Fig. 6). Co-transfection of DNTCF-pMES together with pWnt1, however, reduced TOPflash activity to FOPFlash levels (Fig. 6). These results indicate that DNTCF-pMES can inhibit the expression of Wnt target genes that are activated through the canonical β-catenin-dependent Wnt signaling. In the absence of any Wnt perturbation, TOPflash activity was negligible (Fig. 6). These results indicate that the modified forms of TCF we have engineered should activate or inhibit the expression of Wnt target genes when ectopically expressed in vivo.

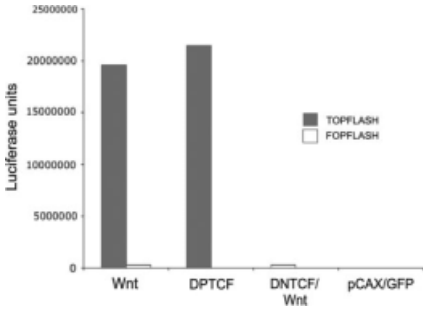

Fig. 6. Modified forms of TCF effectively alter Wnt activity in transfected HEK293 cells. When either Wnt1 or DPTCF and the Wnt-responsive reporter TOPflash are transfected into cells in vitro, both DNAs activated TOPflash to comparable levels. In contrast, if Wnt1 or DPTCF are transfected together with the modified reporter FOPflash, very low levels of luciferase activity were detected. When Wnt1 is co-transfected with DNTCF, luciferase levels were equivalent to those obtained by Wnt1 activation of FOPFlash. Similarly, when pCAX/GFP is transfected with TOPflash, basal levels of luciferase activity were detected.

Previous studies demonstrated that ectopic expression of RCAS-Wnt1- and RCAS-activated β-catenin both resulted in expansion of the Wnt11 expression domain in the chick dermomyotome (Capdevila et al., 1998), supporting the role of the canonical Wnt pathway in the activation of Wnt 11 expression in the DML. In agreement with previous studies (Marcelle et al., 1997; Capdevila et al., 1998), we show that Wnt 11 transcript expression is restricted to the DML of the dermomyotome (Fig. 7A, B). We, therefore, tested whether electroporation of DPTCF-pMES or DNTCF-pMES into the somite influenced the expression of Wnt 11. We examined embryos for Wnt 11 expression by in situ hybridization 24 hr after electroporation of DPTCF-pMES, DNTCF-pMES, or pCAX/GFP into the early somite. When DNTCF-pMES DNA was electroporated into the dorsal somite, significantly reduced expression of Wnt 11 was observed in dorsal cells on the electroporated side (Fig. 7D) compared to non-electroporated somites (Fig. 7A, B) or embryos electroporated with pCAX/GFP (Fig. 7C). When DPTCF-pMES DNA was electroporated into the ventral somite, Wnt 11 expression was detected in ventral regions of the somite (Fig. 7E; note this is a lateral view). Although Capdevila et al. (1998) demonstrated that ectopic expression of RCAS activated β-catenin and RCAS Wnt-1 also resulted in expansion of Wnt 11 expression in the dermomyotome, it is unclear whether different dermomytomal regions respond differently to activation of the β-catenin-dependent Wnt signaling pathway.

The above data argue that electroporation of DNTCF-pMES and DPTCF-pMES into somites should constitutively repress or activate targets of the canonical Wnt pathway, respectively, and can be used to modify the response of somitic cells to external signals. To further examine how β-catenin-mediated Wnt signaling influences myotome formation, somites VII–X were examined following electroporation of DNTCF-pMES or DPTCF-pMES DNA at HH stages 13–14. In somite electroporation experiments, the DML cells were specifically targeted by electroporation and, in contrast to the TOPGFP electroporations, GFP expression after electroporation of DPTCF-pMES or DNTCF-pMES is indicative not only of the cells that were electroporated, but also the expression of the dominant-negative or dominant-positive forms of the TCF protein in the electroporated cells. The pMES plasmid contains the β-actin promoter and an IRES to allow co-expression of GFP and modified TCF proteins constitutively and simultaneously in electroporated cells (Swartz et al., 2001).

After electroporation of DNTCF-pMES or pCAX/GFP, GFP-positive cells were detected in the myotome as demonstrated by the co-expression of GFP and MF20 (Fig. 8B and 8C). In contrast, when DPTCF-pMES was electroporated, many GFP-positive cells were detected in the dermomyotome adjacent to the myotome, but not in the myotome (Fig. 8D). The number of GFP-positive cells in the dermomyotome and the myotome were determined after electroporation

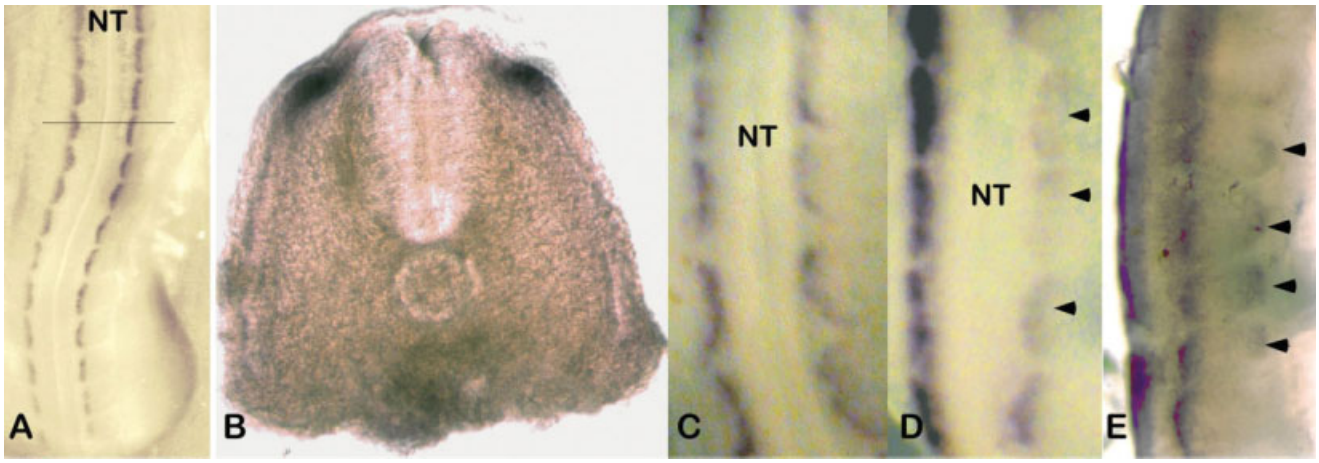

Fig. 7. Wnt11 expression in somites is altered after electroporation of DNTCF-pMES or DPTCF-pMES. In a non-electroporated embryo, Wnt11 is expressed in the DML in somites on both sides of the neural tube (**A**). A transverse section of the non-electroporated embryo shown in A (at the level of the dotted line) shows Wnt11 expression was highest in the DML of the somite (**B**). After electroporation of pCAX/GFP (**C**), Wnt11 expression was similar to that observed in a non-electroporated embryo. Wnt11 expression was greatly diminished in those somites where DNTCF-pMES was electroporated (**D**, arrowheads), while Wnt11 expression was observed in the DMLs of all the somites on the contralateral side of the embryo. Following electroporation of DPTCF-pMES, Wnt11 is expressed ectopically in the ventral somite (**E**, arrowheads) as shown in a lateral view of the DPTCF electroporated embryo.

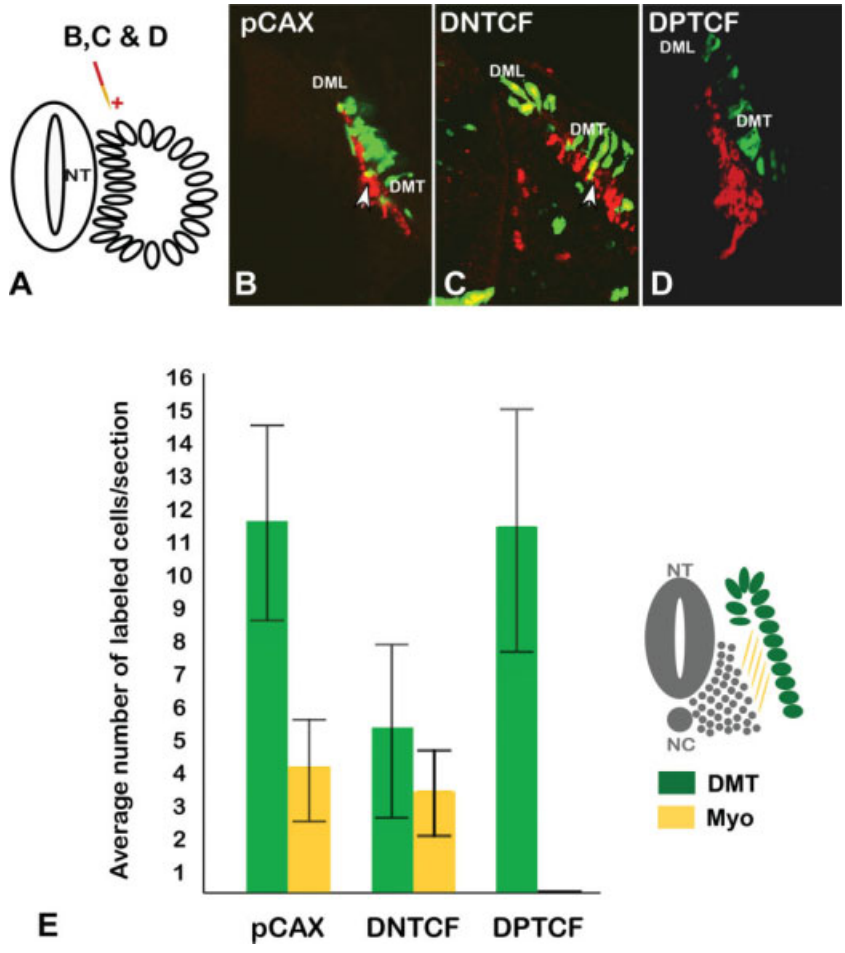

Fig. 8.

of each plasmid. Electroporation of DPTCF-pMES resulted in 100% of the electroporated (GFP-positive) cells in the dermomyotome and no GFP-positive cells in the myotome (Fig. 8E). In contrast, after electroporation of pCAX/GFP or DNTCF-pMES, an approximately equivalent number of GFP-positive cells was observed in the myotome (Fig. 8E).

DISCUSSION

It previously was proposed that signaling through the canonical Wnt

Fig. 8. Effects of constitutively activating or repressing Wnt activity on DML progeny. To investigate the consequences of activating or repressing signaling through the canonical Wnt pathway in DML cells, DNTCF-pMES and DPTCF-pMES were targeted to the DML of stage-VII–X somites and the number of GFP-positive cells in the myotome were counted after 24 hr. Electrodes were positioned as illustrated in the cartoon in **A** and sections stained with MF20 to identify cells in the myotome (MYO). Electroporation of both pCAX/GFP (**B**) and DNTCF-pMES (**C**) resulted in GFP-positive cells in both the DMT and MYO (arrows in B, C). After electroporation of DPTCF-pMES, GFP-positive cells were detected in the DMT, but not in the MYO (**D**). The mean number of GFP-expressing cells in the DMT (green) versus the MYO (yellow) for each construct electroporated is shown in **E**.

Developmental Dynamics

pathway plays a proliferative role in the somite (Galli et al., 2004), but it was not known if all or a subpopulation of somitic cells were Wnt responsive or when during somitogenesis or myogenesis they respond. We show that β-catenin/Wnt-responsive cells can be detected throughout the early dermomyotome and while cells in the dermomyotome of older somites maintain a degree of responsiveness, the greatest response is observed in the cells of the DML. Wnt-responsive cells in both the early and compartmentalized somite also expressed Pax7 (Figs. 2–4). In later somites, the β-catenin/Wnt-responsive cells are enriched in the DML of the dermomyotome, and these cells have been shown to co-express Pax7 and Pax3 (Galli et al., 2008). While some studies have reported that ectopic expression of Pax7 promotes withdrawal from the cell cycle (Olguin and Olwin, 2004), others have found that ectopic expression of Pax7 maintains proliferation and prevents the precocious differentiation of myoblasts (Zammit et al., 2006). Other recent studies show that Pax3 and Pax7 expression in the somite is inversely correlated with that of MyoD, and that Pax7 expression positively correlates with proliferation and negatively correlates with myogenic differentiation (Galli et al., 2008). These data combined with our results argue that β-catenin-mediated Wnt signaling acts to maintain cells in the DML of the dermomyotome in a proliferative and undifferentiated state. However, it remains to be determined whether β-catenin/Wnt signaling is directly responsible for the maintenance of Pax3 or Pax7 expression in cells of the DML.

Using multiple strategies, we found that myotomal cells no longer respond to signaling via the canonical Wnt pathway. When cells are in the myotome, they no longer contain nuclear β-catenin or transcriptionally activate the β-catenin-dependent responsive reporter TOPGFP despite close proximity to the dorsal neural tube, a major source of Wnt signals. Because it is thought that β-catenin-mediated Wnt signaling is needed for the formation of the myotome and the activation of myogenic regulatory factors (Munsterberg and Lassar, 1995; Dietrich et al., 1997; Schmidt et al., 2000), lack of β-catenin staining in the nuclei of myotomal cells and the absence of TOPGFP expression were unexpected.

Lack of β-catenin-dependent Wnt responsiveness in myotomal cells does not exclude the possibility that the cells responded to a Wnt signal in their recent history. However, it does suggest that cells might be required to cease responding to β-catenin-dependent Wnt signaling in the myotome. To address this possibility, we electroporated modified forms of the Wnt effector, TCF, designed to either block or activate downstream events in the β-catenin-dependent Wnt signaling pathway. In these experiments, the cells expressing the modified TCFs could be identified by the co-expression of GFP from the same plasmid. This allowed determination of the effect of constitutively activating or repressing β-catenin-dependent Wnt signaling in a cell-autonomous manner.

We found that no GFP-expressing cells were identified in the myotome if the canonical Wnt pathway was constitutively activated in DML cells by electroporation of a DNTCF-pMES. However, comparable numbers of GFP-expressing cells were found in the myotome when DML cells were electroporated with DNTCF-pMES or the control plasmid, pCAX/GFP. We observed a decrease in the overall number of cells expressing GFP in both the dermomyotome and the myotome following electroporation of DNTCF-pMES (Fig. 8). This argues that proliferation in the dorsal somite is adversely affected by repressing the ability of cells to respond to β-catenin-mediated Wnt signaling. This is consistent with the results obtained following electroporation of Wnt3a (Galli et al., 2004), which led to an increase in the number of cells in both the myotome and dermomyotome.

The shift between myoblast proliferation and differentiation in vitro has been shown to require stimulation of N-cadherin-mediated adhesion (George-Weinstein et al., 1997; Cinnamon et al., 2006) and that the interaction of β-catenin with cadherins antagonizes β-catenin and Lef/TCF interactions (Goichberg and Geiger, 1998; Goichberg et al., 2001). Similarly, nuclear β-catenin is observed in proliferative myoblasts, but β-catenin translocates to cell membranes upon myogenic differentiation (Goichberg et al., 2001). Conversely, elevation of β-catenin levels in myogenic cell lines by LiCl treatment or β-catenin overexpression reduced intercellular adhesion and inhibited myogenesis (Goichberg et al., 2001). These studies support our data suggesting that the transcriptional role of β-catenin must be suppressed prior to myoblast differentiation. We and others (Linask et al., 1998) show that a high level of membrane-associated β-catenin is found in the myotomal cells in vivo and despite high levels of β-catenin protein expression (Fig. 1), no nuclear β-catenin is detected.

Recent results suggest involvement of signaling through the non-canonical Wnt pathway in myotomal cell specification (Chen et al., 2005; Brunelli et al., 2007). We (Fig. 7) and others (Marcelle et al., 1997; Capdevila et al., 1998) have shown that Wnt 11 is a target of signaling through the canonical Wnt pathway in the DML. Our data are consistent with a role for the canonical Wnt pathway in the maintenance of the DML myogenic precursor pool and a role for signaling through the non-canonical Wnt signaling pathway in their differentiation possibly mediated by Wnt 11. The non-canonical Wnt pathway has been shown to inhibit signaling through the canonical Wnt pathway (Weidinger and Moon, 2003; Veeman et al., 2003) and could account for the loss of β-catenin/Wnt responsiveness in DML cell progeny. Electroporation of a dominant-negative form of Wnt5b, another Wnt that acts through the non-canonical pathway, resulted in the loss of MyoD expression in the medial somite (Linker et al., 2003), and Wnt5b, working through the non-canonical Wnt pathway, can lower the cellular response to canonical Wnt signaling in other cell types (Kanazawa et al., 2005). Thus, the reduced response of cells in the medial somite to canonical Wnt signaling may be through activation of Wnt5b. Although dorsal signaling through the canonical Wnt pathway may not be needed for the initial expression of MyoD in the medial somite, it is needed for the continued expression of MyoD. If all dorsal Wnt-expressing tissue is removed, MyoD

expression is lost within 24 hr (Pownall et al., 1996; Dietrich et al., 1997). One possible explanation is that Wnts working through the canonical pathway are needed to induce or maintain the expression of Wnts working through the non-canonical Wnt pathway. Further analysis of Wnt 5b and Wnt 11 function specifically in the DML cells is needed to reconcile the roles for canonical versus non-canonical Wnt pathways in vivo.

Our data support the proposed model that the DML is a stem cell center (Venters and Ordahl, 2002; Venters and Ordahl, 2005) and suggest that signaling through the canonical pathway acts to maintain cells in the DML as progenitor cells. It is also consistent with the role of signaling through the canonical Wnt pathway in the adult muscle stem cell, the satellite cell. Satellite cells express β-catenin as the cells activate and undergo proliferation and constitutive expression of wild type or stabilized β-catenin promotes a self-renewal pathway, while down-regulation of β-catenin promotes myogenic differentiation (Perez-Ruiz et al., 2008). Thus, β-catenin-dependent signaling in proliferating satellite cells directs these cells towards the self-renewal pathway and maintains this stem-cell pool in adult skeletal muscle. The canonical Wnt pathway may, therefore, function to control the self-renewal of stem cells in the DML whose progeny contribute to both the myotome and dermomyotome.

EXPERIMENTAL PROCEDURES

Chicken Eggs and Embryo Preparation

Fertilized White Leghorn chicken eggs were obtained from Ideal Poultry (Cameron, TX) or Hy-Line North America, LLC (Elizabethtown, PA) and incubated at 38°C in a humidified incubator until needed (G.Q.F, Savanna, GA). For electroporation, embryos were incubated until they reached HH stages 13–14 (Hamburger and Hamilton, 1992). After electroporation, embryos were re-incubated for specific times as described below, then fixed and processed. Embryos were dissected from the eggs and placed in Ringer's solution. Before fixation they were washed 3 times for 10 min each in Ringers at room temperature (RT) on a rotating shaker.

Immunohistochemical Localization of β-Catenin

For β-catenin immunolocalization, embryos were fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) overnight (O/N) at 4°C on a rotating shaker. After fixation, embryos were washed three times in PBS for 5 min. Fixed embryos were dehydrated through a graded alcohol series followed by two changes of xylene for 30 min each at RT. Embryos were incubated in fresh paraffin at 56°C for 2 times 1 hr each. Embryos were embedded in paraffin and oriented to yield transverse sections. A standard microtome was used to cut 5-μm transverse sections. Sections were mounted on slides and deparaffinized using 2 changes of xylene for 5 min each, rehydrated through a graded alcohol series and washed 2 times for 5 min in PBS. Sections were placed in a chamber containing 10 mM sodium citrate buffer (pH 6), 0.1% Tween 20 (Sigma, St. Louis, MO), and treated 30 min in a 2100 Retriever (Pick Cell Laboratories, Amsterdam, The Netherlands) and allowed to incubate for an additional 2 hr for antigen retrieval. All subsequent steps were carried out in a humidified slide chamber. Propidium iodide (PI) was used to stain nuclei, but first sections were incubated in a solution of 10 μg/μl RNaseA (Sigma, St. Louis, MO) in PBS for 30 min at 37°C to eliminate cytoplasmic RNA. After RNase treatment, sections were washed 3 times for 5 min in PBS, and blocked in 4% normal goat serum (NGS) in PBS with 0.1% Tween 20 (PBST) for 30 min. Sections were incubated in monoclonal anti-β-catenin antibody (Transduction Labs, San Jose, CA; 250 μg/ml) at 1:100 dilution in PBST for 1 hr at 37°C. Sections were washed 3 times for 5 min in PBS and then incubated for 1 hr at RT in secondary antibody and 1 mg/ml PI was diluted 1:1,000 in PBST. Sections were incubated in goat anti-mouse Alexa Fluor 488 conjugated secondary antibody (2 mg/ml) diluted 1/500 in PBST for 1 hr at RT and the sections were then washed an additional 3 times in PBS and mounted with Pro-long Antifade Gold (Molecular Probes, Eugene, OR). Images were obtained using a Zeiss MrC5 Axiocam digital camera attached to a Zeiss Axioskop2 fluorescence microscope (Carl Zeiss, Inc., Thuringia, Germany).

Electroporation

In ovo electroporation was used for focused introduction of various DNAs into developing chicken somites at HH stages 13–14 (Scaal et al., 2004). To enhance contrast and visualize the embryo, a solution of 3% India ink (Pelikan, Germany) diluted in Ringer's solution was injected below the blastoderm and the vitelline membrane overlying the embryo was removed. Plasmids were ethanol precipitated and resuspended to 5–7 μg/μl in sterile PBS. For two plasmid electroporations, single plasmids were mixed so that the final concentration of each plasmid was 5–7 μg/μl in sterile PBS. DNA was prepared for injection by mixing 4.75 μl of single or double plasmid DNA with 0.25 μl of 20% Fast Green (Sigma, St. Louis, MO) in sterile PBS. In all cases, DNA was microinjected into the somitocoel using an MPPI-2 Pressure Injector (Applied Scientific Instrumentation, Inc., Eugene, OR) so that all injections introduced comparable amounts of DNA. Each somite received approximately 0.15 μl of DNA for a total quantity of 0.9 μg of DNA/somite for single plasmid injections and 1.8 μg of DNA/somite for double plasmid injections. Following electroporation, approximately 0.5 ml of Ringer's solution was placed on top of the embryo. Electrode position was used to target individual quadrants of the epithelial somite during electroporation, as defined by the cells' orientation relative to the neural tube (Scaal et al., 2004). The anode and the cathode were oriented so that the targeted quadrant was adjacent to the anode (Scaal et al., 2004). In all cases, the

anode was placed approximately 180° opposite to the cathode. Routinely, 5 pulses of 50-ms duration and 9–15 V were delivered using two 1-mm platinum wires connected to a square pulse electroporator (BTX, San Diego, CA). Electroporations were performed with the control plasmid pCAX/GFP, which constitutively expresses GFP from the chicken β-actin promoter/CMV-IE enhancer (Swartz et al., 2001; Osumi and Inoue, 2001) to determine the best placement of electrodes for expression in individual quadrants and the amount of injected DNA that could be tolerated without causing morphological damage. All electroporations were performed on one side and transverse sections of fixed and sectioned embryos were stained with Pax7, so that the electroporated side could be compared to the non-electroporated side and only embryos demonstrating comparable morphology and levels of protein expression on each side were used for further analyses. Successful electroporations targeted a subset of cells adjacent to the positive electrode and were identified by the expression of the respective fluorescent proteins.

To examine endogenous Wnt activity in the somite, one or a combination of the following plasmids was electroporated: TOPGFP, FOPGFP, pCAX/GFP, or pDsRED. DNA was electroporated into the dorsal medial, dorsal lateral, ventral medial, and ventral lateral quadrants of epithelial somites. To obtain somites at two different stages of myotome formation, somites were examined at either 6 or 24 hr post-electroporation. Somites II–IV of HH stages 13–14 were injected with single or multiple plasmids, followed by targeted electroporation.

Plasmids Electroporated

TOPGFP, consisting of 4 consensus TCF/LEF binding sites and a minimal 94-bp promoter derived from the mouse cFos gene driving destabilized GFP (Dorsky et al., 2002), was electroporated as described above to identify Wnt-responsive cells in different regions of developing somites. FOPGFP consisting of 6 mutated TCF/Lef binding sites upstream of GFP (from A. Munsterberg, University of East Anglia, Norwich, UK) was used as a negative control. FOPGFP was generated by cloning an XbaI fragment containing the mutated TCF/Lef binding sites from FOPFlash and inserting this into NseI and XhoI digested pEGFP.N3 (Clontech, Mountainview, CA). Either pDsRED (Clontech, Mountainview, CA) expressing DsRED under the control of a CMV viral promoter or pCAX/GFP, which expresses GFP under direction of a chicken β-actin promoter/CMV-IE enhancer (Swartz et al., 2001), was used as an indicator of all electroporated cells since they should be expressed constitutively in all cell types.

Dominant-Positive (DPTCF-pMES) and Dominant-Negative (DNTCF-pMES) TCFs were constructed using modified forms of a mouse/human TCF-1 cDNA (from H. Clevers, University of Utrecht, The Netherlands), mhTCF-1 in pcDNA1 (van de Wetering et al., 1996). For DNTCF, the sequence encoding the β-catenin-binding domain of mhTCF-1 (amino acids 1–56) was removed to generate a TCF protein that cannot interact with β-catenin, ΔNTCF-1 in pcDNA1. ΔNTCF-1 cDNA was excised with HindIII and XbaI, filled in, and ligated into SmaI-digested pMES. For DPTCF, the β-catenin-binding domain of mhTCF-1 was replaced with the activation domain of β-catenin (amino acids 652–781) to generate a protein that is constitutively active (Aoki et al., 1999), DPTCF-1 in pcDNA1. Coding sequence of DPTCF-1 was excised with BamHI and XbaI, filled in, and ligated into the SmaI site of pMES. The pMES expression plasmid (Swartz et al., 2001) contains an IRES-EGFP sequence that allows reliable co-expression of EGFP and the protein of interest. All constructs were sequenced to assure that the DNA was inserted in frame. The expression of ectopic DNTCF and DPTCF-1 protein was verified by immunostaining sections of electroporated somites with antibody to human TCF-1 (Santa Cruz Biotechnology, Santa Cruz, CA). Expression of DNTCF-1 or DPTCF-1 proteins was observed only in the nuclei of somitic cells co-expressing GFP (data not shown) after electroporation of DNTCF-pMES or DPTCF-pMES.

In Vitro Transfections

The Wnt reporter construct TOPflash (Upstate, Charlottesville, VA), containing 6 TCF/Lef binding sites upstream of the Luciferase gene was used as a measure of the functional efficacy of the DNTCF-pMES and DPTCF-pMES DNAs. FOPflash (Upstate), which contains 6 mutated TCF binding sites upstream of luciferase, was used as a negative control. Wnt1 in pCDNA3 (Nam et al., 2006) and LacZ in pCDNA3 (from Drs. J.K. Yoon and J. M. Verdi, respectively, MMCRI, Scarborough, ME) were transfected together with either DNTCF-pMES orDPTCF-pMES DNAs into human embryonic kidney 293 (HEK293) cells. HEK293 cells were plated (at ~60,000 cells/well) into Costar 12-well plates (Corning, Acton, MA). Cells were incubated at 37°C in 5% CO_2 for an additional 24 hr and then transfected using GeneJuice Transfection Reagent (Novagen, San Diego, CA) at 1.5 μl of GeneJuice Transfection Reagent containing 0.5 μg of total DNA. For each transfection, 100 ng of reporter construct, 200 ng of the indicated expression plasmid, and 20 ng of pRL-TK normalization plasmid, containing a herpes simplex virus thymidine kinase promoter driving a Renilla luciferase gene, were used per single well of a 12-well plate. Empty pCS2-LacZ vector was used to adjust the total amount of DNA to 500 ng. Transfection experiments were performed in duplicate, and results were normalized to the expression of the Renilla luciferase transfection control (Promega, Madison, WI). The cells were lysed in Promega Passive Lysis Buffer, and 20 μl of each lysate was monitored for luciferase activity using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI). Light units were measured using a Zylux FB12 Luminometer (Oak Ridge, TN) set for 1-sec delay and 1-sec reading.

Visualization of Structures Following Electroporation

Electroporated embryos were fixed as described above for immunofluorescent labeling and embedded in 7.5% Gene Pure low-melting agarose (ISC Bioexpress, Kaysville, UT). Embryos

were oriented in the agarose to obtain transverse sections. Embryos were sectioned at 100-μm thickness using a Leica VT1000s Vibratome (Leica Microsystems, Germany). After vibratome sectioning, sections were permeablized with 0.5% Triton X 100 (Sigma) in PBS for 15 min. Sections were blocked for 30 min in PBS with 0.1% Tween-20 (PBST; Bio-Rad, Hercules, CA) and 4% normal goat serum (NGS; Invitrogen, Carlsbad, CA).

To determine which cells in somites express reporters after electroporation, embryo sections were stained with antibodies to the transcription factor, Pax7, to label dorsal somitic cells (Chi and Epstein, 2002) or to myosin heavy chain (MF20) to visualize myotomal cells. The Pax7 antibody was used at a 1:50 dilution and the MF20 antibody was used at 1:100. Both monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank (under the auspices of the NICHD, and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242). Alexa Fluor 405, 488, or 568 anti-mouse secondary antibodies (Molecular Probes; 2 mg/ml) were applied at 1:500 in PBST depending on which plasmids were electroporated.

Sections were incubated overnight in primary antibodies at 4°C on a shaker. Sections were incubated at RT in the appropriate secondary antibody for 1 hr on a shaker. Sections were washed again as described above with PBST and mounted in Prolong Gold mounting media (Molecular Probes).

Confocal Microscopy

Electroporated embryos were initially assessed on a Zeiss StemiSv6 fluorescent dissecting microscope to determine the success of electroporation. After fixation, sectioning, and/or immunolabeling where appropriate, the stained embryo sections were imaged using a Bio-Rad Radiance 2000 Confocal Microscope. Each channel was imaged sequentially to ensure that signal was not due to cross-excitation of wavelengths. Images were processed using Adobe Photoshop 7.0.

Quantification of Electroporated Cells

To count the number of cells responding to Wnt signaling during myotome formation in different parts of the somite, embryos (n=19) were electroporated with TOPGFP and pDsRED, incubated 24 hr, then fixed, sectioned, and imaged as previously described. Cells were counted in the following regions: (1) the *dermomyotome*, the lateral-most epithelial layer; (2) *dorsal DML*, those cells of the dermomyotome, as seen in cross-section, that primarily demonstrate a dorsal-to-ventral orientation; (3) *ventral DML*, the medial-most cells of the DML that primarily demonstrate a medial-to-lateral orientation; and (4) the *myotome*, those cells that lie beneath both the dermomyotome and the ventral DML (Fig. 5). A comparable method previously was used to classify cells of the dermomyotome (Venters and Ordahl, 2005). Cells were counted twice, once with only the green channel visible and once with only the red channel visible. Cell counts were tallied for each region in each of the two channels. Binomial confidence values were calculated using Microsoft Excel X.

In Situ Hybridization

In situ probes were generated from a 1-kb chicken Wnt11 clone encompassing the entire coding region (Marcelle et al., 1997). DNA was linearized with SalI (New England Biolabs, Ipswich, MA) and sense cRNA was in vitro transcribed using the Sp6 Megascript Kit (Ambion, Austin, TX) and digoxigenin-11-UTP (Roche, Germany) according to the manufacturer's protocol. Following electoroporation of either DPTCF-pMES or DNTCF-pMES, embryos for which GFP expression was detected in the appropriate region of the somite were chosen for further analysis by whole mount in situ hybridization. The embryos were processed for in situ hybridization as described by Nieto et al. (1996). The embryos were incubated in antisense probe (1 μg/ml in hybridization buffer) for 1 hr at 70°C. Alkaline phosphatase conjugated anti-digoxigenin antibody (Roche, Indianapolis, IN) used was at 1:2,000 dilution in Tris-buffered saline with 1.5% blocking powder (Roche, Indianapolis, IN). The embryos were incubated for 2 hr in BM purple substrate solution (Roche, Indianapolis, IN) at RT. Images were obtained using a Zeiss MrC5 Axiocam digital camera attached to a Zeiss StemiSv6 stereoscope.

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