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Wnt/Lef1 signaling acts via Pitx2 to regulate somite myogenesis

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ABSTRACT

Wnt signaling has been implicated in somite, limb, and branchial arch myogenesis but the mechanisms and roles are not clear. We now show that Wnt signaling via Lef1 acts to regulate the number of premyogenic cells in somites but does not regulate myogenic initiation in the limb bud or maintenance in the first or second branchial arch. We have also analysed the function and regulation of a putative downstream transcriptional target of canonical Wnt signaling, Pitx2. We show that loss-of-function of Pitx2 decreases the number of myogenic cells in the somite, whereas overexpression increases myocyte number particularly in the epaxial region of the myotome. Increased numbers of mitotic cells were observed following overexpression of Pitx2 or an activated form of Lef1, suggesting an effect on cell proliferation. In addition, we show that *Pitx2* expression is regulated by canonical Wnt signaling in the epaxial somite and second branchial arch, but not in the limb or the first branchial arch. These results suggest that Wnt/Lef1 signaling regulates epaxial myogenesis via Pitx2 but that this link is uncoupled in other regions of the body, emphasizing the unique molecular networks that control the development of various muscles in vertebrates.

Introduction

During vertebrate embryogenesis, the striated musculature of the head and trunk arises from two distinct regions: the somitic mesoderm gives rise to the axial, appendicular, and tongue musculature whilst the unsegmented paraxial mesoderm, together with the prechordal plate, forms the majority of the craniofacial musculature. These distinct origins are also reflected by different molecular signatures, for example, the axial skeletal musculature expresses the transcription factor *Pax3*, which is essential for myogenesis, but the developing craniofacial musculature does not (Bajard et al., 2006; Bothe and Dietrich, 2006; Brunelli et al., 2007; Buckingham and Relaix, 2007) reviewed in Noden and Francis-West (2006). Despite these differences, commitment to the myogenic pathway in all regions of the body is controlled by myogenic regulatory factors (MRFs) Myf5, MyoD, Mrf4, and myogenin (Rudnicki et al., 1993; Tajbakhsh et al., 1996). This is followed by terminal differentiation characterised by the expression of myosin heavy chains (MyHC).

In response to extrinsic patterning signals, each somite divides into two main compartments: a dorso-lateral epithelial compartment called the dermomyotome and a ventral mesenchymal compartment called the sclerotome, the progenitor of the vertebrae and ribs. The dermomyotome is a transient structure, which contributes cells to the epaxial and hypaxial myotome in successive phases. The primary myotome is generated by cells that enter first from the medial dermomyotome border, in a second phase myocytes are produced from all four dermomyotome edges. Finally, the central portion of the dermomyotome undergoes an epithelial-to-mesenchymal transition (EMT) leading to an influx of muscle progenitor cells into the primary myotome (Gros et al., 2004; Manceau et al., 2008). Recent work has shown that the expansion of the latter population of myogenic progenitors is influenced by notch and myostatin signaling, which affects their terminal differentiation (Manceau et al., 2008; Schuster-Gossler et al., 2007; Vasyutina et al., 2007). The limb and tongue musculature arises by delamination of the hypaxial dermomyotome in response to hepatocyte growth factor signaling and myogenic cells migrate to their final destination (Dietrich, 1999). Within the developing limb bud, they become committed to the myogenic lineage and form the dorsal and ventral muscle masses.

The Wnt family of growth factors play key roles during embryonic myogenesis. Early studies demonstrated that a combination of Shh protein and Wnt1 or Wnt3a-expressing fibroblasts is sufficient to activate skeletal muscle-specific gene expression in explants of somites or presegmented mesoderm (Münsterberg et al., 1995). Wnt signaling has also subsequently been implicated in myogenic commitment within the developing limb bud; however, it has been shown to be inhibitory for craniofacial myogenesis (Anakwe et al., 2003; Geetha-Loganathan et al., 2005; Tzahor et al., 2003). The Wnt ligands can signal through a

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number of pathways including the β-catenin-dependent pathway, planar cell polarity and calcium pathways (reviewed by Gordon and Nusse, 2006). The best characterised is the pathway involving β-catenin, the glycogen-synthase kinase 3β (GSK3β) destruction complex and the T-cell factor/lymphoid enhancing factor-1 (Tcf/Lef1) transcription factors. In an uninduced cell, β-catenin is phosphorylated and removed by the GSK3β destruction complex and Tcf/Lef1, together with Groucho, acts as a transcriptional repressor. However, Wnt binding to the serpentine frizzled receptor results in the inhibition of the GSK-3β destruction complex allowing the accumulation of β-catenin, which is subsequently translocated to the nucleus. Here, β-catenin binding converts Tcf/Lef1 to a transcriptional activator (reviewed by Stadeli et al., 2006; Willert and Jones, 2006). Canonical Wnt signaling also requires a co-receptor, the low-density lipoprotein receptor-related proteins 5 and 6 (LRP5/6) (Niehrs, 2006).

Wnt signaling is modulated by a number of antagonists including extracellular antagonists such as the secreted frizzled related proteins/frizbees (Sfrps/Frzb) and Dkk1. The Sfrps have homology to the extracellular domain of the frizzled receptors and bind to Wnt proteins preventing their binding to the frizzled receptors. The Sfrps are, therefore, not specific to a particular Wnt pathway. In contrast, Dkk1 acts by binding directly to the extracellular domain of LRP-5/6 and therefore interferes specifically with canonical Wnt signaling.

The canonical Wnt signaling pathway is essential for the development of the epaxial musculature, consistent with the expression of β-catenin and Lef1 in the dorso-medial lip of the dermomyotome (Schmidt et al., 2004; Schmidt et al., 2000). The importance of Wnt signaling for the activation of myogenesis is further illustrated by the finding that transplacental delivery of Frzb1 inhibits somite myogenesis. This study suggested that Wnt signals may act by regulating both myogenic commitment and expansion of committed cells (Borello et al., 1999). In addition, the detailed analyses of Myf5 regulatory elements revealed important Tcf/Lef sequences, which mediate the correct spatiotemporal expression of Myf5 in the epaxial domain of the somite (Borello et al., 2006; Teboul et al., 2002). Consistent with this, experiments using targeted electroporation of activated β-catenin showed that Wnt/β-catenin signaling is crucial for myogenic specification (Gros et al., 2009).

The transcription factor, *Pitx2*, is a target of the β-catenin-dependent Wnt pathway (Kioussi et al., 2002). Pitx2 can also be an effector of this pathway, for example, in epithelial cells it forms a functional complex with β-catenin and Lef1 to synergistically induce transcription from the Lef1 promoter (Amen et al., 2007; Vadlamudi et al., 2005), thus establishing a positive feedback loop. It is known that *Pitx2*, *Pitx1*, and *Pitx3*, are expressed in the developing musculature (Dong et al., 2006; Golding et al., 2004; Poulin et al., 2000; Shih et al., 2007a,b). Pitx2 has been shown to regulate proliferation in cardiac neural crest cells, the pituitary gland, and the myogenic cell lines, C2C12 and Sol8 (Kioussi et al., 2002; Martinez-Fernandez et al., 2006). In the first branchial arch, *Pitx2* overexpression increases the number of differentiating myocytes. Pitx2 also regulates cell survival, as gene inactivation in mice results in the apoptosis of first branchial arch myogenic cells (Dong et al., 2006; Shih et al., 2007a).

Here, we examined the relationship between *Lef1* and *Pitx2* and show that *Pitx2* expression is regulated by Wnt/Lef1 signaling in the myotome and second branchial arch. In addition, we investigated the function of *Lef1* and *Pitx2* during somite and limb myogenesis.

Materials and methods

Embryo manipulations, RCAS infection, electroporation, and bead implants

Fertile white Leghorn chicken eggs were obtained from Henry Stuart (Lincolnshire) and incubated at 37.5 °C and were staged according to Hamburger and Hamilton (HH) (Hamburger and Hamilton, 1992). Stage 12 embryos were used for retrovirus (RCAS) infection and embryos at HH16 were used for somite electroporations. After manipulations, eggs were sealed and incubated for 24 or 48 hours, depending on the experiment. Embryos were harvested in DEPC PBS, fixed overnight in 4% paraformaldehyde at 4 °C, and processed for *in situ* hybridisation. The timing of somite infections should predominantly affect the primary myotome.

Concentrated retrovirus was prepared following standard protocols (Morgan and Fekete, 1996). The following RCAS constructs were used: RCAS-ΔNLef1, RCAS-ΔNLef1-VP16, RCAS-ΔN34Lef1, RCAS-Pitx2a, RCAS-Pitx2a-En, and RCAS-GFP. Pitx2 viruses were kindly provided by Yi-Ping Chen (Tulane University, New Orleans; Yu et al., 2001). Concentrated RCAS virus was injected into presegmented mesoderm of HH stage 12 embryos and the embryos were allowed to develop for 48 hours before harvesting. The contralateral, uninfected side, and/or RCAS-GFP-infected somites served as control. The spread of infection was examined using *in situ* detection of viral gag mRNA. Alternatively, pellets of O-line cells infected with the retroviruses were grafted into the developing limb bud at stages 17/18 and embryos were allowed to develop until day 8 (Anakwe et al., 2003).

Electroporation was carried out as described in Sweetman et al. (2008). Briefly, HH16 epithelial somites 2–6 were injected with plasmid DNA at a concentration of 1.5 mg/ml. Positive and negative platinum electrodes were kept 2 mm apart and placed on either side of the somites. A TSS20 Ovodyne electroporator (Intracel) was used to apply 5 pulses of 15 V for 15 ms. PBS buffer was added before and after electroporation.

For the Dkk1 manipulation studies, affigel beads were soaked in 500 ng/μl solution of recombinant mouse Dkk1 protein (carrier-free, R&D Systems) for 1 hour at 37 °C. They were applied to either the first or the second branchial arches or the developing limb buds at HH stages 20/21 of embryonic development. Control beads were soaked in 0.1% BSA in PBS. The embryos were allowed to develop for 24 hours and were then analysed for changes in gene expression by whole-mount *in situ* hybridisation.

Micromass cultures

Micromass cultures from HH stage 19/20 chick wing buds were prepared as described (Anakwe et al., 2003). Briefly, the ectoderm was dissociated from the limb mesenchyme in 2% trypsin and the mesenchymal cells were disaggregated to a single-cell suspension. A total of 2×10^5 cells were resuspended in 10 μl of concentrated RCAS retrovirus and plated as a droplet. After 1 hour, the micromass cultures were flooded with DMEM containing 10% FCS, 1% chick serum, 1% penicillin/streptomycin. Micromasses were cultured for 3 days, fixed in methanol for 1 minute at room temperature and analysed for Pan-MyHC expression using the A4.1025 antibody (DSHB). Each assay consisted of at least 3 micromasses and the experiments were performed in triplicate. Statistical significance was determined using the Student's *t*-test.

In situ hybridisation and immunohistochemistry

Whole-mount *in situ* hybridisations were carried out as previously described (Anakwe et al., 2003; Schmidt et al., 2000). Anti-sense Lef1, Pitx2, MyoD, Myf5, Mgn, MyoR, Pax3, Pax7, and anti-gag probes were used (Dastjerdi et al., 2007; Smith et al., 2005; Yu et al., 2001). After *in situ* hybridisation, embryos were fixed in 4% PFA, washed with PBS, and transferred into gelatin for vibratome sectioning or into 30% sucrose/PBS and finally OCT for cryosectioning and immunoanalysis. Ten-micrometer sections were subjected to a double immunostaining using phospho-histone H3 and MF-20 antibodies (DSHB, 1:500). Briefly, sections were incubated with 0.1% Triton and treated with 1:10 H_2O_2/PBS for 10 minutes. After washing twice in PBS, sections were blocked in 10% goat serum and primary antibody was applied overnight at 4 °C. Sections were blocked in 10% goat serum before applying secondary antibodies, anti-rabbit Alexa Fluor 488 to detect phospho-histone H3 (green) (Molecular Probes), at 1:1000. Sections were treated with DAPI to stain nuclei and mounted in Mowiol. Pictures were taken on an Axioscope microscope using Axiovision software (Zeiss, Germany).

Statistical analyses

The number of phospho-histone H3-positive cells was counted in the dermomyotomes and myotomes of RCAS-ΔNLef1-VP16- or RCAS-Pitx2a-infected and contralateral, non-infected somites. SPSS was used to calculate means and standard errors. Counts from infected and non-infected sides were treated as paired readings (Lef1, $n=137$, Pitx2, $n=47$) and statistical analysis was carried out to confirm the significance of the observed differences (Wilcoxon test).

Results

Canonical Wnt signaling via Lef1 affects the size of the myotome

Wnt signaling via β-catenin promotes myogenesis. In order to elucidate its role in more detail we infected presegmented mesoderm at HH12 with RCAS retrovirus expressing a mutant form of Lef1, RCAS-ΔNLef1, which retains the HMG box that binds to DNA, but lacks the β-catenin co-activator and Groucho co-repressor binding domains (Fig. 1G). One side of the embryo was targeted allowing the contralateral side to be used as control. After 48 hours, successful transfection and targeting was determined by *in situ* detection of viral *gag* transcripts (Figs. 1A and A′). Effects of ΔNLef1 on myogenic differentiation were investigated by *in situ* hybridisation analyses of a series of marker genes. This showed that transfected somites had a thickened myotome characterised by an increased number of cells expressing *Pax-7*, a premyogenic marker gene ($n=12$; Figs. 1B and B′). In addition, expression of the myogenic bHLH transcription factors, *MyoD* ($n=17$) and *myogenin* ($n=7$) was increased on the transfected side (Figs. 1A, A′, C, and C′). As the ΔN-Lef1 amino-terminal deletion protein cannot interact with the Groucho co-repressor, these results are consistent with the idea that loss of Lef1-mediated repression was sufficient to activate myogenesis. Furthermore, the increased number of cells expressing Pax-7 and Pax-3 suggests that Wnt signaling via Lef1 acts early during myogenic specification.

To extend these observations, we used the same deletion construct fused to the strong transcriptional activator VP16 (Fig. 1G). ΔNLef1-VP16 was targeted to the developing somite by RCAS-mediated infection (Figs. 1D–F). Alternatively, newly formed epithelial somites were electroporated with an expression vector, pCAβ encoding the same protein: ΔNLef1-VP16. The pCAβ vector also expresses GFP from an IRES (Supplemental Figs. 1A–D). After 24 hours, *in situ* detection of

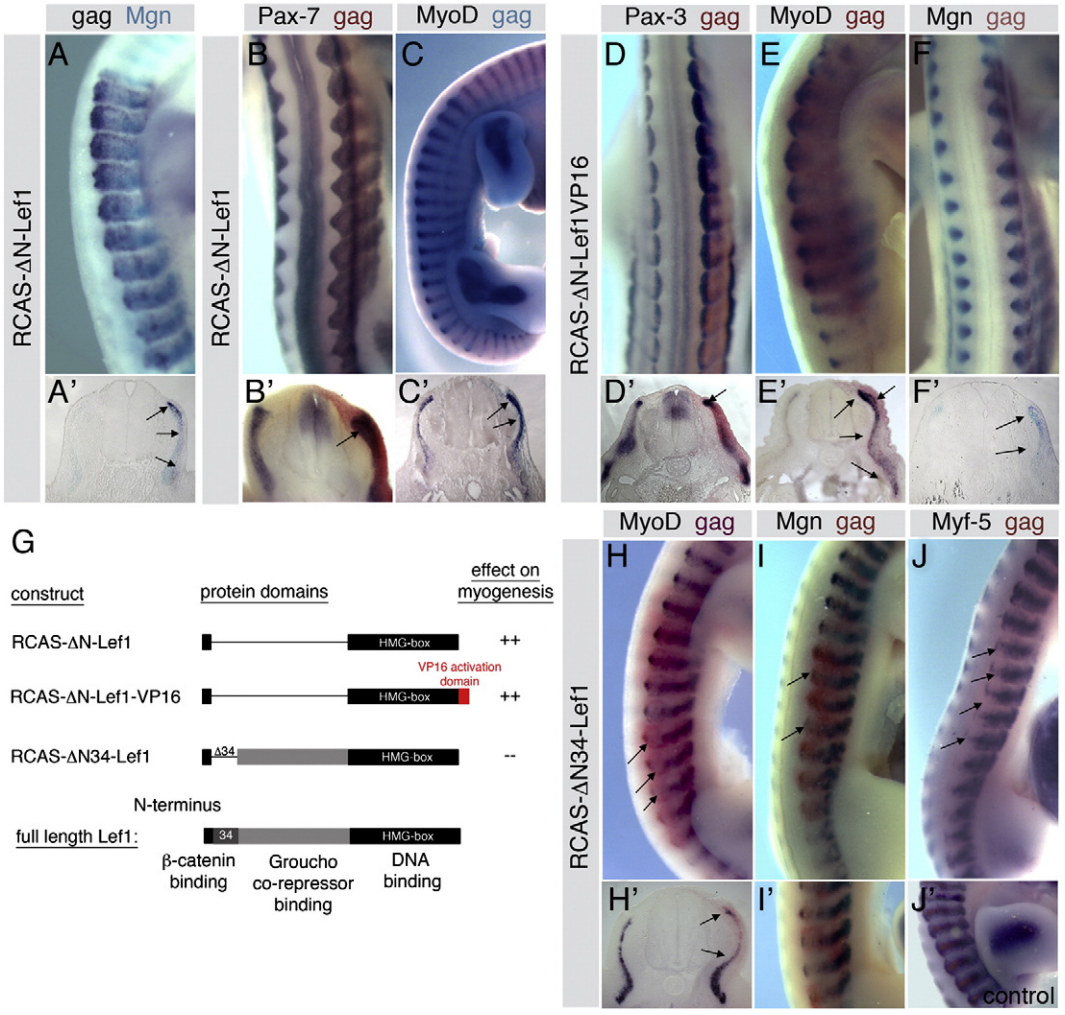

Fig. 1. Manipulating Lef1 activity in somites affects the number of myogenic cells. The presegmented mesoderm of chicken embryos was infected with avian retrovirus (RCAS) expressing ΔN-Lef1 (A–C′), ΔNLef1-VP16 (D–F′), ΔN34-Lef1 (H–J′), the structure of the proteins expressed are indicated schematically in (G). *In situ* hybridization for viral *gag* transcripts (A) (dark purple) confirmed efficient transfection of somites. Double *in situ* hybridization was performed with gag and a panel of markers. This showed that the number of cells expressing Pax-7 (B), Pax-3 (D), MyoD (C, E), and Mgn (A, F) increased on the manipulated side after targeted misexpression of ΔNLef1 and ΔNLef1-VP16. In contrast, misexpression of ΔN34-Lef1 decreased the number of cells expressing MyoD (H), Mgn (I) and Myf-5 (J). The probes used are indicated above each panel, whole-mount embryos and corresponding sections are shown, except (I′), which shows another example similar to (I) and (J′) which shows the contralateral side of (J). The black arrows indicate the affected regions, gag probe was developed with INT/BCIP or Fast Red (red) with the myogenic marker in NBT/BCIP (purple), except in (A, F) where gag is shown in purple (NBT/BCIP) and Mgn is turquoise (BCIP).

ectopic GFP expression determined successful targeting (Supplemental Figs. 1A and A′). The phenotype observed with ΔNLef1-VP16 was similar to that observed with ΔNLef1. Sections through the transfected region showed that ΔNLef1-VP16 resulted in increased expression of premyogenic markers *Pax-3* ($n=6$; Figs. 1D and D′), Pax7 ($n=3$; data not shown) and myogenic markers *MyoD* ($n=9$; Figs. 1E and E′) and *Mgn* ($n=8$; Figs. 1F and F′). The same results were obtained after electroporation of pCAβ-ΔNLef1-VP16 (Supplemental Figs. 1A–D); however, phenotypes after RCAS-mediated overexpression were usually stronger than after electroporation.

Next, we examined the effects of loss of Lef1-mediated transcriptional activation on myogenesis by targeted misexpression of RCAS-ΔN34-Lef1. In this deletion, the β-catenin binding domain was specifically removed, whilst the binding domain for the Groucho co-repressor was retained, leading to a dominant negative effect (Fig. 1G). Expression of RCAS-ΔN34-Lef1 in presegmented mesoderm resulted in a marked decrease of *MyoD* ($n=11$; Figs. 1H and H′), *Mgn* ($n=10$; Figs. 1I and I′), and *Myf5* ($n=11$; Figs. 1J and J′) in somites on the infected side of the embryo.

Lef1 interacts with Pitx2 to regulate myogenic differentiation in somites

Pitx2 is a direct transcriptional target of the Wnt–β-catenin signaling; in addition, it acts with Lef1 to regulate the *Lef1* promoter, creating a positive feedback loop (Ai et al., 2007; Amen et al., 2007; Vadlamudi et al., 2005). In the mouse embryo, *Pitx2* is expressed in the developing myotome, branchial arch mesoderm, and limb myogenic cells (Kioussi et al., 2002; L'Honore et al., 2007; Marcil et al., 2003; Shih et al., 2007b). Expression of *Pitx2* in chick embryonic musculature has, to date, only been reported in the branchial arches (Dong et al., 2006). Therefore, to investigate a potential interaction of *Pitx2* with *Lef1* during somite myogenesis, we analysed *Pitx2* expression in chick somites.

Pitx2 transcripts were first detected in the hypaxial myotome of HH20 embryos (Fig. 2A). By HH22, *Pitx2* was expressed in committed myogenic cells throughout the myotome, but with lower levels in the central region (Figs. 2B and B′). In posterior somites, expression was still limited to the hypaxial domain (Fig. 2B). Expression of Pitx2 is overlapping with MRFs, *Lef1*, *Tcf1*, and *β-catenin* (Schmidt et al., 2004), and to investigate the relationship between canonical Wnt signaling and *Pitx2* during embryonic myogenesis, we examined the effects of RCAS-ΔNLef1-VP16 and RCAS-ΔN34Lef1 on *Pitx2* expression in developing somites (Figs. 2C–D′). Targeted misexpression of ΔNLef1-VP16 by RCAS infection of epithelial somites at HH17 led to an increase in *Pitx2* transcripts, particularly in the epaxial myotome ($n=16$) (Figs. 2C and C′). In contrast, infection with a retrovirus encoding ΔN34Lef1 caused reduced *Pitx2* expression ($n=7$) (Figs. 2D and D′). The negative effect of ΔN34Lef1 on Pitx2 expression was seen in epaxial and central myotomes, but not in the hypaxial domain.

To examine whether Lef1 may affect the myotome via Pitx2, at least in part, we determined the phenotypes resulting from RCAS-mediated expression of either a Pitx2a full-length protein or Pitx2a-En where the engrailed repressor is fused to Pitx2a (Figs. 2E–H). Targeted expression of Pitx2a resulted in a dramatic increase in myotome thickness as indicated by expression of MyoD ($n=14$) and Mgn ($n=13$) (Figs. 2E–F′). The phenotype was most apparent in, but not limited to, the epaxial myotome. Conversely, RCAS-Pitx2a-En resulted in a loss of transcripts for the myogenic markers Myf5 ($n=12$, not shown), MyoD ($n=20$), and Mgn ($n=4$), throughout myotomes (Figs. 2G–H′).

Lef-1 and Pitx-2 expression increased cell proliferation in dermomyotomes and myotomes

Next we investigated the potential mechanism by which Lef1 and Pitx2a affect the size of the myotome. In particular, we determined whether proliferation of myogenic cells could be affected in developing somites, as has been demonstrated previously for Pitx2a in cardiac neural crest cells and the myogenic satellite C2C12 and Sol8 cell lines (Kioussi et al., 2002; Martinez-Fernandez et al., 2006). Labelling with phospho-histone-H3 was used to quantify the number of mitotic cells 48 hours after transfection with RCAS-ΔN-Lef1 (Fig. 3A) or RCAS-Pitx2a (Fig. 3C). The position of the myotome was identified in brightfield images and the number of mitotic cells within infected dermomyotomes and myotomes was counted and compared with the number of mitotic cells in non-infected contralateral somites (Figs. 3B and D). After infection with RCAS-ΔNLef1 or RCAS-Pitx2a more cells in mitosis were detected (30%), suggesting that proliferation may be increased (Fig. 3 compare panels A and B with C and D; Fig. 3E, $P<0.01$). This conclusion is supported by the overall increase

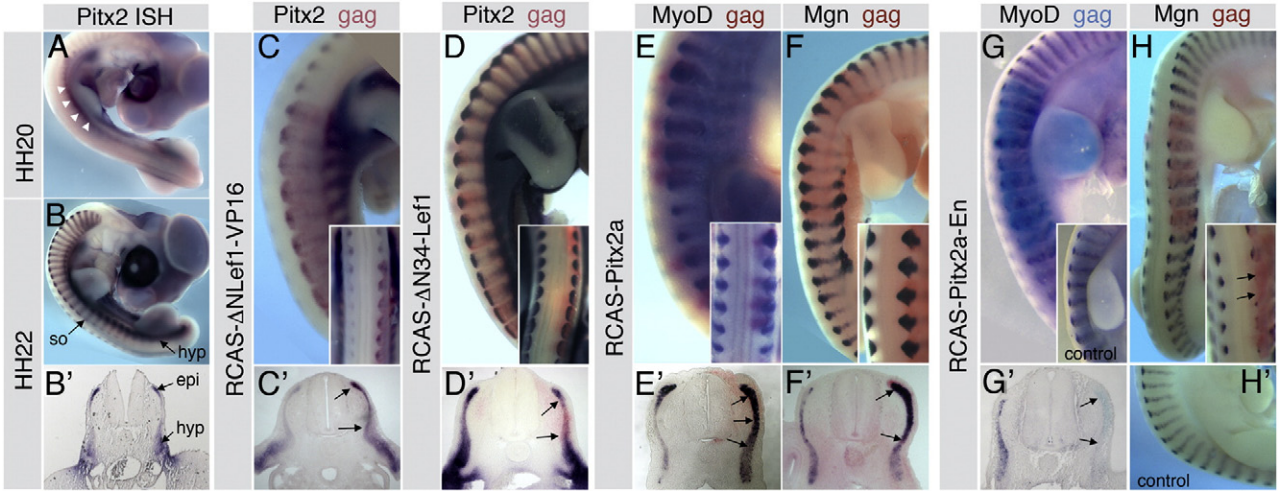

Fig. 2. Targeted misexpression of RCAS-ΔNLef1-VP16 or RCAS-ΔN34-Lef1 increased or decreased the myotomal expression of Pitx2 and Pitx2 itself affects the number of myogenic cells. Whole-mount *in situ* hybridization was performed with the probes indicated above each panel. At HH20, Pitx2 transcripts were detected in hypaxial myotomes (A, white arrowheads), by HH22 Pitx2 expression was seen throughout the myotome except in posterior somites (B, B′). (C–H) Double whole-mount *in situ* hybridization with Pitx2 and gag. Infection with RCAS-ΔNLef1-VP16 (C) resulted in an increase of Pitx2 transcripts particularly in the epaxial and central myotomes (C′ black arrows). Infection with RCAS-ΔN34-Lef1 (D) resulted in loss of Pitx2 transcripts particularly in the epaxial and central myotomes (D′ black arrows). Infection with RCAS-Pitx2a resulted in an increase of MyoD and Mgn transcripts (E, F) particularly in the epaxial and central myotomes (E′, F′ black arrows). Infection with RCAS-Pitx2a-En resulted in loss of MyoD and Mgn transcripts (G, H). Gag probe was developed with INT/BCIP or Fast Red (red) or BCIP (light blue); other probes were developed with NBT/BCIP (purple). Lateral views are shown in (A–H) with dorsal view shown in the insets. Relevant sections are shown below the whole mounts (B′–G′); (H′) shows contralateral control. so, somite; hyp, hypaxial mytome; epi, epaxial myotome.

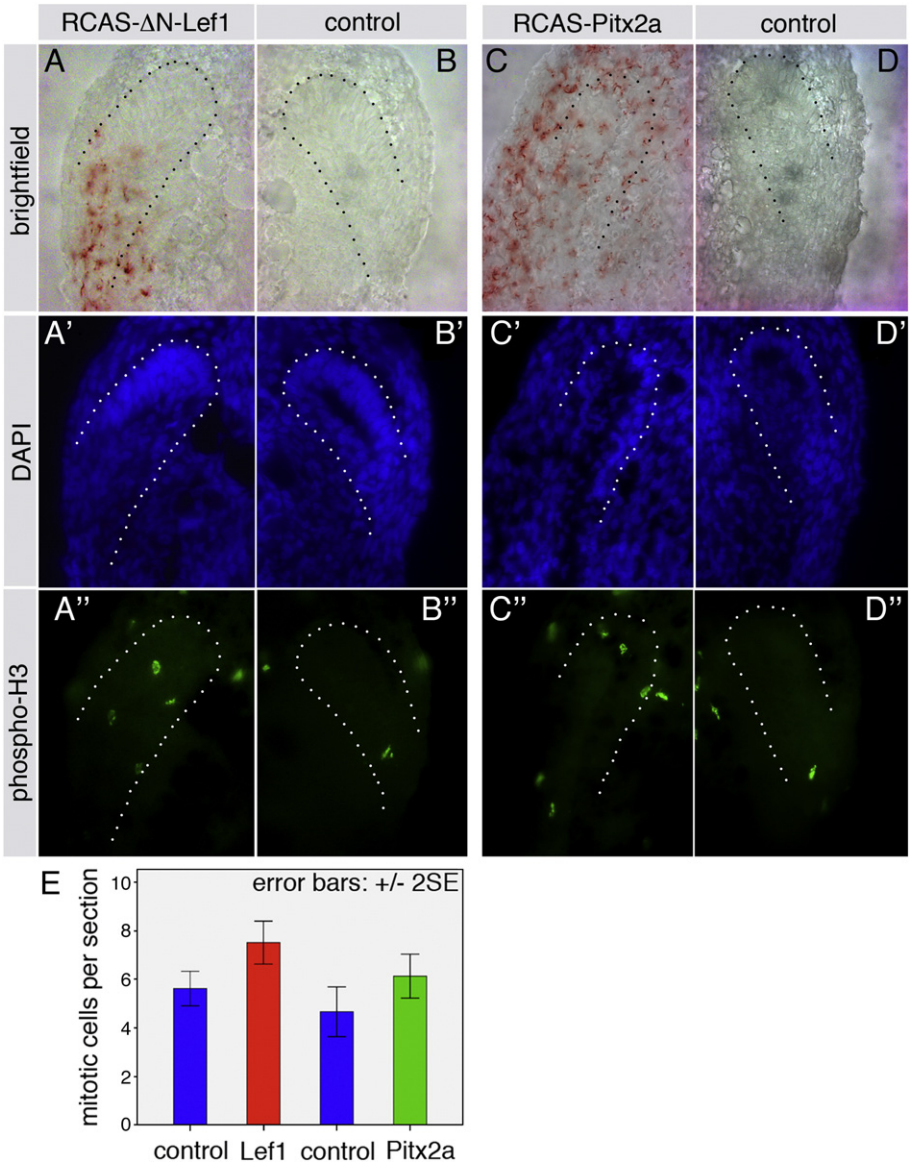

Fig. 3. Targeted misexpression of ΔN-Lef1 or Pitx2a led to an increased number of mitotic cells in the dermomyotome and myotome. Brightfield and fluorescent images of sections (10 μm) are shown. (A, B) Infection of presegmented mesoderm with RCAS-ΔN-Lef1 resulted in an increase of mitotic cells, stained with anti-phospho-histone H3, compare the infected (A″) with the contralateral side (B″). (C, D) Infection of presegmented mesoderm with RCAS-Pitx2a resulted in an increase of mitotic cells, stained with anti-phospho-histone H3; compare the infected (C″) with the contralateral side (D″). RCAS-gag transcript was detected by *in situ* hybridization (A, C; INT/BCIP, red). Sections were stained with DAPI (A′–D′, blue) or anti-phospho-histone H3 (A″–D″, green). SPSS analysis confirmed that the differences in the means of paired counting ($n = 137$ for Lef1, $n = 47$ for Pitx2a) were statistically significant ($P < 0.01$). The diagram in (E) shows a graphical representation of this data. The dotted lines indicate the dermomyotome and myotome area.

in myotome size. The statistical significance of the observed differences was confirmed using the SPSS Wilcoxon test (number of sections counted/paired counting: ΔNLef1, $n = 137$; Pitx2, $n = 47$).

The molecular regulation of limb and branchial arch myogenesis differs from somite myogenesis

In the limb bud, the temporal and spatial expression pattern of *Pitx2* is consistent with that previously reported in the mouse (Kioussi et al., 2002; L'Honore et al., 2007; Marcil et al., 2003; Shih et al., 2007b) (Supplemental Fig. 2). *Pitx2* transcripts were first observed at stage 21. Comparison of *MyoD* versus *Pitx2* expression on adjacent sections through limb buds showed that the *Pitx2* expression domain encompasses both that of *MyoD* and *Pax3* (Supplemental Figs. 2D and E). This indicates that *Pitx2* is expressed in developing myogenic cells both prior to and after myogenic commitment (Supplemental Figs. 2A, B, D, and E). This is in contrast to the somite where *Pitx2* is expressed after myogenic commitment.

The previous experiments showed that Lef1/Pitx2 regulates myogenesis within the somite and we provided evidence that proliferation of myogenic progenitors is affected. As myogenesis is differentially controlled in different regions of the body, we extended our analysis to the developing limbs and branchial arches (Fig. 4). First, we analysed possible effects of canonical Wnt signaling on *Pitx2* expression. Application of beads soaked in Dkk1, an antagonist that specifically blocks this pathway, did not alter *Pitx2* expression in the limb bud (Fig. 4A, $n = 6$). Likewise, infection of the limb buds with RCAS retrovirus expressing dominant negative ΔN34-Lef1 had no effect on *Pitx2* expression ($n = 5$, Figs. 2O and P). However, RCAS-ΔNLef1 infection of limb buds led to a slight increase in Pitx2 transcripts (Figs. 4M and N; $n = 3$). These findings suggest that Wnt signaling can positively affect *Pitx2* expression in the limb bud mesenchyme; however, it is not required.

To further investigate the relationship between Pitx2 and limb myogenesis together with the role of the canonical Wnt pathway during limb myogenic differentiation, we analysed the expression of

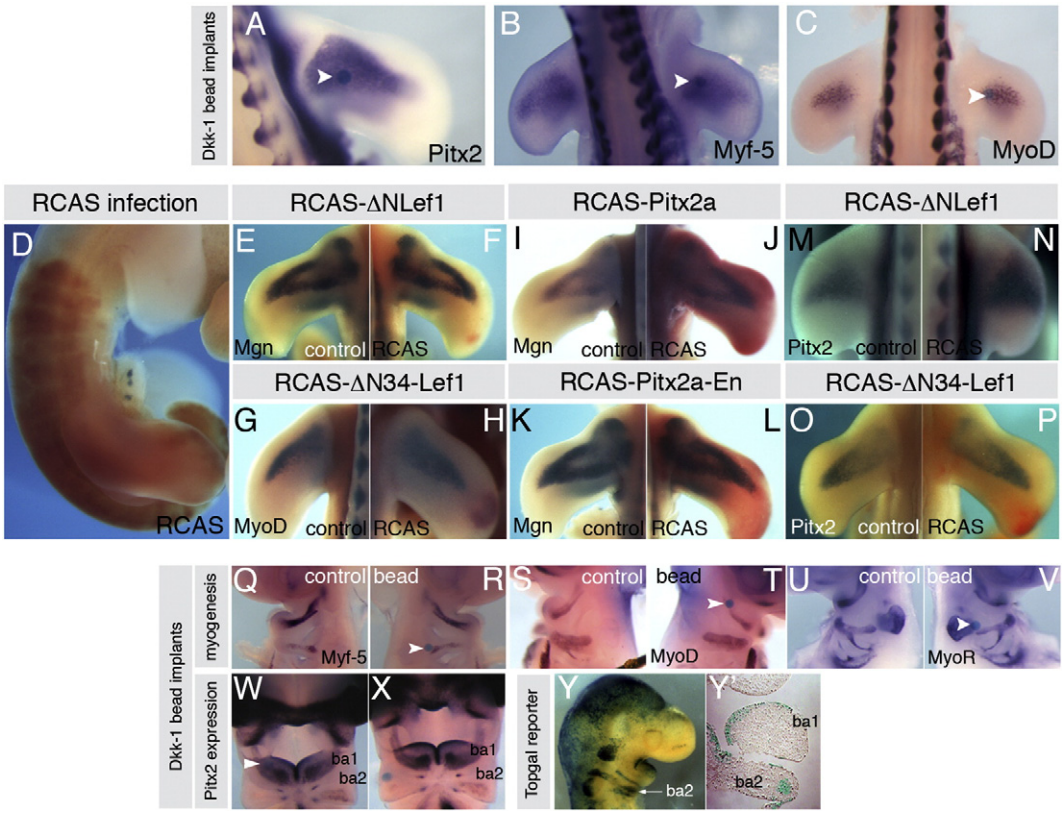

Fig. 4. Manipulation of Wnt signaling does not affect limb or branchial arch myogenesis. (A–C) Beads soaked in Dkk-1 were implanted into the limb bud at HH19–20, and after 24 hours, embryos were processed for *in situ* hybridization for (A) Pitx2, (B) Myf-5, and (C) MyoD. (D–P) RCAS infection of embryos followed by whole-mount *in situ* hybridization for the indicated markers. (D) Detection of viral transcripts demonstrates successful infection of limb buds. Active Lef1 (E, F) or dominant-negative Lef1 (G, H) has no effect on myogenic markers. Dominant-negative Lef1 did not affect Pitx2 expression (O, P); however, active Lef1 slightly increased Pitx2 transcripts (M, N). Targeted misexpression of Pitx2a (I, J) or the dominant-negative Pitx2a-En (K, L) had no effect on myogenic gene expression (I–L). (Q–X) Chick embryos showing expression of indicated markers in the manipulated and contralateral control branchial arch 24 hours after application of a Dkk1 soaked bead (indicated by a white arrowhead). Q–V are lateral views of the control (Q, S, U) and manipulated (R, T, V) arch whilst W, X show frontal views. (Y, Y') Analysis of an E9.5 TOPGAL reporter mouse embryo that has been stained for LacZ activity. (Y) Lateral view of the head showing labelling in the first and second branchial (ba) arch. Mesodermal expression is arrowed. (Y') Frontal section of 1/2 of the embryo shown in Y showing expression in the neural crest derived mesenchyme in the first branchial arch (ba1) and in the mesoderm of the second branchial arch (ba2). Probes used are indicated on each panel, control = contralateral limb, RCAS = infected limb.

the myogenic markers, *Myf5*, *MyoD*, and *Mgn* following activation or inhibition of Wnt signaling by misexpression of ΔNLef1 or ΔN34Lef1 proteins (Figs. 4E–H; $n=4$, $n=7$). Alternatively we applied Dkk1 beads (Figs. 4B and C). These studies demonstrated that myogenic commitment within the limb bud is not regulated by the canonical Wnt pathway (Dkk1: Myf5, $n=5$; MyoD, $n=5$), a conclusion consistent with recent studies in the mouse (Hutcheson et al., 2009).

To determine whether Pitx2, which in limb bud mesenchyme seemed to be Wnt-independent, could affect myogenesis, we used RCAS to misexpress Pitx2a (Figs. 4I and J; $n=30$) or Pitx2a-En (Figs. 4K and L; $n=16$). Embryos were harvested 48 hours after infection. At this time, we could not detect any differences in expression of *MyoD* and *Mgn* transcripts between infected and non-infected limb buds, suggesting that limb bud myogenesis was independent of Pitx2a, at least in the early phase.

We also extended these studies to the branchial arches, where myogenic differentiation is differentially regulated. In the head, application of Dkk1 soaked beads inhibited *Pitx2* expression in the second, but not in the first, branchial arch mesoderm and ectoderm (Figs. 4W and X), indicating that canonical Wnt signaling may be involved. This was consistent with analysis of the TOPGAL reporter mouse at E9.5, which showed LacZ reporter activity, indicative of canonical Wnt signaling, within the mesoderm of the second but not the first branchial arch (Figs. 4Y and Y′). However, as in the limb bud, application of Dkk1 beads did not alter the expression of myogenic markers (Figs. 4Q–V; ba1: *Myf5*, $n=7$; *MyoD*, $n=8$; *MyoR*, $n=1$; ba 2: *Myf5*, $n=2$; *MyoD*, $n=3$; *MyoR*, $n=3$).

Late myogenic differentiation in the limb is affected by Pitx2

Although we did not detect an effect of Pitx2 on early limb myogenesis, we have previously shown that Pitx2 determines the number of terminally differentiated myocytes in the first branchial arch (Dong et al., 2006). Therefore, we next investigated a potential role of Pitx2 during terminal myogenic differentiation in the limb bud. RCAS(BP) retroviruses encoding either Pitx2a-En or Pitx2a proteins were used to infect developing limb cells in micromass cultures, a simple assay which allows quantification of myocyte differentiation (Figs. 5G and H). Limb bud cells were isolated from HH19/20 embryos and plated at a high density in the presence of high-titer RCAS retrovirus. Terminal myogenic differentiation was determined after 3 days by staining with the pan-MyHC antibody. The effect of both isoforms of Pitx2, Pitx2a and Pitx2c, was examined, as it has been shown that these can have differential effects (Yu et al., 2001). In the micromass assay, misexpression of Pitx2a or Pitx2c significantly increased the number of differentiated myocytes compared to RCAS-GFP-infected controls. In contrast, blocking Pitx2a or Pitx2c function had the converse effect and reduced myocyte number significantly (Figs. 5G and H; $P<0.05$).

We extended this *in vitro* analysis to *in vivo* studies. Here we grafted pellets of RCAS-infected cells into a HH18/19 limb bud. This results in viral spread throughout the majority of the limb (Duprez et al., 1996). RCAS-infected embryos were incubated until day 8 of development when primary myogenesis is complete. The limbs were fixed and immunostained with the pan-MyHC antibody to analyse

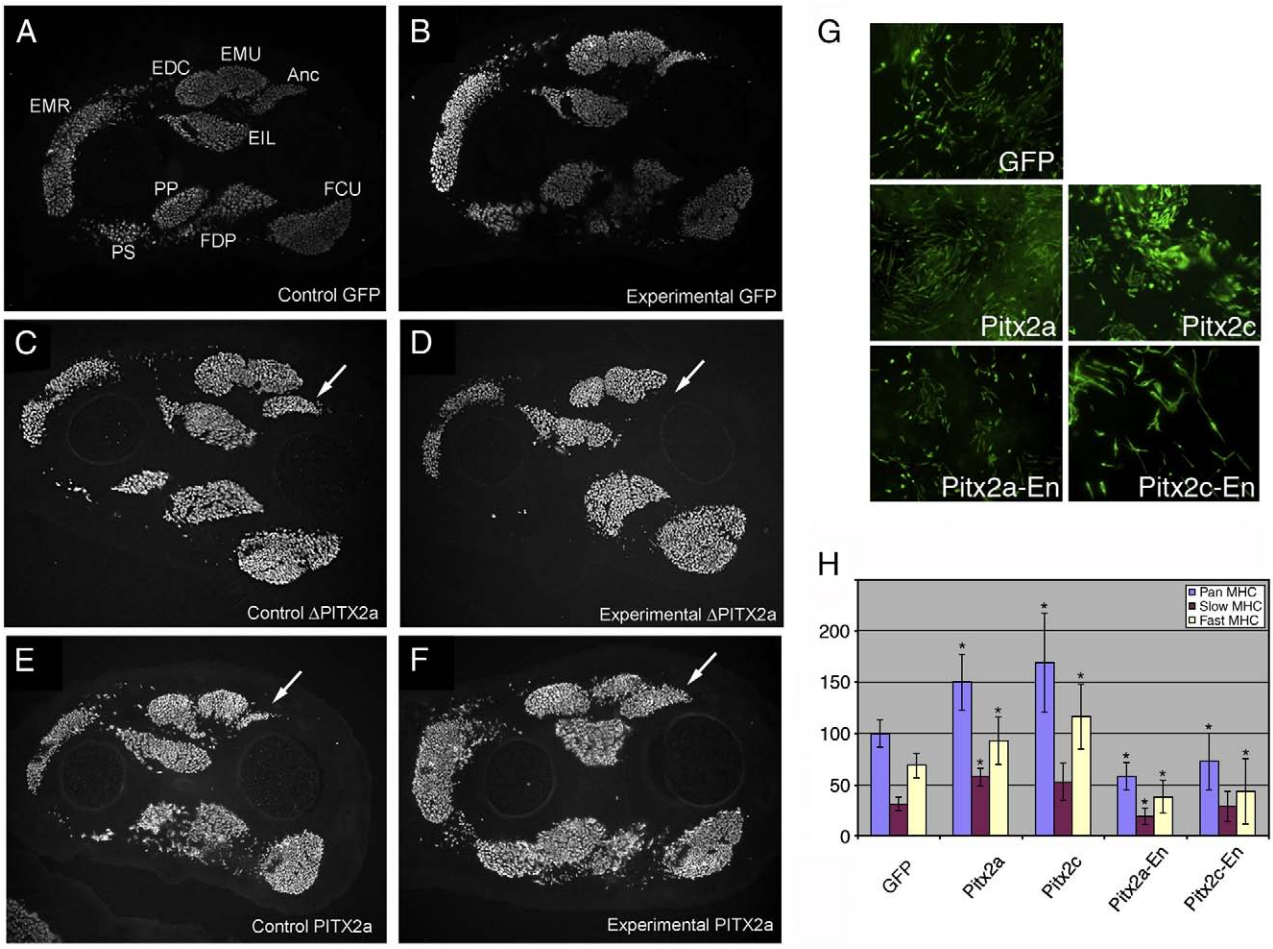

Fig. 5. Pitx2 regulates myogenic differentiation in the limb bud. (G) Fluorescent images of micromass cultures infected with retroviruses encoding Pitx2a and Pitx2c or dominant-negative Pitx2a and Pitx2c constructs. The micromasses were stained with 1025, a pan-MyHC antibody, to visualise the myocytes. (H) Quantification of the number of myocytes in the micromass cultures ($^{*}P<0.05$, $^{***}P<0.005$). (A–F) Fluorescent images of transverse cryosections of control (A, C, E) and retrovirally infected (B, D, F) day 8 chick wings infected with RCAS expressing GFP, ΔPitx2a, or Pitx2a and labelled with the pan-MyHC 1025 antibody. Sections were taken at the same level of the chick wing. The arrows indicate the location of the Anc muscle. In the Pitx2a-En-infected limb, the Anc muscle is absent and the EDC and EMR muscles are decreased in size. In the Pitx2a-infected limb, the Anc, EIL, EDC and EMR muscles are increased in size. Anc, anconeus; EDC, extensor digitorum communis; EIL, extensor indicis longus; EMR, extensor metacarpi radialis; EMU, extensor metacarpi ulnaris; FDP, flexor digitorum profundus; PP, pronator profundus; PS, pronator superficialis.

terminal differentiation. Muscle development was compared to the uninfected contralateral limb. The number of myofibers was counted every 40 μm through the zeugopod of the limb and the flexor and extensor muscles were analysed separately. We found that blocking Pitx2a function *in vivo* by expressing Pitx2a-En virus reduced the number of differentiated myocytes/myofibers (Figs. 5C and D; $n=4$ limbs; average number in control extensor muscles, $n=159$ versus 77 in infected extensor muscles, $P<0.01$; average number control flexor muscles, $n=280$ versus 234 in infected flexor muscles, $P<0.01$). Conversely, overexpression of Pitx2a significantly increased the number of differentiated myocytes/myofibers and hence increased muscle size (Figs. 5E and F; flexor muscles: control $n=265$ versus 339 in the infected side, $P<0.05$; extensor muscles, control, $n=158$ versus 219 in infected limbs, $P<0.01$). Infection with the control GFP virus did not significantly alter the number of myofibers or patterning of the muscles (extensors, control, $n=162$ versus GFP-infected, $n=158$, Figs. 5A and B). This demonstrated that within the limb bud, Pitx2 affected the number of myocytes/myofibers and thus final muscle size.

Discussion

The canonical Wnt signaling pathway has been implicated in epaxial, limb and branchial arch myogenesis: in the epaxial somite and limb bud Wnt signaling has been proposed to be involved in myogenic commitment whereas in the head, it has been shown to be inhibitory (reviewed by Noden and Francis-West, 2006). Here, we have uncovered differential requirements and functions of Wnt/Pitx2 in these regions and identified the stages in the myogenic pathway when canonical signaling acts. By targeted misexpression of the ΔNLef1 mutant construct, we uncover that de-repression of canonical Wnt signaling is sufficient to promote myogenesis in the somite (Figs. 1A–C). Strong activation of somite myogenesis was also observed following RCAS-ΔNLef1-VP16 infection consistent with the documented effect of canonical Wnt signaling on myogenic commitment (Borello et al., 2006; Gros et al., 2009). Interestingly, the increased expression of the myogenic markers, Pax-7 and Pax-3, suggests that Wnt/Lef1 may act early during myogenic commitment at least in somites. In the limb and branchial arch muscles, however, manipulation of Lef1 activity or application of the Wnt antagonist, Dkk1, showed that myogenic commitment/maintenance does not require the canonical Wnt signaling pathway (Fig. 4). This is in keeping with observations reported in transgenic mice, where conditional activation or inactivation of β-catenin was used to demonstrate that early and late limb muscle progenitors have distinct requirements for β-catenin (Hutcheson et al., 2009).

We also analysed the expression and function of a putative downstream target of canonical Wnt signaling, Pitx2, which in

epithelial cells can act in synergy with Lef1 to promote Lef1 transcription (Vadlamudi et al., 2005). We have shown that *Pitx2* is regulated by the canonical Wnt pathway in the epaxial somite and second branchial arch, but not in the first branchial arch (Figs. 2 and 4). However, in all regions, misexpression of Pitx2 increases the number of myogenic cells (Figs. 2 and 5; Dong et al., 2006).

We previously proposed that canonical Wnt signaling may regulate myogenic differentiation in the developing limb bud. Evidence to support this was the ability of the dominant-negative Lef1 construct to reduce myocyte number as assayed by the expression of MyHC chain when misexpressed in limb cultures (Anakwe et al., 2003). Furthermore, the Wnt antagonist, Sfrp2, reduced myocyte number *in vivo* and *in vitro* (Anakwe et al. 2003). Here, we show that canonical Wnt signaling does not regulate *MyoD* or *Myf5* expression in developing limb buds, indicating that, in contrast to the epaxial musculature of somites, limb myogenesis is not initiated via the Wnt/β-catenin pathway. Therefore, the role of canonical Wnt signaling in the development of the embryonic limb musculature is presently unclear and may occur at later stages of differentiation after myogenic commitment. Alternatively, canonical Wnt signaling may regulate cell proliferation and/or survival.

Inhibition of Wnt signaling in the early cranial paraxial mesoderm results in the premature and ectopic activation of *MyoD* expression (Tzahor et al., 2003). The Wnt inhibition studies reported here in which we show that the expression of *MyoD*, together with *Myf5* and *MyoR* (Fig. 4) is not regulated by canonical Wnt signaling at first appear to be at odds with the previous study. There are two obvious explanations for this discrepancy. First, myogenic cells in the proximal versus distal first branchial arch have distinct molecular signatures reflecting their different origins; the cranial paraxial and lateral splanchnic mesoderm, respectively (Nathan et al., 2008). These distinct regions may be differentially controlled by the canonical Wnt pathway: to date, it has been shown that the distal musculature is regulated by Wnt signaling at early stages (8–10) of development (Nathan et al., 2008). The studies described here focus on the proximal region. Second, the experiments presented here were at a later stage of development (stage 20/21 versus stage 8/10). Regardless of the explanation for the differences, what the data does conclusively show is that once myogenic commitment is initiated within the proximal branchial arch, canonical Wnt signaling is not required to maintain myogenic differentiation.

The Pitx2 promoter contains Tcf/Lef binding sites and expression can be induced by LiCl, which activates the canonical Wnt signaling pathway (Vadlamudi et al., 2005). Our analysis here shows that Pitx2 expression is differentially regulated by Wnt/Lef1 signaling in the different regions of the body: canonical Wnt signaling does control *Pitx2* expression in the epaxial myotome (Figs. 2C and D) and in the second branchial arch mesoderm and ectoderm (Figs. 4W–Y), but is not required for Pitx2 expression in the first branchial arch (Fig. 4W). In the developing limb bud, we have found that RCAS-ΔNLef-VP16 at early stages led to upregulation of Pitx2 in infected forelimb buds (Figs. 4M and N); however, implanting Dkk1-inhibitor beads at HH stage 20/21 did not result in loss of *Pitx2* expression (Fig. 4A). This suggests that migratory muscle progenitors are sensitive to increased Wnt signaling, whereas differentiating myoblasts no longer require Wnt to maintain *Pitx2* expression.

Targeted misexpression was used to examine the function of Pitx2 in somites and limb buds. This showed that Pitx2 is important for primary myogenesis, in particular in the epaxial somite. Both Lef1-VP16 and Pitx2 infection increased the number of myogenic cells (Figs. 1, 2, and 5). The effects observed with Pitx2-En and Pitx2a are highly reminiscent of those seen following the manipulation of Wnt/Lef1 signaling. We considered the following possibilities for how Lef1 and Pitx2 may regulate myogenic cell number. Pitx2 and Lef1 may increase the number of myogenic cells by (a) increasing commitment of premyogenic cells to the myogenic lineage, (b) promoting cell proliferation, and/or (c) decreasing apoptosis. There is no evidence of significant apoptosis within the developing dermomyotome and myotome, thus making the last scenario unlikely. However, the first scenario is consistent with our finding that targeted misexpression of activated or de-repressed Lef1 mutant proteins (ΔN-Lef1-VP16, ΔN-Lef1), i.e., activation of the canonical Wnt pathway, increased the number of cells expressing early myogenic markers (Figs. 1B and D; Supplemental Fig. 1B). Counting the number of mitotic, phospho-histone H3-positive cells in the dermomyotome and myotome suggests that canonical Wnt signaling via Lef1 and Pitx2 also stimulates the proliferation of myogenic progenitor cells by 30% (Fig. 3), which overall leads to a thickened myotome. A previous study showed that Wnt-3a regulates proliferation in HH10 somite explants. These authors also showed that ectopic expression of Wnt-3a in the neural tube *in vivo* results in enhanced proliferation of dorsal/dermomyotomal cells and the subsequent expansion of the dermomyotome and myotome (Galli et al., 2004). Our results are consistent with this and further demonstrate that Lef1 and Pitx2 are the effectors of Wnt signaling cues. Other recent studies have implicated roles for the notch signaling pathway and myostatin in controlling the balance between proliferation and differentiation of secondary myogenic progenitors that invade the primary myotome following the epithelial-to-mesenchymal transition of the central part of the dermomyotome. Mutations led to premature differentiation and thus the depletion of the progenitor pool, resulting in muscle hypotrophy (Manceau et al., 2008; Schuster-Gossler et al., 2007; Vasyutina et al., 2007). Together with the data presented here, this indicates that different pathways are involved in regulating the number of primary and secondary myogenic progenitor cells.

The ability of Pitx2 to increase proliferation, in part by inducing the expression of cell cycle regulators such as cyclin D1 and D2, has previously been shown in cardiac cells and in the Sol8 and C2C12 myogenic cell lines (Kioussi et al., 2002; Martinez-Fernandez et al., 2006). *Pitx2* is also expressed in proliferating satellite cells, the progenitor cells of the adult musculature (data not shown). Therefore, a unifying theme of Pitx2 appears to be to increase proliferation.

Analysis of the *Pitx2* null mouse embryo has shown that in the absence of *Pitx2*, differentiation of the first branchial arch musculature, but not the second branchial arch, is affected (Dong et al., 2006; Shih et al., 2007a). This demonstrated that differential molecular programmes regulate myogenesis in the first and second branchial arch and is consistent with the identification of an enhancer that initiates *Myf5* expression specifically in the second branchial arch (Summerbell et al. 2000). The Dkk1 bead implants (Fig. 4) also seem to uncouple a role for *Pitx2* and myogenic differentiation in the second branchial arch mesoderm.

Overall, this study provides insight into the molecular players downstream of Wnt signaling, which are involved in embryonic myogenesis, and uncovers some striking differences in different muscle groups.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.10.023.

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