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Wnt signalling regulates myogenic differentiation in the developing avian wing

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SUMMARY

The limb musculature arises by delamination of premyogenic cells from the lateral dermomyotome. Initially the cells express *Pax3* but, upon entering the limb bud, they switch on the expression of *MyoD* and *Myf5* and undergo terminal differentiation into slow or fast fibres, which have distinct contractile properties that determine how a muscle will function. In the chick, the premyogenic cells express the Wnt antagonist Sfrp2, which is downregulated as the cells differentiate, suggesting that Wnts might regulate myogenic differentiation. Here, we have investigated the role of Wnt signalling during myogenic differentiation in the developing chick wing bud by gain- and loss-of-function studies in vitro and in vivo. We show that Wnt signalling changes the number of fast and/or slow fibres. For example, in vivo, Wnt11 decreases and increases the number of slow and fast fibres, respectively, whereas overexpression of Wnt5a or a dominant-negative Wnt11 protein have the opposite effect. The latter shows that endogenous Wnt11 signalling determines the number of fast and slow myocytes. The distinct effects of Wnt5a and Wnt11 are consistent with their different expression patterns, which correlate with the ultimate distribution of slow and fast fibres in the wing. Overexpression of activated calmodulin kinase II mimics the effect of Wnt5a, suggesting that it uses this pathway. Finally, we show that overexpression of the Wnt antagonist Sfrp2 and ΔLef1 reduces the number of myocytes. In Sfrp2-infected limbs, the number of Pax3 expressing cells was increased, suggesting that Sfrp2 blocks myogenic differentiation. Therefore, Wnt signalling modulates both the number of terminally differentiated myogenic cells and the intricate slow/fast patterning of the limb musculature.

Key words: Wnt, Limb, Myogenic differentiation, Fibre type, Chick

INTRODUCTION

The limb myogenic progenitors arise from the ventrolateral dermomyotome of the somite in response to signals from the adjacent lateral plate mesoderm (reviewed by Buckingham et al., 2003; Francis-West et al., 2003). Following delamination, the premyogenic cells, which express the transcription factors *Pax3*, *Lbx1* and *Msx1*, migrate towards the distal tip of the limb bud, where they become committed to myogenic differentiation, as shown by the onset of the expression of the myogenic determination helix-loop-helix factors, *MyoD* and *Myf5* (reviewed by Buckingham, 2003; Francis-West et al., 2003). The premyogenic (*Pax3* expressing) and early myogenic cells (*MyoD*/*Myf5* expressing) form the pre-muscle masses, which are loose collections of cells scattered within the subectodermal mesenchyme (Christ and Ordahl, 1995; Amthor et al., 1998).

The onset of myogenic differentiation is repressed by a number of growth factors, allowing the expansion of the premyogenic pool and ultimately the number of terminally differentiated myoblasts within the limb bud. These repressive signals include scatter factor, which is expressed by the mesenchyme, and fibroblast growth factors (FGFs), which are expressed by the apical ectodermal ridge and ectoderm. In addition, bone morphogenetic protein (BMP) signalling from both the ectoderm and mesenchyme plays a repressive role, as demonstrated by the ability of the BMPs to maintain *Pax3* expression in the developing limb bud (Amthor et al., 1998; Scaal et al., 1999; Edom-Vovard et al., 2001). Sonic hedgehog (Shh) also maintains the ventral muscle precursors in an

undifferentiated state, possibly acting via the maintenance of BMP expression (Duprez et al., 1998; Krüger et al., 2001; Bren-Mattison and Olwin, 2002). It is not yet totally clear whether the onset of myogenic differentiation within the limb bud is just a default or passive state following the release of the premyogenic cells from their inhibitory cues, or whether positive inductive factors are needed. However, recent work has suggested that inductive signals from the FGF family are required for differentiation (Marics et al., 2002). Thus, FGF signalling is initially repressive but is later inductive or permissive for myogenic differentiation, emphasizing the complexity of the molecular regulatory network that controls myogenesis in the limb bud.

Myoblasts subsequently coalesce to form the dorsal and ventral muscle masses, which are the template of the future muscles (Schramm and Solursh, 1990). Myoblasts also start to differentiate terminally by switching on the expression of the terminal differentiation factors, the myosin heavy chains (MyHCs). These terminally differentiated myoblasts then fuse, forming multinucleated fibres that can contract (Hilfer et al., 1973; Sweeney et al., 1989). This period of primary fibre development is followed by secondary fibre formation. The secondary fibres align on the surface of the primary fibres, starting at day 7 in the chick embryo, and grow to constitute the bulk of skeletal muscle at birth (Fredette and Landmesser, 1991).

Each muscle is characterized by a unique profile of slow and fast fibre types that will determine how that muscle will function (Miller and Stockdale, 1986a; Miller and Stockdale, 1986b). Fast fibres express one of the fast MyHC isoforms and usually use glycolytic metabolism. They can generate high force but fatigue easily. By contrast, slow fibres use oxidative metabolism and express slow isoforms of the MyHC (Hughes and Salinas, 1999). These fibres contract slowly and are able to maintain a contraction for longer without fatigue.

When and where fibre-type commitment occurs has been a running debate. A recent elegant study in which the somitic precursors of the quail pectoralis muscle were grafted into the equivalent position in a chick host suggested that commitment occurs within the somite (Nikovits et al., 2001). In these studies, the slow/fast patterning of the pectoralis muscle was characteristic of the donor and not the host. Clonal analysis studies have also shown that myogenic cells are heterogeneous in their slow/fast MyHC expression and are committed to their different fibre-type fates by stage 24/25 in the quail (DiMario et al., 1993) (reviewed by Stockdale, 1990). This is in contrast to fate-labelling studies in which individual premyogenic clones were marked with a specific nucleotide tag (Kardon et al., 2002). These studies showed that a single premyogenic cell could give rise to both slow and fast myoblasts in addition to a distinct lineage (endothelial cells). These latter data suggest that environmental cues, presumably within the limb bud, control fibre-type patterning and are consistent with other data in which clones of foetal or satellite myogenic cells were shown to differentiate or to modify their fate when grafted into a new host (Hughes and Blau, 1992; DiMario and Stockdale, 1997; Robson and Hughes, 1999). One way of reconciling this data is to argue that different muscles in the limb can be governed by a different set of signalling interactions. An alternative, and equally plausible, argument is that the premyogenic cells are biased to one fibre-type fate as they leave the somite but that they exhibit plasticity (i.e. that they are not committed) and their ultimate fate is determined or modified by local environmental signals (reviewed by Francis-West et al., 2003).

Factors that specify limb myogenic fibre-type differentiation are unknown. In chick somites and zebrafish adaxial musculature, Shh or hedgehog signalling promotes and is essential for slow fibre-type formation. Therefore, loss of Shh signalling inhibits slow fibre development, whereas excess Shh promotes slow fibre formation (Currie and Ingham, 1996; Blagden et al., 1997; Cann et al., 1999; Lewis et al., 1999; Barresi et al., 2000). However, in the limb bud, Shh does not appear to determine myogenic cell fate but does initially prevent differentiation of a subpopulation of the presumptive slow muscle precursors, maintaining them in a proliferative state and, ultimately, increasing the number of slow fibres (Bren-Mattison and Olwin, 2002).

The role of the Wnt family of secreted factors during limb myogenic development has to date been neglected, yet members of this family initiate myogenic differentiation in the epaxial and hypaxial musculature, substituting for the neural tube and ectodermal signals, respectively (Ikeya and Takada, 1998; Cossu et al., 1996; Tajbakhsh et al., 1998). In addition, overexpression of the Wnt antagonist Sfrp3 blocks myogenic differentiation in mouse somites (Borello et al., 1999). The Wnt family consists of 19 members, which can act through one of three pathways that might depend on the Frizzled receptor profile of the receiving cell – first, through the classical β-catenin pathway, second, through a calcium protein kinase C (PKC)-mediated pathway and, finally, through a novel Jun kinase pathway (reviewed by Church and Francis-West, 2002).

Several members of this family are expressed in the limb, where they control patterning, outgrowth and/or differentiation (reviewed by Church and Francis-West, 2002). *Wnt5a*, *Wnt11* and *Wnt14* are expressed in the mesenchyme, whereas *Wnt4*, *Wnt6* and *Wnt7a* are expressed in the ectoderm, the last of these being restricted to the dorsal surface, where it controls dorsal ventral patterning (reviewed by Church and Francis-West, 2002). In addition, *Wnt3a* is expressed in the apical ectodermal ridge (AER). Therefore, within the limb bud the pre- and differentiating myogenic cells are within range of Wnt signalling, which is thought to propagate over 11-12 cell diameters, from the ectoderm and mesenchyme. Thus, it is possible that, as in the somites, Wnts might regulate myogenic differentiation. Finally, *Sfrp2* is expressed in the migrating muscle precursors in the chick, whereas *Sfrp1* is expressed in the lateral dermomyotome in the mouse, again suggesting that modulation of Wnt signals might control limb myogenic differentiation (Ladher et al., 2000b; Lee et al., 2000). Here, we show by gain- and loss-of-function studies that different members of the Wnt family have distinct effects on limb muscle development, controlling the number of terminally differentiated cells and the number expressing either slow or fast MyHCs. Thus, we identify novel functions of Wnt signalling during limb myogenic differentiation.

MATERIALS AND METHODS

Embryos

Fertilized Ross White chicken eggs were supplied by Poyndon Farm (Goff's Oak, UK) or SPF-free eggs were obtained from Lohman

Tierzucht, Germany. The eggs were incubated at 38±1°C and the embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951).

In situ hybridization

In situ hybridization to whole embryos was carried out as described by Francis-West et al. (Francis-West et al., 1995). cDNA and ribroprobes were made as described previously: MyoD (Lin et al., 1989), cWnt5a (Kawakami et al., 1999) and cWnt11 (Tanda et al., 1995).

Retroviral constructs and culture

Concentrated retroviral stocks and retrovirally infected chicken embryonic cells for grafting were prepared as described by Logan and Francis-West (Logan and Francis-West, 1999). The Wnt3a, Wnt5a, Wnt7a, Wnt14, activated β-catenin, Sfrp2 and dominant-negative Lef1 (ΔLef1) retroviruses are as described previously: Wnt3a, activated β-catenin and ΔLef1 (Kengaku et al., 1998), Wnt5a (Kawakami et al., 1999), Wnt7a (Rudnicki and Brown, 1997), Wnt14 (Hartmann and Tabin, 2001) and Sfrp2 (Ellies et al., 2000). The other retroviruses were constructed in RCAS(BP) and encode *Xenopus* Wnt4, mouse Wnt6, a partial chick Wnt11 cDNA equivalent to the *Xenopus* Wnt11 construct described by Tada and Smith (Tada and Smith, 2000), which acts as a dominant-negative, activated rat calmodulin kinase II (Kühl et al., 2000a), *Xenopus* Dsh, which lacks the PDZ domain (DshΔPDZ) (Tada and Smith, 2000), enhanced-green fluorescent protein (eGFP; Clontech), or in RCAS-L14, which encodes chicken Wnt11 (Tanda et al., 1995).

Retroviral misexpression studies

Grafting of retrovirally infected cells into stage 18-21 limb buds was as described in Francis-West et al. (Francis-West et al., 1999). Stage 19/20 and 21/22 wing bud micromass cultures were prepared as described in Francis-West et al. (Francis-West et al., 1999) except that they were plated in the presence of high titre (>10^8 pfu) RCAS(BP) retroviruses and were cultured in the absence of ascorbate. The micromasses were cultured for three days.

Immunohistochemistry

Embryos were dissected and placed into 20% sucrose in PBS at 4°C. They were embedded in OCT compound (BDH Lab Supplies) and cryosectioned at 15 μm. Micromass cultures were fixed in methanol for 2 minutes and were washed twice for 5 minutes with PBS. Muscle development was analysed using the following primary antibodies diluted in PBS: A4.1025 (1 in 100), which recognizes all terminally differentiated muscle cells and A4.840 (1 in 50), which recognizes cells expressing the slow MyHC isoforms SM3 and SM1 (from the developmental hybridoma bank) (Webster et al., 1988; Hughes and Blau, 1992). The Pax3 antibody (1 in 100) was a gift from C. Ordahl, C. Marcelle and M. Bronner-Fraser, and is described by Baker et al. (Baker et al., 1999). The GAG antibody (1 in 5) is as described in Logan and Francis-West (Logan and Francis-West, 1999). Incubation with the primary antibodies was followed by incubation with horse anti-mouse IgG (γ specific) conjugated to FITC (Vector; 1:400) and donkey anti-mouse IgM (μ specific) conjugated to Cy3 (Jackson; 1:800) for at least 1 hour at room temperature. Cultures and sections were mounted under coverslips with PBS:glycerol (1:9) with 0.1% phenylenediamine as an antifade reagent. They were then viewed and the images were captured using a Leica DMRD microscope and the HiPic32 program. The data was analysed using Student's *t* test.

RESULTS

Correlation of Wnt expression with myogenic differentiation

To determine potential roles of Wnt signalling during limb myogenic differentiation, we analysed the expression of Wnts by whole-mount in situ hybridization and compared their expression, both temporally and spatially, to that of the muscle determination factor MyoD. As the limb myogenic precursors enter and differentiate in the limb bud, they come into contact with the mesenchymal signals Wnt5a and Wnt11. *Wnt5a* is initially expressed throughout the mesenchyme at stage 18, later becoming predominantly confined to the distal tip with lower expression levels proximally (Fig. 1A-C,J-M and data not shown) (see also Dealy et al., 1993; Kawakami et al., 1999). Between stages 25 and 27, *Wnt5a* expression is also found in the central core next to the developing cartilage elements and muscle masses (Fig. 1C,M). By contrast, *Wnt11* is not expressed until after the onset of *MyoD* expression and hence myogenic commitment (Fig. 1D,G). *Wnt11* is first expressed in the proximal dorsal subectodermal mesenchyme

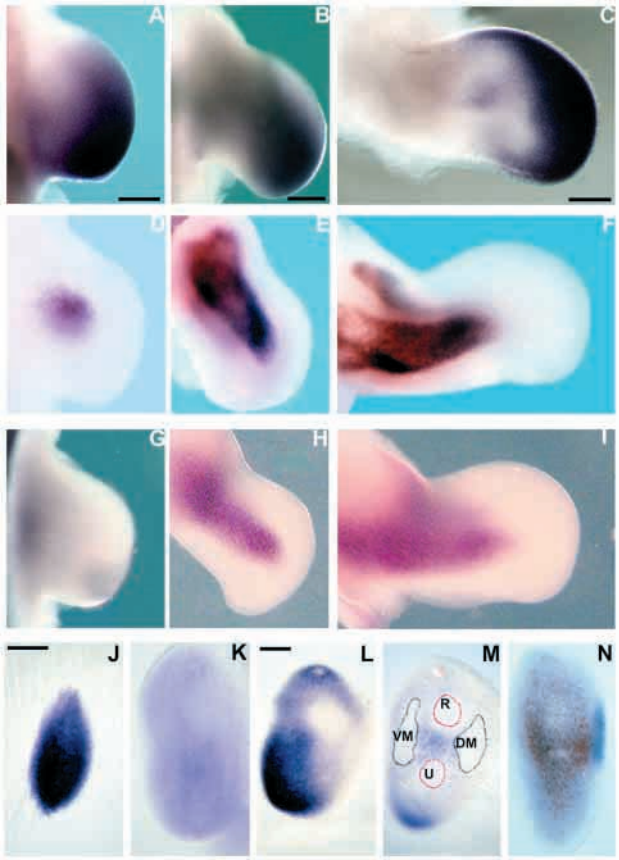

Fig. 1. Correlation of *Wnt5a* and *Wnt11* expression with myogenic differentiation. The expression of *Wnt5a* (A-C,J-M), *MyoD* (D-F,N) and *Wnt11* (G-I,N) in stage 22 (A,D,G), 25 (B,E,H) and 27 (C,F,I) wing buds was determined by whole-mount in situ hybridization. (J-N) Transverse vibratome sections of wing buds expressing *Wnt5a* (J-M) and *Wnt11* and *MyoD* (N) at stages 22 (J,K), 26 (N) and 27 (L,M). In (A-M), expression is shown in purple/red. In (N), *MyoD* expression is shown in red and *Wnt11* expression is shown in purple. In (A-N), anterior is uppermost and, in (A-I), distal is to the right. In the vibratome sections, (J) is more distal than (K), and (L) is more distal than (M). In (M), developing cartilage is circled in red, whereas developing muscle is circled in black. DM, dorsal muscle mass; R, radius; U, ulna; VM, ventral muscle mass. Scale bars: 100 μm in A,D,G; 150 μm in B,C,E,F,H,I; 125 μm in J,K; 150 μm in L,M.

overlying the myogenic cells at late stage 22 in the wing (data not shown) (see Tanda et al., 1995). By stage 24, *Wnt11* transcripts are clearly detectable in the dorsal mesenchyme and are also found in the ventral subectodermal mesenchyme, again overlying the developing myogenic precursors (Fig. 1E,H and data not shown). Between stages 24 and 30, *Wnt11* expression extends distally over the developing myogenic cells and is slightly more advanced on the dorsal side in correlation with the advanced rate of myogenic differentiation (Fig. 1E,F,H,I,N and data not shown). At stage 26, *Wnt14* is also expressed in the mesenchyme around the developing cartilage elements and forming joint regions, where it might influence primary muscle fibre development, which is not yet complete (V.C. and P.F.-W., unpublished) (Hartman and Tabin, 2001).

In addition to the mesenchymal signals, particularly at early stages of differentiation, the myogenic precursors also come into close proximity with ectodermal signals, which are known to influence the rate of myogenic differentiation (Amthor et al., 1998). *Wnt4*, *Wnt6* and *Wnt7a* are expressed in the ectoderm, the latter being confined to the dorsal surface, whereas *Wnt3a* is expressed in the AER (data not shown) (reviewed in Church and Francis-West, 2002). The relationship between the myogenic cells and Wnt expression in a stage 24 wing is illustrated in Fig. 2A. In summary, the premyogenic cells are within range of Wnt signals from the ectoderm and are in contact with or in close proximity to the mesenchymal signals Wnt5a and Wnt11. Overall, the different expression of *Wnt5a* and *Wnt11* between stages 23 and 27 correlates with the ultimate distribution of slow and fast fibres in the primary muscle fibres in the wing. In general, slow fibres are concentrated towards the centre of the limb bud, where *Wnt5a* transcripts are found, whereas the fast fibres are found closer to the ectoderm, where *Wnt11* transcripts are located (Fig. 2B,C).

Wnt signalling affects myogenic differentiation in vitro

To analyse the potential roles of Wnt signalling during myogenic development, we first overexpressed these factors using the replication-competent retrovirus RCAS(BP) in an in vitro micromass culture that recapitulates myogenic differentiation in vivo (Swalla and Solursh, 1986; Archer et al., 1992). In addition, it has the advantage that the effect of Wnts on patterning are uncoupled from their effects on myogenic development. For example, overexpression of Wnt7a dorsalizes the ventral limb, whereas Wnt3a overexpression induces ectopic AER formation and hence additional regions of outgrowth, which would induce secondary changes in muscle patterning and differentiation. Changes in muscle differentiation were assessed by double-labelling using the antibodies A4.1025, which is a pan-MyHC marker, and A4.840, which recognizes the slow MyHC SM3 and an embryonic MyHC, SM1. SM1 is initially expressed by most myogenic cells but is downregulated rapidly in fast myogenic cells while being maintained in slow-MyHC-expressing cells (Webster et al., 1988; Hughes and Blau, 1992). The A4.840 antibody will recognize slow MyHCs present in mixed slow/fast fibres and fibres that exclusively express slow MyHC; for ease of reading, these myogenic cells will be referred to as ‘slow’. To determine the number of myoblasts expressing exclusively fast MyHCs, the number of myogenic cells recognized by the antibody A4.840 was subtracted from the number recognized by the pan-A4.1025 antibody. For each Wnt, at least three independent experiments were carried out, consisting of at least three micromass cultures. Control micromasses were infected with a retrovirus encoding green fluorescent protein (GFP).

Control cultures typically possessed 1060±64 MyHC-expressing cells, of which 94% were mononucleate. As for the control micromass cultures, 92% or greater of MyHC-expressing cells in Wnt-infected cultures were mononucleate (Wnt3a, 100%; Wnt4, 92%; Wnt5a, 96%; Wnt6, 94%; Wnt7a, 99%; Wnt11, 92%; Wnt14, 97%). Overexpression of different members of the Wnt gene family had two distinct effects on myogenic differentiation. First, Wnt signalling could change the number of terminally differentiated myogenic cells. Second, a change in the number of slow and/or fast-MyHC-expressing cells was observed (Figs 3, 4, Table 1).

Wnt5a and Wnt6 had no significant effect on the number of myocytes, whereas Wnt3a significantly decreased the number of terminally differentiated myogenic cells (Fig. 3A,B,D,E, Fig. 4, Table 1). By contrast, Wnt4, Wnt7a, Wnt11 and Wnt14 overexpression increased the number of MyHC-expressing cells (Fig. 3A,C,F-H, Fig. 4, Table 1). These changes in number were linked with different changes in the number of fast and/or slow myocytes. In Wnt3a transfected cultures, the

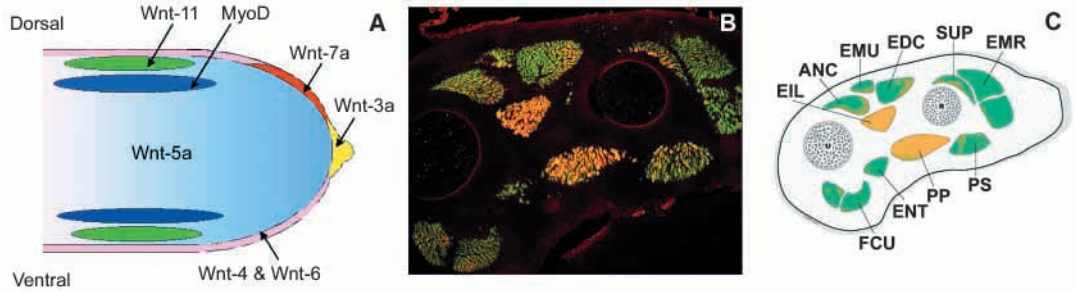

Fig. 2. Correlation of Wnt expression with muscle differentiation. (A) A sketch through the dorso-ventral axis of a stage 24 wing bud showing the relationship between *Wnt* expression and the developing myogenic cells. Initially, *Wnt7a* is expressed throughout the dorsal ectoderm but, by stage 24, its expression is restricted to the dorsal ectoderm overlying the progress zone. (B) Transverse section through an 8-day limb showing the expression of slow MyHC (orange) versus fast MyHC (green). (C) Diagrammatic sketch of (B). ANC, anconeus; EDC, extensor digitorum communis; EIL, extensor indicis longus; EMR, extensor metacarpi radialis; EMU, extensor metacarpi ulnaris; Ent, entepicondyloulnaris; FCU, flexor carpi ulnaris; PP, pronator profundus; PS, pronator superficialis; SUP, supinator.

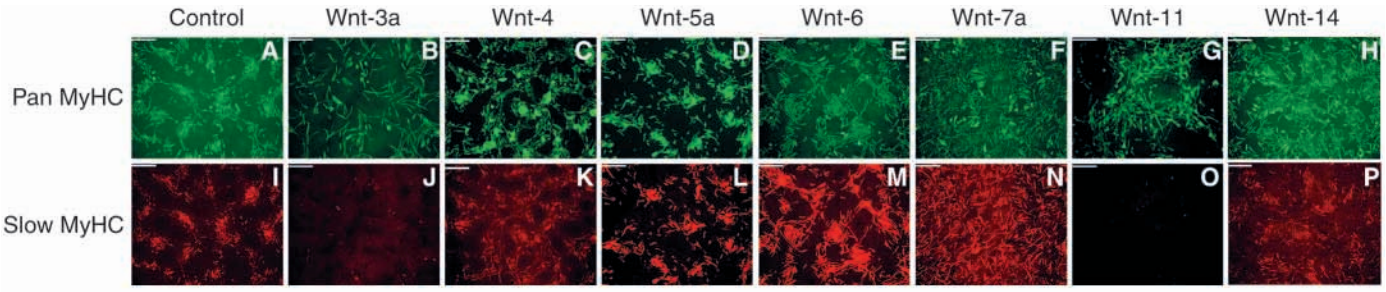

Fig. 3. Effects of Wnt overexpression on fibre-type differentiation in vitro. (A-P) Fluorescent images of stage 21/22 wing micromass cultures showing terminally differentiated myogenic cells that have been visualized with antibody A4.1025, which recognizes both slow and fast MyHCs (A-H, green), and antibody A4.840, which specifically recognizes slow MyHCs (I-P, red). The micromass cultures have been infected with control RCAS(BP) virus (A,I) or retroviruses expressing Wnt3a (B,J), Wnt4 (C,K), Wnt5a (D,L), Wnt6 (E,M), Wnt7a (F,N) Wnt11 (G,O) or Wnt14 (H,P). Scale bars, 100 μm.

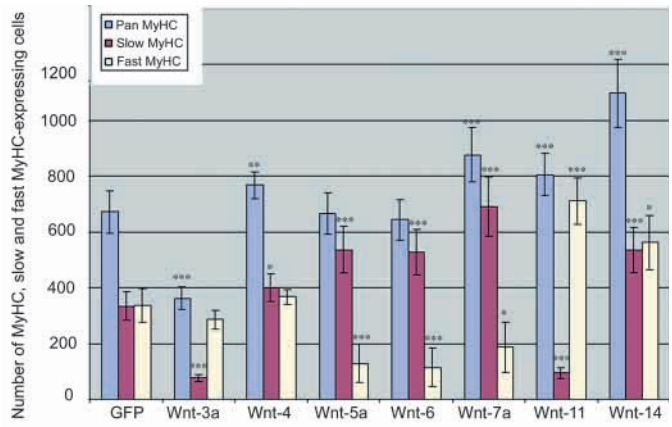

Fig. 4. The effects of Wnt overexpression on fibre-type differentiation in vitro. The bar chart shows the total number of differentiated myogenic cells, and the number expressing fast or slow MyHCs in 3-day limb micromass cultures that have been infected with either a control RCAS(BP) virus or retroviruses expressing Wnt3a, Wnt4, Wnt5a, Wnt6, Wnt7a, Wnt11 or Wnt14, as shown in Fig. 2. The slow population of myoblasts (red) might express either exclusively slow MyHC or both slow and fast MyHCs, whereas the fast myogenic population (yellow) only expresses fast MyHC isoforms. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

Table 1. Summary of the effect of Wnt signalling on limb myogenic differentiation

Protein	Pan MyHC	Slow MyHc	Fast MyHC
Stage 21/22			
Wnt3a	↓<0.001	↓<0.001	–
Wnt4	↑<0.010	↑<0.050	–
Wnt5a	–	↑<0.001	↓<0.001
Wnt6	–	↑<0.001	↓<0.001
Wnt7a	↑<0.001	↑<0.001	↓<0.050
Wnt11	↑<0.001	↓<0.001	↑<0.001
Wnt14	↑<0.001	↑<0.001	↑<0.050
ΔWnt11	↓<0.001	↓<0.050	↓<0.001
β-Catenin	↓<0.001	↓<0.050	↓<0.001
CamKII	↓<0.001	↑<0.001	↓<0.001
ΔLef1	↓<0.050	↑<0.050	↓<0.001
Dsh/ΔPDZ	↓<0.010	↑<0.050	↓<0.001
Stage 19/20			
β-Catenin	↓<0.001	–	↓<0.010
ΔLef1	↓<0.050	–	↓<0.050
Dsh/ΔPDZ	↓<0.001	↓<0.050	↓<0.001

The effect of overexpression or loss of function of the Wnt signalling pathway on myogenic differentiation in stage 21/22 and stage 19/20 micromass cultures.

decrease in the total number of MyHC-expressing cells was linked to a reduction in the number of slow myocytes, whereas the number of fast-MyHC-expressing cells was not significantly changed (Fig. 3A,B,I,J, Fig. 4, Table 1). By contrast, the increase in the number of terminally differentiated cells in Wnt4- and Wnt7a-transfected micromasses was associated with a significant increase in the number of slow MyHC-expressing cells (Fig. 3I,K,N, Fig. 4, Table 1). In the Wnt7a micromasses there was a small reduction in the number of fast myocytes, whereas the number of fast myocytes was unaffected in Wnt4-transfected micromasses (Fig. 4, Table 1). In contrast to Wnt7a, the increase in the number of terminally differentiated myogenic cells in Wnt11-transfected micromasses was linked to an increase in the number of fast-MyHC-expressing cells (Fig. 3A,G, Fig. 4, Table 1). There was also a dramatic decrease in the number of slow MyHC-expressing cells (Fig. 3I,O, Fig. 4, Table 1). In contrast to the other Wnts, Wnt14 increased both the number of slow and fast myocytes (Fig. 3A,H,I,P, Fig. 4, Table 1). Finally, although misexpression of Wnt5a and Wnt6 did not change the number of myocytes, the ratio of fast to slow myocytes was dramatically altered: there was an increase in the number of myocytes expressing slow MyHCs with a simultaneous decrease in the number of terminally differentiated myogenic cells expressing fast MyHCs (Fig. 3A,D,E,I,L,M, Fig. 4, Table 1).

Wnt5a and Wnt11 can modulate fibre-type development in vivo

We next investigated whether misexpression of Wnts can change fibre-type differentiation in vivo, focusing on Wnt5a and Wnt11, which are expressed around the developing muscles as they start to differentiate. These Wnts were also initially chosen because their presence gives dramatic effects on slow/fast differentiation in vitro and their expression correlates with the slow/fast fibre distribution in the wing (Fig. 2A-C). Thus, we misexpressed these Wnts in the developing chick limb using the RCAS(BP) retrovirus. Following in vivo infection between stages 18 and 20, the embryos were

subsequently allowed to develop until day 7 or 8, when they were fixed and the virally infected and unmanipulated limbs were sectioned in parallel to obtain equivalent sections along the proximodistal axis. This route of retrovirus infection resulted in all the muscles being infected with no consistent or obvious dorsoventral or proximodistal bias (Fig. 5A,B) [see also Duprez et al. (Duprez et al., 1996) for further analyses of the rate of viral spread]. The muscles were then analysed for fast and slow MyHC expression in the primary muscle fibres using the A4.1025 and A4.840 antibodies. At this stage, some of the secondary fibres have also formed, using the primary myotubes as a scaffold. These small fibres are easily distinguished morphologically from the much larger primary fibres and were not included in this analysis. The number of terminally differentiated primary myogenic cells and those expressing slow MyHCs was analysed every 75 μm along the proximodistal axis in each muscle in the autopod and zeugopod. The total number of fibres recognized by either of the antibodies in each section was analysed and compared with the control contralateral limb.

This analysis showed that all muscles in the autopod and zeugopod could be affected by misexpression of Wnt5a and Wnt11. Overall, the effect was not as dramatic as that observed in vitro. In general, there were small pockets of ectopic fast or slow fibres found dotted throughout each muscle (arrowed in Fig. 5F; compare with 5E). In control GFP-infected limbs, the number of terminally differentiated fibres and those recognized by A4.840 was not significantly changed compared to the contralateral limb (n=3; data not shown). By contrast and consistent with the effects observed in the micromass cultures, Wnt5a significantly increased and decreased the number of slow and fast MyHC-expressing cells, respectively (n=5, P<0.05) even though the total number of terminally differentiated fibres was not significantly affected (Fig. 5C-F). In Wnt11-transfected limbs and as in vitro, the total number of terminally differentiated cells was significantly increased (Fig. 5G,H; n=3, P<0.05). Again, as in vitro, there was an significant increase and decrease in the number of fast MyHC and slow MyHC-expressing cells respectively (Fig. 5G-J; n=3, P<0.05).

We also tested the effect of Wnt6 and Wnt14, because these members of the Wnt family had a significant effect in vitro. Furthermore, in addition to the earlier expression adjacent to developing muscle cells, *Wnt14* has been shown to be expressed in developing muscle cells at day 15 in the chick embryo (Hartmann and Tabin, 2001). As in vitro, Wnt6 significantly increased the number of slow-MyHC-expressing cells (n=3, P<0.05) while not significantly affecting the total number of terminally differentiated myogenic cells (data not shown). By contrast, Wnt14 did not give a significant effect on muscle development in vivo (n=3, data not shown). We did not test other Wnts such as Wnt3a and Wnt7a as these would give changes in patterning and/or outgrowth, which would have secondary consequences on muscle development in vivo.

Loss of Wnt function alters muscle differentiation

Effect of the secreted Wnt antagonist Sfrp2

The overexpression studies showed that ectopic Wnt signalling can affect myogenic differentiation, although this did not prove that there is an endogenous role in vivo. To further determine the role of Wnt signalling and to confirm the role of endogenous Wnt signalling, we took a loss-of-function approach. We first focused on Sfrp2 because, as we previously

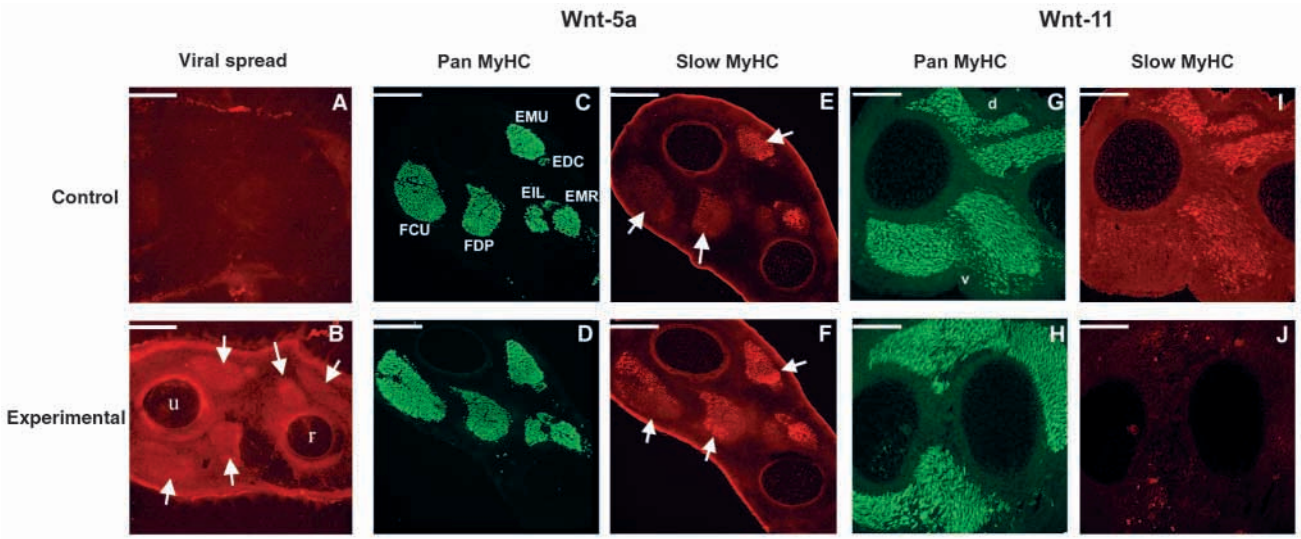

Fig. 5. Effects of overexpression of Wnt5a and Wnt11 on fibre-type differentiation in vivo. Fluorescent images of transverse cryosections of day 8 (A-F) and stage 29 (G-J) chick wings that were infected with retroviruses expressing GFP (B), Wnt5a (D,F) or Wnt11 (H,J) at stage 18-20, and of the contralateral control wings (A,C,E,G,I). Wings have been visualized with antibody A4.1025, which recognizes slow and fast MyHCs (C,D,G,H, green), and antibody A4.840, which recognizes slow MyHC (E,F,I,J, red). (B) The embryo was pathogen free and viral spread has been visualized with an anti-GAG antibody. The arrows in (B) indicate viral spread throughout the limb bud and muscles (compare with A). The arrows in (E) and (F) compare equivalent muscles, showing an increase in slow MyHC expression in the manipulated limb (F). In the Wnt11-transfected limb, there are fewer slow fibres in the dorsal and ventral muscle masses (J) than in the contralateral limb (I). d, dorsal muscle mass; EDC, extensor digitorum communis; EIL, extensor indicis longus; EMR, extensor metacarpi radialis; EMU, extensor metacarpi ulnaris; FCU, flexor carpi ulnaris; FDP, flexor digitorum profundus; R, radius; u, ulna; v, ventral muscle mass. Scale bars: 200 μm in A-D; 100 μm in E-H.

reported, *Sfrp2* is expressed in the migratory premyogenic limb cells and appears to be downregulated as myogenic cells differentiate (Ladher et al., 2000b). Thus, we misexpressed Sfrp2 using the RCAS(BP) retrovirus in the developing limb and in micromass culture.

Following grafting of virally infected cells into stage 18-20 limb buds in vivo, there was a 56% decrease in the number of terminally differentiated myogenic cells and some muscles were reduced to remnants or were entirely absent (Fig. 6A-D, n=3, P<0.001). The percentage of slow-MyHC-expressing cells was similar to that in the controls: 36% in Sfrp2-transfected limbs compared to 41% in the uninfected contralateral limb. Sfrp2 might reduce the number of terminally differentiated muscle cells by changing cell proliferation and/or survival, or it might block myogenic differentiation. To investigate the latter, we determined the number of Pax3-expressing cells (i.e. the number of premyogenic cells) in Sfrp2-transfected limbs and found an average increase of 49% compared with the contralateral control limb (n=3, P<0.05, data not shown). Similarly, Sfrp2 reduced the number of terminally differentiated myoblasts in stage 19/20 micromass cultures (n=9, P<0.001) and, as observed in vivo, the ratio of fast to slow myocytes was not significantly affected (control slow 40%; Sfrp2 slow 43%, data not shown).

Effect of ΔWnt11

We also constructed a retrovirus encoding a truncated Wnt11 protein, which has been shown to act as a dominant negative, to test its effect on myogenic differentiation (Tada and Smith, 2000). Following overexpression of RCAS(BP)/ΔWnt11 in vivo, the number of terminally differentiated cells was not significantly changed, although the limbs appeared much thinner upon fixing (Fig. 6E-H; n=3). Closer analysis showed that the muscles were smaller and the individual fibres were more closely packed than those in the contralateral control limb (compare Fig. 6E and F). The number of fast- and slow-MyHC-expressing myogenic cells was significantly decreased and increased, respectively, which is the opposite of the effect of misexpressing Wnt11 in vivo (Fig. 6G,H, n=3, P<0.05). Similarly, in vitro misexpression of RCAS(BP)/ΔWnt11 significantly reduced the number of fast myocytes in stage 21/22 micromass cultures (n=9, Table 1, P<0.001 and data not shown). However, there was also a small but significant reduction in the number of slow myocytes (Table 1, P<0.0.5, and data not shown). The overall reduction in the number of terminally differentiated cells was not due to fusion of myocytes because all were mononucleate.

Misexpression of intracellular components of the Wnt pathway

To further investigate the role of Wnt signalling during limb myogenic differentiation and to determine the intracellular pathways involved, we misexpressed activated calmodulin kinase II (CamKII), which is implicated in the Wnt5a signal transduction pathway, and β-catenin, which mediates Wnt3a function in the limb (Kengaku et al., 1998, Kühl et al., 2000a; Kühl et al., 2001). In addition, we blocked endogenous Wnt signalling with mutated Lef1 (ΔLef1) and Dsh proteins (DshΔPDZ), which block the β-catenin signalling pathway (Slusarski et al., 1997a; Slusarski et al., 1997b; Kengaku et al., 1998; Kühl et al., 2000a; Kühl et al., 2000b). The mutated Dsh protein also blocks the planar cell polarity pathway, which is activated by Wnt11 signalling. As before, we performed this assay at least three times with at least three micromasses per experiment and determined the number of terminally differentiated myogenic cells and those recognised by the A4.840 antibody. In none of these assays was there a significant change in the percentage of mononucleate cells (GFP, 94%; activated CamKII 95%; ΔLef1, 95%; DshΔPDZ, 96%).

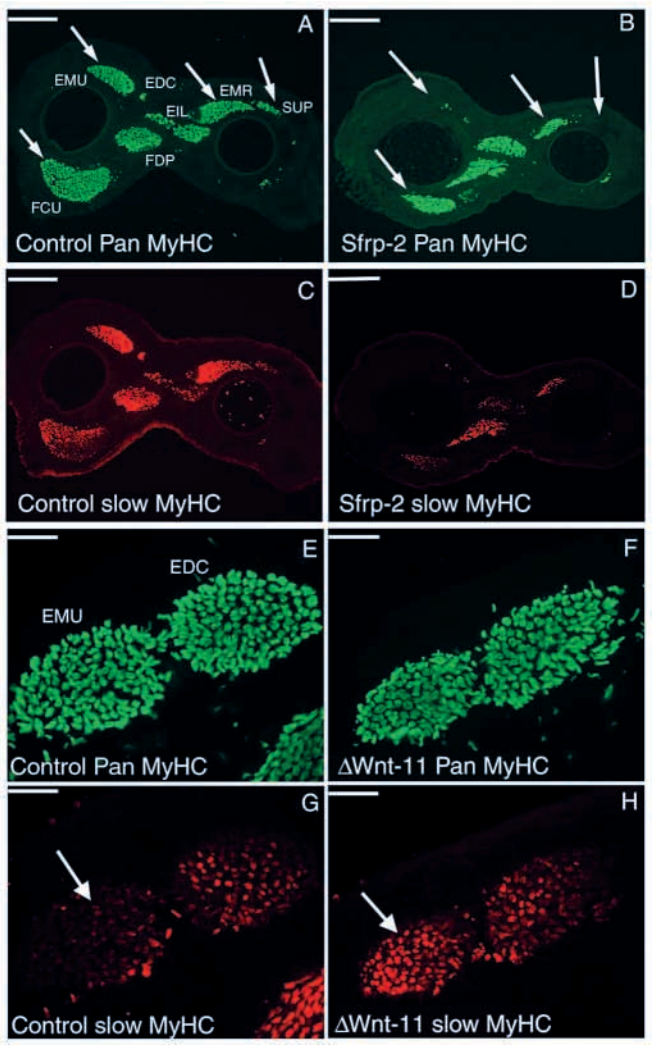

Fig. 6. Effects of loss-of-function of Wnt signalling in vivo. Fluorescent images of transverse cryosections of day 8 chick wings that were infected with retroviruses expressing Sfrp2 (B,D) and ΔWnt11 (F,H) at stage 18-20, and the contralateral control wings (A,C,E,G), which have been visualized with antibody A4.1025, which recognizes slow and fast MyHCs (A,B,E,F, green), and antibody A4.840, which recognizes slow MyHC (C,D,G,H, red). (A-D) Sections through the whole wing. (E-H) High power pictures of the EMU and EDC muscles. The arrows in (A,B,G,H) compare equivalent muscles, showing the changes in muscle development. In (B), the muscles are absent (EMU, SUP) or are decreased in size (EMR, FCU). In H, the EMU muscle has more slow fibres. EDC, extensor digitorum communis; EIL, extensor indicis longus; EMR, extensor metacarpi radialis; EMU, extensor metacarpi ulnaris; FDP, flexor digitorum profundus; SUP, supinator. Scale bars: 200 μm in A-D; 100 μm in E-L.

Overexpression of the activated components in stage 21/22 cultures in general mimicked the effect of the Wnts proposed to signal through them. Thus, activated CamKII promoted slow myocyte formation while decreasing the number of the fast-MyHC-expressing myocytes (Fig. 7A,D,E,H, Fig. 8, Table 1). The latter also resulted in a significant reduction in the number of myocytes, which was not observed in the Wnt5a-transfected cultures (Fig. 3D,L, Fig. 4, Fig. 7D,H, Fig. 8, Table 1). Like Wnt3a, activated β-catenin significantly reduced the number of terminally differentiated myoblasts and those expressing slow MyHCs at stage 21/22 (Fig. 8, Table 1 and data not shown). However, β-catenin also reduced the number of fast-MyHC-expressing cells. A similar result was obtained with stage 19/20 micromasses except that, at this stage, the number of slow myocytes was not significantly changed (Table 1 and data not shown).

Blocking the β-catenin pathway by misexpression of ΔLef1 and DshΔPDZ proteins reduced the total number of myocytes, and this was associated with a significant decrease in the number expressing fast MyHCs (Fig. 7A-C,E-G, Fig. 8, Table 1). By contrast, the number of slow-MyHC-expressing cells was increased (Fig. 7E-G, Fig. 8, Table 1 and data not shown). The morphology of the myocytes in ΔLef1-infected cultures was also affected: they were much smaller and had a rounded appearance, lacking the typical elongated processes, indicating either a delay in development or a requirement for β-catenin signalling in assembly of the myocyte cytoskeleton (compare Fig. 7I,J). A reduction of the total number of myocytes was also observed with stage 19/20 micromasses in ΔLef1- and DshΔPDZ-transfected cultures (Table 1 and data not shown). As at stage 21/22, this reduction was linked to lower numbers of fast myocytes in both cases (Table 1 and data not shown). However, in contrast to the stage 21/22 micromasses, the number of slow myocytes was not significantly affected in the ΔLef1-transfected micromasses and was decreased in the DshΔPDZ-transfected cultures (Table 1 and data not shown).

DISCUSSION

The Wnt family of secreted factors has been shown to be essential for differentiation of the epaxial musculature (reviewed by Buckingham et al., 2003). Here, we have analysed the potential roles of Wnt signalling during limb myogenic differentiation, which to date have been unexplored. We have shown by gain- and loss-of-function studies that members of the Wnt family have distinct effects on myogenic differentiation, controlling the number of terminally differentiated myoblasts and the ratio and/or number of slow and fast myocytes/fibres. Thus, Wnt signalling within the limb bud might initiate the onset of myogenic differentiation as in the somite and, in particular, regulate fibre-type specification and development.

A striking observation in these studies was that modulation of the Wnt signalling pathway changes the number of slow and/or fast fibres both in vivo and in vitro. Of particular importance are the mesenchymal signals Wnt5a and Wnt11, which are expressed adjacent to the developing muscle cells. As premyogenic cells enter the developing limb, they encounter Wnt5a, which is produced throughout the mesenchyme. Later, *Wnt5a* expression becomes predominantly restricted to the progress zone, just distal to the differentiating muscle masses, which are now expressing *MyoD*. However, *Wnt5a* expression is also maintained at higher levels around the developing cartilaginous core adjacent to the developing muscle masses, where the muscles containing most of the slow fibres develop in the chick wing, such as the extensor indicis longus and pronator profundus (Fig. 2B,C). By contrast, *Wnt11* expression is switched on in the subectodermal mesenchyme overlying the developing muscles after the onset of myogenic commitment. Overexpression of these Wnts both in vivo and in vitro had opposing effects, with Wnt5a and Wnt11 enhancing and reducing the number of slow myocytes, respectively. The total number of terminally differentiated cells was relatively unchanged from the controls, suggesting that (as in neural crest development) Wnt signalling acts as a cell fate switch, although this is as yet unproven (Jin et al., 2001). If this was the case, it would be similar to hedgehog signalling in zebrafish adaxial muscle development, in which hedgehog has been proposed to act as a binary switch specifying slow versus fast fibre-type fate (Norris et al., 2000). Whether Wnt5a and Wnt11 act directly on the myogenic cells themselves or signal as a relay via other mesenchymal signals is currently

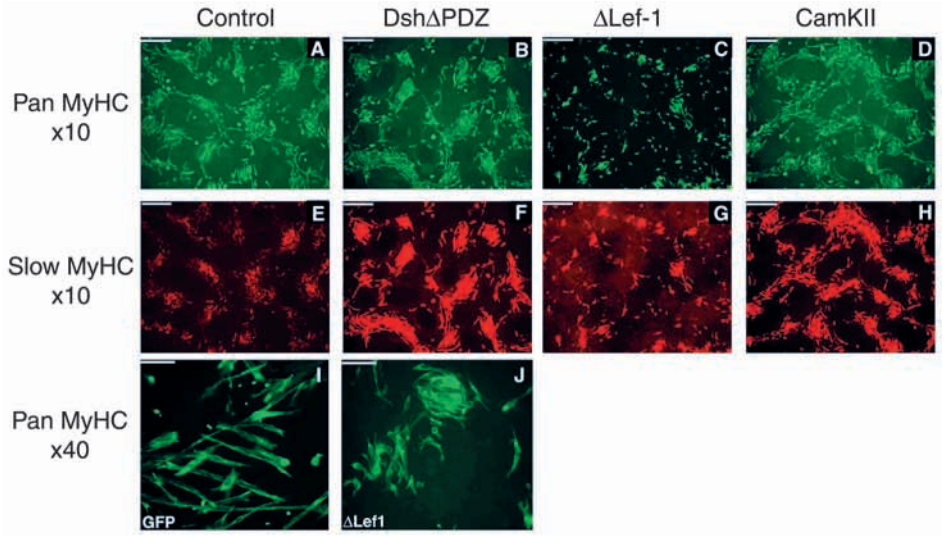

Fig. 7. The effect of overexpression of components and dominant-negative components of the Wnt signalling pathway on fibre-type differentiation in vitro. (A-J) Fluorescent images showing terminally differentiated myogenic cells that have been visualized using antibody A4.1025, which recognizes slow and fast MyHCs (A-D,I,J, green), and antibody A4.840, which recognizes slow MyHC (E-H, red) in stage 21/22 micromass cultures that have been infected with control RCAS virus (A,E,I) or retroviruses expressing DshΔPDZ (B,F), ΔLef1 (C,G,J) or activated CamKII (D,H). Scale bars: 100 μm in A-H; 25 μm in I,J.

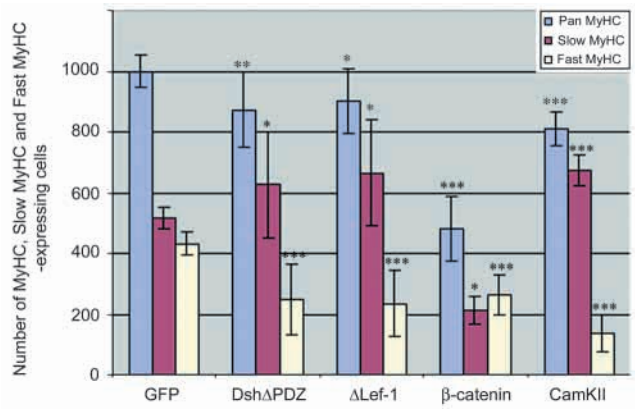

Fig. 8. The effect of overexpression of activated components and dominant-negative components of the Wnt signalling pathway on fibre-type differentiation in vitro. The bar chart shows the total number of differentiated myogenic cells and the number expressing fast or slow MyHCs in 3-day limb micromass cultures that have been infected with either a control RCAS(BP) virus or retroviruses expressing DshΔPDZ, ΔLef, β-catenin or activated CamKII. The slow population of myoblasts (red) might express either exclusively slow MyHC or both slow and fast MyHCs, whereas the fast myogenic population indicated by the yellow bar only expresses fast MyHC. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

unknown but the close proximity of Wnt5a- and Wnt11-expressing cells with developing myogenic cells suggests that the former is highly likely.

In vivo, all of the limb muscles analysed in the zeugopod and autopod could be affected, although none was completely transformed to an exclusively slow or fast fate. This might be related to the variability in and timing of viral spread, such that the Wnts are not misexpressed at a stage when they can affect myogenic differentiation. Alternatively, there might be local extrinsic environmental signals, such as FGFs and BMPs or opposing members of the Wnt family, which modulate the effect of ectopic signalling. Indeed, FGFs have been shown to modulate Wnt activity during limb, otic and neural development in the chick, and BMPs have been shown to affect Wnt regulation of neural crest differentiation (Farrell and Münsterberg, 2000; Jin et al., 2001; Ladher et al., 2000a; Wilson et al., 2001). Furthermore, in *Xenopus*, different members of the Wnt family have been shown to have antagonistic actions (Du et al., 1995; Torres et al., 1996; Kühl et al., 2001). Therefore, in vivo, the effect of Wnt signalling will probably be modulated by other factors.

Wnt5a and *Wnt11* are expressed in very similar domains in the developing leg bud but the ultimate arrangement of slow and fast fibres in the leg is distinct. In the leg, as in the wing, slow fibres are found centrally but they are also found at the periphery (for example, in the sartorius and anterior iliotibialis muscles, which are almost exclusively slow). At first glance, this might suggest that our model is wrong. However, we have found that Wnts have distinct effects in leg and wing micromasses. As in the wing, Wnt5a promotes slow myocyte formation and Wnt11 increases the total number of myocytes (data not shown). However, in contrast to the wing, Wnt11 has no significant effect on the ratio of slow to fast myocytes in the leg (data not shown).

The role of Wnt11 in the regulation of limb fibre-type differentiation was supported by misexpression of ΔWnt11 in vivo, which gave the opposite effect to Wnt11 misexpression: increasing the number of slow fibres while decreasing the number of fast myogenic cells. This shows that endogenous Wnt signalling can change the number of fast and slow fibres. Taken together with the gain-of-function studies, this suggests two possible mechanisms of action. First, Wnt11 signalling might specify myoblasts to a fast fibre-type fate at the expense of slow myoblasts. In this case, Wnt11 would be instructive and it is assumed that all myoblasts are equivalent to respond. This would be consistent with the overall similarity of the numbers seen in the gain-of-function studies. Alternatively, as proposed by others, there might be at least two populations of presumptive myoblasts prespecified to become either slow or fast myocytes (reviewed by Stockdale, 1990). In this scenario, Wnt11 would be permissive for fast myocytes, promoting their differentiation and/or proliferation while inhibiting the development of the slow myoblast populations.

The in vitro data were slightly different but, as in vivo, the number of fast myocytes was decreased, indicating that Wnt11 signalling is required for fast fibre-type differentiation. In contrast to the in vivo data, the numbers of slow myocytes was also slightly decreased, suggesting that endogenous Wnt11 signalling is not inhibitory, and might even be required, for their development, at least in a micromass assay. The same effect on slow myocyte development was observed in stage 19/20 micromasses following overexpression of DshΔPDZ, which blocks both the β-catenin and the JNK pathways, but not following overexpression of ΔLef1, which only blocks β-catenin signalling. This implicates the JNK pathway in the initial regulation of slow myocyte development. A possible explanation for the different effects on slow myocytes in vivo and in vitro is that, as discussed above, limb environmental signals might modulate the effect of Wnt signalling. In micromass culture, these will be different to those present in vivo: the ectodermal signals are absent and this is also associated with the downregulation of mesenchymal signals such as Shh and BMPs (Krüger et al., 2001).

Other members of the Wnt family also changed the number of fast and slow myocytes. In Wnt4- and Wnt7a-transfected micromass cultures, the increase in myogenic cell number was linked to a significant increase in the number of slow myocytes, whereas, in Wnt14-transfected micromasses, there was a significant increase in both slow and fast myocytes. Like Shh, Wnt4, Wnt7a and Wnt14 might delay myogenic differentiation and, in the case of Wnt4 and Wnt7a, have distinct effects on different subpopulations of proliferating myogenic precursors, which would ultimate increase the number of slow and/or fast myocytes (Duprez et al., 1998; Bren-Mattison and Olwin, 2002).

Our results do not resolve the problem of when and where the slow and fast fibre types are specified, nor whether Wnts are acting as permissive or instructive signals. However, they clearly indicate that the number of fast or slow fibres is controlled within the limb bud, as have other recent studies in which Shh has been shown to act selectively on the presumptive slow myoblast population (Bren-Mattison and Olwin, 2002). When and where fibre-type specification occurs is still being debated. The results of Nikovits et al. (Nikovits et al., 2001) have shown that the fibre types are specified within

the somite, at least for the pectoralis muscle, but whether this is true for all limb muscles is currently unclear. Recent fate labelling studies of individual myogenic precursors have strongly suggested that there is no inherent specification of fast and slow muscle precursors as they leave the somite (Kardon et al., 2002) [see Francis-West et al. (Francis-West et al., 2003) for further discussion].

CamKII gave a similar phenotype to Wnt5a, suggesting that, as in *Xenopus*, Wnt5a signals via the PKC pathway in the developing limb bud (Kühl et al., 2000a). Increases in calcium signalling have also been linked to slow fibre formation in adult muscles, suggesting that patterning mechanisms that occur in the distinct adult muscle populations also occur during specification/development of embryonic myoblasts (Chin et al., 1998; Bigard et al., 2000; Delling et al., 2000; Naya et al., 2000; Serrano et al., 2001). Wnt6 had the same effect as Wnt5a, suggesting that Wnt6 might also use the PKC pathway. However, at present, no signalling pathway has been identified for Wnt6, and an equally likely and alternative explanation is that Wnt6 might induce and mediate its effects via Wnt5a expression.

We also found that overexpression of Wnt3a or β-catenin, or blocking the β-catenin pathway with ΔLef1 decreased the number of terminally differentiated myocytes. In addition, misexpression of the Wnt antagonist Sfrp2, which is expressed by uncommitted myogenic precursors, also decreased myocyte number both in vivo and in vitro. The loss-of-function data show that endogenous Wnt signalling determines the number of terminally differentiated cells but does not identify a mechanism. The increase in the number of Pax3-expressing cells observed following misexpression of Sfrp2 in vivo suggests that Wnt signalling is needed for the onset of MRF expression. This proposal is consistent with the data in the embryonic carcinoma cell line P19, in which it has been shown that β-catenin can initiate and is required for myogenic commitment (Petropoulos and Skerjanc, 2002). Furthermore, in somites, overexpression of the secreted Wnt antagonist Sfrp3 blocks myogenesis without affecting *Pax3* expression, suggesting that Wnt signalling acts downstream of Pax3 to induce myogenic commitment (Borello et al., 1999). However, if this proposal is correct, the ligand responsible for this activation is currently unknown. It is unlikely to be Wnt3a, which is restricted to the AER and, in vivo, activates *Fgf8* expression in the ectoderm (FGF8 is an inhibitor of myogenic differentiation) (Kengaku et al., 1998). Wnt3a is also not antagonized by Sfrp2, which must be the candidate molecule that prevents initiation of myogenic differentiation (Ladher et al., 2000b; Lee et al., 2000). An alternative mechanism is that β-catenin might repress myogenic differentiation in the developing limb bud. This has been suggested from studies in the myogenic cell lines L8, C2 and its derivative C2C12 (Goichberg et al., 2001; Martin et al., 2002). However, the situation might be much more complex, with a fine balance of β-catenin signalling regulating myogenic differentiation. For example, it has been found that, in C2 cells, both overexpression and inhibition of β-catenin signalling suppress myogenic differentiation (Goichberg et al., 2001). This complexity is also emphasized by our in vitro data, in which we have found that β-catenin decreases the number of slow myocytes at stage 21/22 but has no effect at stage 19/20. Similarly, blocking β-catenin signalling has distinct effects on slow myocyte development at these two stages. The reasons for this are currently unclear and are under investigation.

Here, we have shown a role for endogenous Wnt signalling during limb myogenic development, showing that Wnts modulate both the number of terminally differentiated myocytes and the number expressing either slow or fast MyHCs. Different members of the Wnt family have very distinct and even antagonistic effects on muscle development. The next challenge will be to dissect out how these opposing effects are mediated and how other signalling factors modulate the effect of Wnt signalling to produce the intricate pattern of slow and fast fibres within each muscle, which is responsible for co-ordinated movement and the maintenance of posture.

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