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Transplacental delivery of the Wnt antagonist Frzb1 inhibits development of caudal paraxial mesoderm and skeletal myogenesis in mouse embryos

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SUMMARY

Axial structures (neural tube/notochord) and surface ectoderm activate myogenesis in the mouse embryo; their action can be reproduced, at least in part, by several molecules such as Sonic hedgehog and Wnts. Recently, soluble Wnt antagonists have been identified. Among those examined only *Frzb1* was found to be expressed in the presomitic mesoderm and newly formed somites and thus its possible role in regulating myogenesis was investigated in detail. When presomitic mesoderm or newly formed somites were cultured with axial structures and surface ectoderm on a feeder layer of C3H10T1/2 cells expressing *Frzb1*, myogenesis was abolished or severely reduced in presomitic mesoderm and the three most recently formed somites. In contrast, no effect was observed on more mature somites. Inhibition of myogenesis did not appear to be associated with increased cell death since the final number of cells in the explants grown in the presence of Frzb1 was only slightly reduced in comparison with controls.

In order to examine the possible function of *Frzb1* in vivo, we developed a method based on the overexpression of the soluble antagonist by transient transfection of WOP cells with a *Frzb1* expression vector and injection of transfected cells into the placenta of pregnant females before the onset of maternofoetal circulation. Frzb1, secreted by WOP cells, accumulated in the embryo and caused a marked reduction in size of caudal structures. Myogenesis was strongly reduced and, in the most severe cases, abolished. This was not due to a generalized toxic effect since only several genes downstream of the *Wnt* signaling pathway such as *En1*, *Noggin* and *Myf5* were downregulated; in contrast, *Pax3* and *Mox1* expression levels were not affected even in embryos exhibiting the most severe phenotypes. Taken together, these results suggest that Wnt signals may act by regulating both myogenic commitment and expansion of committed cells in the mouse mesoderm.

Key words: Myogenic induction, MyoD, Myf5 activation, Wnt, Frzb1

INTRODUCTION

There is increasing evidence for a positive role of Wnt signaling molecules in the activation of myogenesis in amniote embryos (Münsterberg et al., 1995; Stern et al., 1995; Capdevila et al., 1998; Tajbakhsh et al., 1998), but the mechanism of this process remains elusive. At least another molecule, Sonic hedgehog, is also required to initiate myogenesis in the presomitic mesoderm (Borycki et al., 1998). Moreover, Noggin, a target of Wnt signaling, seems to contribute to the process mainly by neutralizing the inhibitory actions of BMPs (Hirsinger et al., 1997; Marcelle et al., 1997; Pourquie et al., 1996).

It has been proposed that Shh and Wnts may initiate myogenesis by activating the myogenic regulatory factor *Myf5* in newly formed somites (Münsterberg et al., 1995); in the mouse, activation of *Myf5* in the dorsomedial lip of the dermomyotome leads to epaxial myogenesis. Studies with explant cultures have shown that other signals from the surface ectoderm, which can be replaced by Wnt7a, appear to preferentially activate *MyoD* in the hypaxial (ventral) domain of somites (Cossu et al., 1996; Tajbakhsh et al., 1998). Interestingly, *Shh*, which is expressed medially, is indispensable for epaxial but not for hypaxial myogenesis (Borycki et al., 1999). Therefore, Wnts together with Shh may contribute to the activation of myogenesis through the activation of *Myf5* or *MyoD* (Tajbakhsh et al., 1998), although, an additional role for Wnts in the maintenance/expansion of the myogenic cell population is also possible.

Recently, putative receptors of Wnts have been identified (Bhanot et al., 1996), both as classic transmembrane proteins, termed Frizzled, and as soluble, presumably secreted

antagonists, differently named by various laboratories (reviewed in Wodarz and Nusse, 1998). The latter molecules are of particular interest for their obvious potential to bind Wnt molecules in the extracellular space and thus prevent their interaction with the target cell.

Attempts to understand ligand-receptor specificity are complicated by lack of solubility of Wnt proteins; much information has been accumulated recently by studying the activation of downstream genes or through biological assays, such as axis duplication, which are induced by certain but not all Wnts and can be enhanced or inhibited by various membrane or soluble receptors. In *Xenopus*, Frzb1 (also known as sFRP3) inhibits axis duplication induced by Xwnt8 and also muscle development (Leyns et al., 1997; Wang et al., 1997; Mayr et al., 1997). In mammalian cells, expression of *SARP1* (also known as *sFRP2*) renders cells resistant to apoptosis while expression of *SARP2* (*sFRP1*) renders cells more susceptible (Melkonyan et al., 1997). The effect of the mammalian equivalent of *Frzb1* (*SARP3/sFRP3*) has not yet been reported. From the current picture, it emerges that in general soluble Wnt binding antagonists inhibit Wnt activity, thus acting as natural dominant negative molecules.

To address the possible role of soluble frizzled-related proteins in regulating myogenesis, we first examined their expression in postimplantation mouse embryos and then focused our study on *Frzb1*, which is expressed in presomitic mesoderm, limb buds and branchial arches at the onset of myogenesis. Here we show that, in vitro, murine Frzb1 inhibits myogenesis in organ cultures of presomitic mesoderm and newly formed somites. When overexpressed in vivo, using a transplacental delivery system, *Frzb1* interferes with the development of caudal paraxial mesoderm and, in less severely affected embryos, an effect on myogenesis can be distinguished.

MATERIALS AND METHODS

Whole-mount in situ hybridization

Embryos were prepared for whole-mount in situ hybridization as previously described (Tajbakhsh et al., 1997). β-galactosidase staining, before the whole-mount in situ hybridization of the *Myf5*^{a2} embryos, in which the *Myf5* locus is targeted with *nLacZ*, was performed as described (Houzelstein and Tajbakhsh, 1998). The following probes were used: *En1* (Acampora et al., 1998), *Pax3* (Goulding et al., 1991), *Mox1* (Candia et al., 1992), *BMP4* (Jones et al., 1991) and *Noggin* (McMahon et al., 1998). Photographs of whole-mount stained embryos were taken with a Leica MZ8 stereomicroscope using color reversal film Ektachrome 64T. Stained embryos were embedded in 7% gelatin/15% sucrose and 30 μm cryostat sections were cut. An average number of 10 embryos were analyzed for each marker.

Embryonic explant cultures

Myf5^{a2} embryos at E9.5 (20-24 somites) were used for these experiments (Tajbakhsh et al., 1996). The explant culture method was as previously described (Cossu et al., 1996; Tajbakhsh et al., 1998).

C3H10T1/2 cells, used as a feeder layer, were transiently transfected with the pcDNA3/Frzb1 vector (a derivative of the invitrogen pcDNA3 vector containing the full cDNA of the murine *Frzb1* gene; Leyns et al., 1997). 2×10^5 cells were transfected with 10 μg of DNA using the pEi (polyethylenimine) method (Boussif et al., 1995).

At the end of the culture period (3 or 5 days), the explants were fixed, stained for β-galactosidase and incubated with an anti-MyoD antibody (Tajbakhsh et al., 1998). The total cell number was inferred by calculating the total DNA content of the isolated explant (Labarca and Paigen, 1980). Sonicated salmon sperm DNA was used as a standard. Acridine orange staining was used to detect apoptotic cells (Graham et al., 1993).

Placenta injection

WOP cells (Without Origin of Polyoma DNA replication, a 3T3 derivative transformed by a replication-defective polyomavirus: Dailey and Basilico, 1985) were transiently transfected (1.8×10^5 cells with 10 μg of DNA, using the pEi protocol) with the pcDNA3/Frzb1 expression vector, with control empty vector, with a pcDNA3/*lacZ* vector or with the pcDNA3/mShhΔ5′UTR vector expressing full-length murine *Sonic hedgehog* cDNA (a gift from A. McMahon). 1 day after the transfection, 2×10^4 cells in 5 μl of PBS were injected into the maternal side of the placenta through the uterine wall, using a syringe with a 30 GA1/2 needle. Mice had been anaesthetized by intramuscular injection of a 3:1 mixture of Ketamine/Xilazyne (Parke-Davies/Bayer) in a volume of 1 μl/g of body weight. Usually one uterine horn of the embryos (at approximate age of E8.5, E8.75; 10-14 somites) was injected. We noticed that injection in both horns requires extensive manipulation of the uterus and this frequently causes abortion. For the same reason, only 5 or 6 placentas were injected in mice carrying many embryos in one horn, thus avoiding injection into the placentas closer to the ovary. The mice survived surgery, and, under these conditions, abortion occurred in about 20% of operated animals. Mice were killed after 2 additional days and embryos were collected and processed for morphological and biochemical analysis.

Immunocytochemistry

Immunocytochemistry on tissue sections and cultured cells was carried out as described (Tajbakhsh et al., 1994) using the following antibodies: anti-MyoD (Koishi et al., 1995), anti-myosin heavy chains (Salvatori et al., 1995), anti-nestin (Tajbakhsh et al., 1994) and anti-PECAM antibody (Santa Cruz Biotechnology).

Immunoprecipitation

To study the transfer of secreted proteins from cells injected into the placenta to the embryo, WOP cells were metabolically labeled for 2 hours with 1 mCi/ml of [^{35}S]methionine (NEN, specific activity 1 mCi/mmol) in complete medium. Labeled cells were injected into the placenta as described above and after 24 hours isolated embryos were collected and the protein extracted as described below. The cleared supernatant was separated on a Sephadex G-50 fine gel filtration column. Radioactivity was eluted in fractions corresponding to molecular mass ranging from 60 to 5 kDa. The supernatant of labeled cells was also separated on the same column and, in this case, radioactivity was eluted in all fractions, from void volume to low molecular mass. In order to detect Frzb1 in embryos following placental injection, an HA-tagged construct was generated and the protein detected by immunoprecipitation. WOP cells were transfected with the pCS2mFrzbHA vector, containing the HA epitope tag between the alanine 159 and the aspartic acid 160, or with the pCS2 vector alone. Pools of eight Frzb1-HA placentally injected or control embryos were collected after 24 or 48 hours and homogenized by passage through a G25 needle in Modified RIPA Lysis buffer (50 mM Tris-HCl pH 7.4, 300 mM NaCl, 10 mM KCl, 1 mM EDTA, 5 mM DTT, 0.25% sodium deoxycholate, 1% Nonidet P40, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml pepstatin, 1 mM PMSF). The homogenate was centrifuged at 2000 ***g*** and the supernatant was diluted to 150 mM NaCl (final concentration). Protein G-agarose (Santa Cruz Biotechnology) was washed with Modified RIPA buffer and incubated with the anti-HA.11 monoclonal antibody (BABCO; diluted at 1:150) for 1 hour at 4°C. The mixture was washed in Modified RIPA buffer

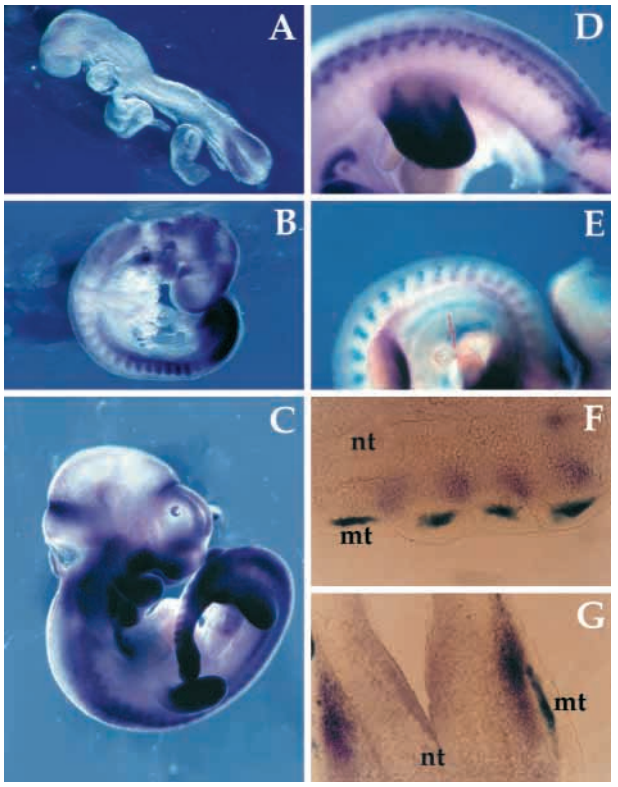

Fig. 1. *Frzb1* is expressed, although not exclusively, in the myogenic areas of postimplantation embryos. Whole-mount in situ hybridization reveals expression (A) in the presomitic mesoderm and neural folds of E8.5 embryos, (B) in presomitic mesoderm and cranial halves of newly formed somites and lateral mesoderm of E9.5 embryos, (C) at mesencephalon/metencephalon and diencephalon/telencephalon junctions, in the limbs and in the mandibular arches of E10.5 embryos and (D) in the mesenchyme adjacent to the neural tube. (E) Analysis of *Frzb1* expression in similarly aged, β-galactosidase stained *Myf5*^{a2} embryos revealed contiguous but non-overlapping areas of expression of *Frzb1* and *Myf5*. This is also illustrated in (F) coronal and (G) transverse sections of the same embryo where purple labeling is *Frzb1* and blue is *Myf5*. nt, neural tube; mt, myotome.

and incubated for 1 hour at 4°C with the experimental samples. The immunoprecipitate was washed 5 times in RIPA buffer, resuspended in SDS loading buffer and run on a 10% SDS-PAGE gel. The Frzb1 protein band was visualized by western blot using the anti-HA.11 monoclonal antibody diluted at 1:500.

RESULTS

Expression of *Frzb1* during somitogenesis

As a first approach to investigate the possible role of soluble frizzled-related proteins in mouse myogenesis, we analyzed their expression pattern in postimplantation mouse embryos, ranging in age from E8.5 (10-12 somites) to E10.5 (35-40 somites). Fig. 1A shows that at E8.5, *Frzb1* is expressed at high levels in the presomitic mesoderm and in the neural folds; at E9.5 *Frzb1* is highly expressed in the presomitic mesoderm and in the cranial half of newly formed somites (Fig. 1B). At E10.5 *Frzb1* is expressed in the dorsal intersomitic space between more mature somites, in the forming limb buds, in the brain

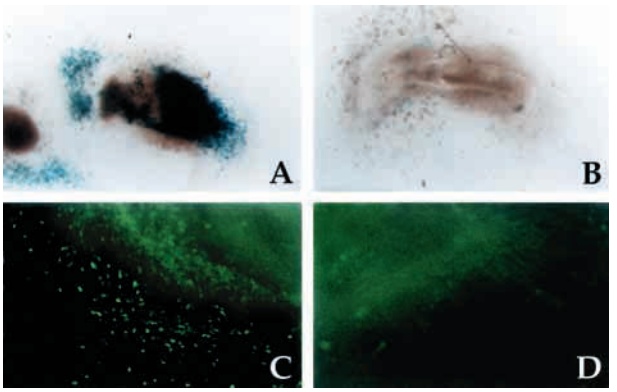

Fig. 2. Frzb1 inhibits expression of both *Myf5* and *MyoD* in vitro. Explants of presomitic mesoderm from E9.5 *Myf5*^{a2} embryos were divided in half along the axis and co-cultured for 3 days (with their half neural tube and surface ectoderm but without lateral mesoderm) (A,C) on control C3H10T1/2 cells or (B,D) on C3H10T1/2 cells expressing *Frzb1*; the cultures were then stained (A,B) for β-galactosidase activity and (C,D) with anti-MyoD polyclonal antibody. (C,D) The large tissue mass shows a background (non nuclear) staining.

and in the branchial arches (Fig. 1C,D). In embryos in which one allele of the *Myf5* locus has been targeted with *nLacZ* (*Myf5*^{a2} embryos; Tajbakhsh et al., 1996), at E10.5, *Frzb1* is expressed in mesenchymal cells in an area adjacent (dorsomedially) to newly formed myotomes (revealed by β-galactosidase staining, Fig. 1E) and this can be seen in sections (Fig. 1F,G). Of the soluble Frizzled-related soluble molecules studied so far (*sFRP1-4*; Hoang et al., 1998; Leimeister et al., 1998; our unpublished results) only *Frzb1* appears to be expressed in premyogenic regions of the mouse embryo. We therefore investigated its possible role in myogenesis.

Frzb1 inhibits myogenesis in explants from mouse somites

A full-length cDNA encoding murine *Frzb1* was cloned in a retroviral vector and recombinant virus was produced in BOSC-23 cells. Initial attempts to infect directly explants of presomitic mesoderm yielded inefficient transduction (≤1% of the population as evaluated by expression of an HA tag). C3H10T1/2 fibroblasts were therefore transfected with a *Frzb1*-expressing vector and used as a feeder layer for the somite cultures.

Explant cultures were prepared from presomitic mesoderm (PSM), somites I-III and somites V-VIII of E9.5 *Myf5-nLacZ* embryos (somite I being the most recently formed; Ordahl, 1993). Presomitic mesoderm with associated axial structures (neural tube/notochord) was mechanically dissociated from the lateral mesoderm and cultured with its contiguous overlaying surface ectoderm. Each portion was divided in two symmetrical halves, following a craniocaudal section along the midline of the neural tube. The left structures were cultured on control C3H10T1/2 fibroblasts while the corresponding right fragments were cultured on *Frzb1*-expressing C3H10T1/2 fibroblasts. We had shown previously that the presence of axial structures and surface ectoderm promotes myogenesis under these conditions (Cossu et al., 1995, 1996). At the end of the culture period, cultures were stained for β-galactosidase activity (which reveals *Myf5*-expressing cells) and with anti-

MyoD antibodies. Fig. 2 shows that many *Myf5*- and *MyoD*-expressing cells were detected in cultures grown on control C3H10T1/2 fibroblasts. In contrast, only a few *Myf5*- or *MyoD*-positive cells (less than 1% of controls) were detected in cultures of presomitic mesoderm grown on C3H10T1/2 fibroblasts expressing *Frzb1*. In cultures of the most recently formed (I-III) somites, myogenesis was also inhibited by Frzb1 but less dramatically. The number of positive cells ranged from 30 to 50% of controls (data not shown). In cultures from older (V-VIII) more anterior somites, inhibition by Frzb1 could no longer be detected (data not shown). In parallel cultures, the total number of cells was measured at the end of the culture period and, in all cases, was found to be similar in explants grown on control C3H10T1/2 fibroblasts and in explants grown on C3H10T1/2 fibroblasts expressing *Frzb1*. In addition, analysis of the extent of apoptosis by acridine orange staining did not reveal significant differences between these cultures (data not shown). In order to test whether differentiation was inhibited and not simply delayed several cultures were grown for 5 or 7 days; also in these cases the number of *Myf5*- or *MyoD*-expressing cells did not increase significantly in explants grown on C3H10T1/2 fibroblasts expressing *Frzb1* (data not shown).

The in vivo effect of Frzb1 on myogenesis and mesoderm development

In order to investigate a possible role for *Frzb1* in regulating myogenesis in vivo, we developed a gain-of-function approach based on transplacental delivery of molecules secreted by cells transfected with the appropriate expression vector and injected into the maternal side of the placenta. For this purpose, WOP cells were transiently transfected with a vector expressing the full-length murine *Frzb1*. Under our conditions, tranfection efficiency ranged from 70 to 90% of the cell population, rendering selection unnecessary. 2 days after transfection 2×10^4 WOP cells were injected through the uterine wall into the maternal side of the placenta of embryos at E8.5-E8.75 (Fig. 3A). About 20 % of injected cells (see Methods) could be recovered from the placenta 2 days after the injection, although there was variability among different placentas injected. Cryostat sections of placentas injected with *lacZ*-expressing WOP cells, revealed numerous β-galactosidase-positive cells predominantly localized to one site (probably the injection site) and a few positive cells at a moderate distance (Fig. 3B). We never observed β-galactosidase cells in the amniotic fluid or in the embryo, suggesting that, under these conditions, WOP cells do not cross the placental barrier. When transfected with a *Frzb1-HA* expression vector, WOP cells secreted high levels of Frzb1-HA protein which could be immunoprecipitated from the corresponding embryos (Fig. 3C). Indeed, gel filtration analysis of radioactive proteins recovered from the embryos, injected with metabolically labeled WOP cells (cultured with [^{35}S]methionine), revealed that proteins smaller than 60 kDa crossed the placental barrier (data not shown).

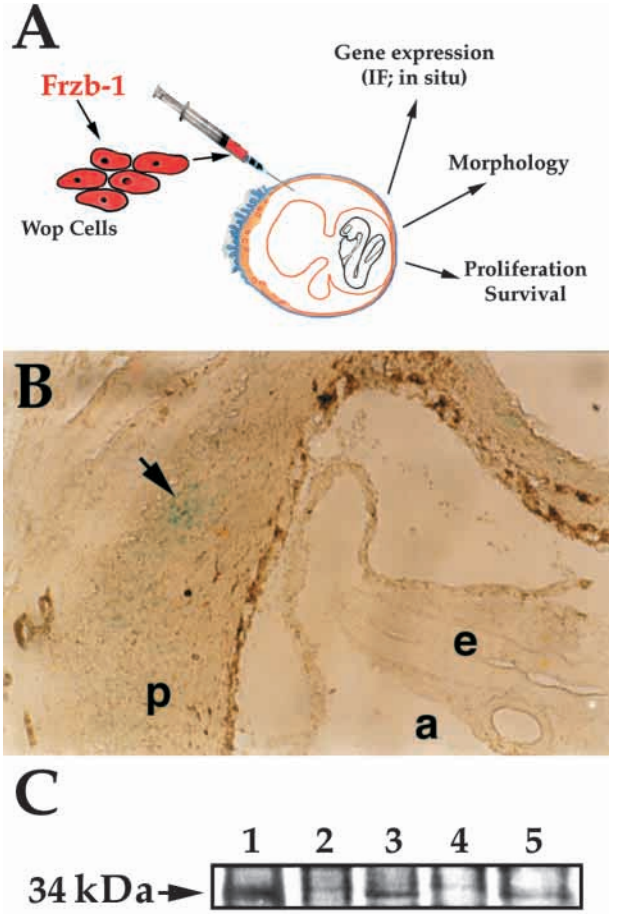

Fig. 3. Transplacental delivery of Frzb1 to postimplantation mouse embryos. (A) WOP cells were transiently transfected with a *Frzb1* expression vector and after one day injected (2×10^4 cells in 5 μl of PBS) into the decidua of E8.5, E8.75 embryos. IF, immunofluorescence. (B) A cryostat section of a placenta 24 hours after the injection of WOP cells previously transfected with an expression vector for the *lacZ* gene, revealed numerous β-galactosidase-positive cells located on the maternal side and occasionally along the internal side of the uterine smooth muscle border, but never inside the embryo or in the amniotic fluid. P, placenta; e, embryo; a, amnion. (C) Embryos from injected and control placentas were isolated 24 hours after the injection of WOP cells previously transfected with an expression vector expressing a tagged (HA) *Frzb1*. Pools of eight injected and control embryos were lysed in RIPA buffer and immunoprecipitated with an anti-HA antibody; the immunoprecipitate was separated on SDS-PAGE, transferred onto nitrocellulose and blotted with the same antibody. A band corresponding to the expected molecular size (arrow) can be detected in the supernatant of transfected WOP cells (lane 1) and in samples of Frzb1-injected embryos after 24 hours (lane 3) but not in the supernatant of control WOP cells (lane 2) nor in samples from mock-injected embryos (lane 4). 48 hours after injection a faint band can still be detected in samples from injected embryos (lane 5).

Embryos that had developed in the presence of excess amounts of Frzb1 protein exhibited growth retardation and severe malformations in the caudal region of the body (Fig. 4). The extent of this phenomenon was roughly proportional to the number of injected WOP cells, ranging from apparently normally developed embryos (less than 10^4 cells) to complete resorption (more than 5×10^4 cells). Injection of cells transfected with the empty vector or with a vector expressing full-length murine *Sonic hedgehog* did not appear to alter embryonic development (data not shown).

Morphological analysis of Frzb1-treated embryos revealed a retarded development with marked reduction of the caudal

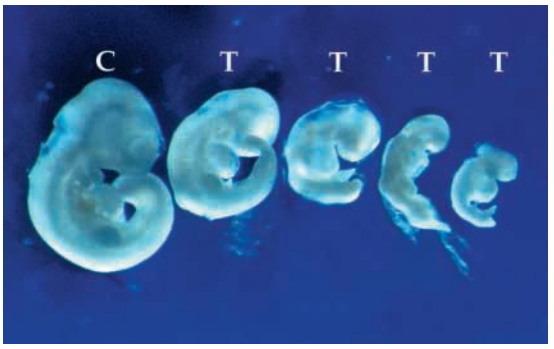

Fig. 4. Frzb1 causes severe malformations of the caudal region of injected embryos. Morphology of control (C) and treated (T) embryos, revealing different severity of malformations in different embryos.

mesoderm. Somites formed in an apparently normal fashion but their number and size were reduced. Immunofluorescence analysis of sections just caudal to the forelimb level revealed that, in moderately affected embryos, the expression of nestin (Fig. 5E), which marks both neurons and the myotome was only slightly affected at both sites whereas, in severely affected ones, it was virtually undetectable (Fig. 5F); skeletal muscle markers such as myosin heavy chains were modestly affected in myotomes of moderately affected embryos (Fig. 5H) and lacking in severely affected ones (Fig. 5I). In contrast, the expression of the endothelial marker, PECAM, was unaffected even in the most severely perturbed embryos (Fig. 5L) where it labeled the vessels that appeared markedly dilated, possibly due to the reduced cellularity of the mesoderm. The epithelial gut and mesonephros appeared less affected or unaffected (data not shown).

In order to monitor whether overexpression of *Frzb1* would specifically interfere with Wnt signaling, we examined injected embryos for the expression of genes whose expression is known to be activated by Wnts. Fig. 6A shows that *Myf5* expression is apparently normal in the cranial somites but cannot be detected in all the somites that had formed caudally to the forelimb in Frzb1-treated embryos. In contrast, *Pax3* (Fig. 6B) or *Mox1* (Fig. 6G) expression levels appear normal in the paraxial mesoderm even of severely malformed embryos. In embryos labeled for both *Pax3* and *Myf5* expression (Fig. 6C), sections immediately caudal to the forelimb revealed expression of both genes (Fig. 6D) while more caudal sections (at an approximate distance of three somites) revealed expression of *Pax3* but not of *Myf5* (Fig. 6E). *En1* is expressed normally in the isthmus and in cranial somites but, much like *Myf5*, is not detectable in the more caudal somites of injected embryos (Fig. 6F).

It has been shown that *Noggin* is one of the target genes of Wnt signaling in presomitic mesoderm and somites (Hirsinger et al., 1997; Marcelle et al., 1997). Much as in amphibians (Zimmerman et al., 1996), Noggin is thought to bind BMP4 and thus neutralize its inhibitory action on dorsal structures of postimplantation mouse embryos. Therefore Frzb1-treated embryos were analyzed for the expression of *Noggin* and *BMP4*. Fig. 7A shows that, in control embryos, *Noggin* is expressed axially and in the dorsal somites; in contrast, expression of *Noggin* is maintained axially but abolished in somites of Frzb1-injected embryos (Fig. 7B). The expression of BMP4 appeared to be unaffected (data not shown).

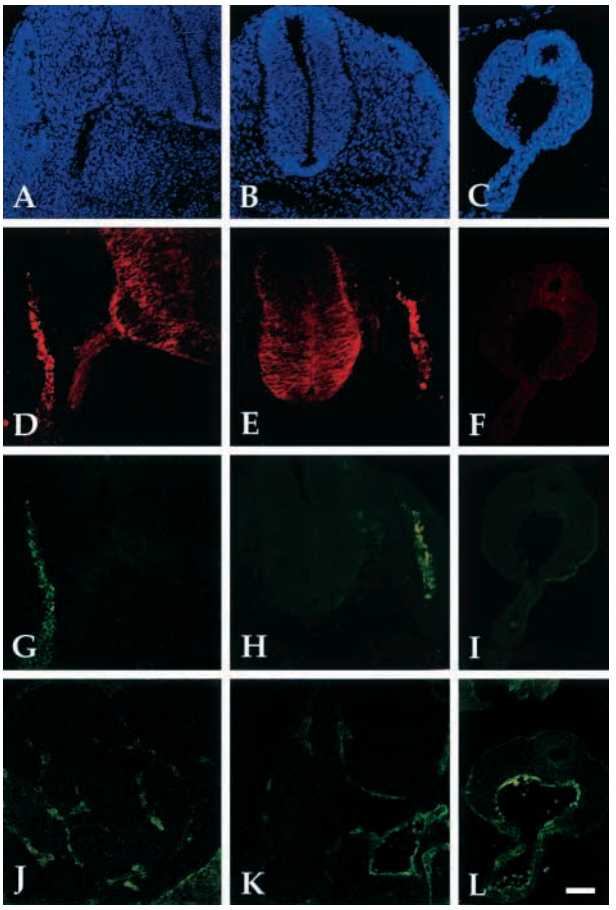

Fig. 5. Frzb1 inhibits expression of differentiation markers in proportion to the severity of induced malformation. Immunofluorescence analysis of (D,G,J) control, (E,H,K) moderately affected and (F,I,L) severely affected embryos stained with antibodies against (D-F) nestin, (G-I) myosin heavy chains and (J-L) PECAM. All sections are at the same level of the interlimb region. Note modest reduction of nestin and myosin expression in moderately affected embryos and complete absence of expression in severely affected ones. In contrast, PECAM is normally expressed in all the embryos. Hoechst staining is shown in A, B and C. Bar corresponds to 50 μm.

DISCUSSION

Frzb1 inhibits myogenesis in vitro

The results reported in this paper show that the Wnt antagonist Frzb1 inhibits the activation of mammalian myogenesis both in vitro and in vivo. We had previously shown that the activation of epaxial and hypaxial myogenesis could be mimicked by Wnt1 and Wnt7a (and to a minor extent also by other Wnts such as Wnt4 and Wnt5a), which can replace the neural tube and the surface ectoderm in activating preferentially *Myf5* or *MyoD*, respectively (Tajbakhsh et al., 1998).

Frzb1, unlike the related genes *sFRP1* (Hoang et al., 1998), *sFRP2* and *sFRP4* (Leimeister et al., 1998) is expressed at high levels in virtually all the premyogenic areas of the embryo, such as presomitic mesoderm, newly formed somites, limb buds and branchial arches (this manuscript and Hoang et al.,

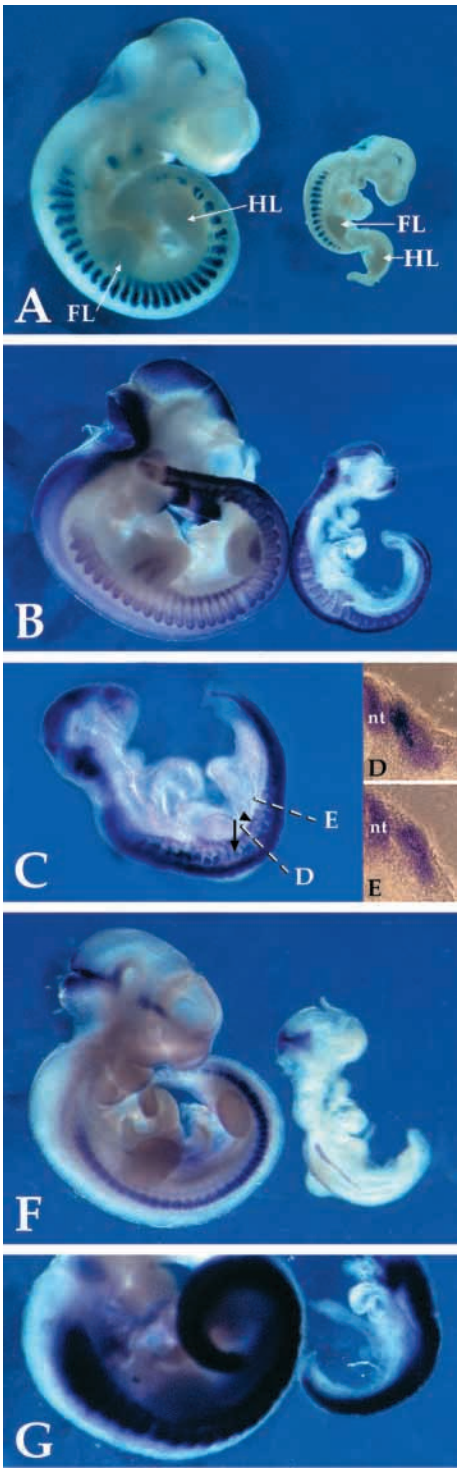

Fig. 6. Frzb1 inhibits expression of *Myf5* and *En1* but not of *Pax3* and *Mox1*. Whole-mount in situ hybridization of control (on the left) and Frzb1-treated (on the right) embryos reveals the expected expression of (A) *Myf5* and (F) *En1* in the head and somites of control embryos; in Frzb1-treated embryos expression is maintained in the brain and in cranial somites but it is lost in more caudal somites. In contrast, expression of (B) *Pax3* and (G) *Mox1* is unchanged in Frzb1-treated embryos. In *Myf5*^{a2} Frzb1-treated embryos, double stained for *lacZ* (arrow) and *Pax3* (arrowhead) expression (C), *Pax3* (purple) and *Myf5* (blue) are expressed in cranial inter-limb somites (insert D) while only *Pax3* can be detected in more caudal ones (insert E). The craniocaudal level of the sections is indicated by dashed lines. FL, forelimb; HL, hindlimb; nt, neural tube.

1998). These findings suggest a possible role for *Frzb1* in regulating skeletal myogenesis. When explants of presomitic mesoderm (with neural tube and surface ectoderm) were cultured on Frzb1-producing fibroblasts, the activation of myogenesis was dramatically (over 90%) inhibited and expression of both *Myf5* and *MyoD* was severely reduced. Under the conditions employed, explants grown on Frzb1-producing cells and on control fibroblasts were a similar size and neither proliferation nor cell death appeared to be significantly altered by Frzb1. The effect observed was less dramatic in explants of newly formed somites and there was virtually no effect in more mature somites. Indeed, overexpression of *Frzb1* does not inhibit differentiation of the established myogenic cell line C2C12 (U. B., G. C., unpublished observations). Together these results suggest that sequestering Wnts mainly results in blocking the consequent activation of myogenic regulatory genes but does not interfere significantly with terminal differentiation.

A novel method allows the study of the effect of Frzb1 in vivo

Information on the phenotype of *Frzb1* null embryos is not yet available. Furthermore, transgenic manipulation is currently complicated by the lack of a suitable promoter expressed at the right time and place to direct overexpression of *Frzb1*. In order to study the possible action of Frzb1 on myogenesis, in vivo, we therefore adapted a method of transplacental delivery described 20 years ago (Fleischman and Mintz, 1979). WOP cells, transfected with a *Frzb1* expression vector, were injected into the maternal side of the placenta where they could be detected up to 2 days after injection. At variance with hemopoietic cells employed in the original work, injected WOP cells remain in the placenta and do not invade the embryo proper or appear in the amniotic fluid.

Frzb1 was secreted by WOP cells and transferred to the embryo where it caused dose-dependent malformations, ranging from slight alterations of somitogenesis and neural tube closure to complete shrinking of the caudal mesoderm. This method allows delivery to the embryo of biologically active extracellular molecules, which are thought to play a role in a given morphogenetic or histogenetic event, by both overexpression of the active molecule or expression of modified molecules with a dominant negative function. The temporal window but not the area of expression can be selected and, in this respect, the method described here differs from a recently reported method that allows direct intraembryonic injection of biologically active molecules (Liu et al., 1998). It should be noted, however, that intraplacental injection is straightforward and does not require instrument-assisted manipulation thus allowing the testing of a given molecule before generating a specific mouse mutant strain.

Given the novelty of the method employed, we determined whether the effect observed was specifically due to overexpression of *Frzb1*. This conclusion is supported by several lines of evidence: first, the absence of any effect when

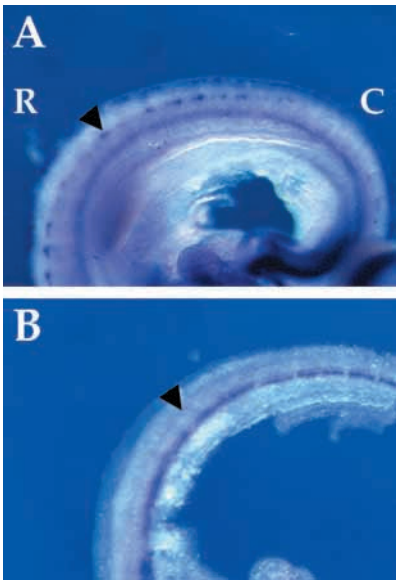

Fig. 7. Frzb1 inhibits expression of *Noggin*. Whole-mount in situ hybridization of (A) control and (B) Frzb1-treated embryos revealing the expected expression of *Noggin* in the axis and dorsomedial edge of somites (arrowhead) of control embryos; in Frzb1-treated embryos expression is maintained axially but it is lost in somites. R, rostral, C, caudal.

cells expressing the empty vector were injected into the placenta (thus ruling out a possible toxicity of the surgical procedure); second, the absence of a teratogenic effect when cells engineered to express *Sonic hedgehog* were similarly injected into the placenta. The lack of an effect due to Shh was unexpected but it may be explained by the specific temporal window selected. Early inductive events caused by Shh probably occur before a significant amount of Frzb1 protein has reached the embryo. This is in agreement with our observation for a requirement of Shh together with Wnt for myogenesis in explants of early (E8.5) presomitic mesoderm (Tajbakhsh et al., 1998). Lastly, we were able to show selective inhibition of genes that are probably downstream of the Wnt signaling pathway, such as *Noggin* (Hirsinger et al., 1997), *Myf5* (Tajbakhsh et al., 1998) and *En1* (Ikeya and Takada, 1998) but not of other genes expressed in the same region of the embryo such as *Mox1* or *Pax3*. *Pax3* is thought to be regulated by surface ectoderm via Wnt signaling (Maroto et al., 1997; Fan and Tessier-Lavigne, 1994), but it is already expressed in the presomitic mesoderm, long before the onset of *Wnt* expression, thus suggesting that, much as in the case of *Shh*, timing may be important. Alternatively, the Wnts that are involved in the expression of *Pax3* in somites may not be inhibited by Frzb1. *Xenopus* Frzb1 has been shown to bind to XWnt8 and Wnt1 (Leyns et al., 1997), but its full spectrum of action has not been determined.

Frzb1 overexpression affects predominantly presomitic mesoderm and inhibits myogenesis in vivo

The effect of Frzb1 appears mainly directed towards mesoderm development, although the neural tube closure appears to be delayed. Indeed, the modest effect on the cranial structures may be due to the fact that, by the time placenta-derived Frzb1 accumulates in the embryo, critical morphogenetic events in brain and face development have already occurred whereas caudal structures are still undergoing critical events such as somitogenesis. The effects of Frzb1 described here on mouse somitogenesis are congruent with the proposed role of Wnts in *Xenopus* muscle development. Microinjection of *Xwnt8* cDNA expressed at gastrula and later stages promotes ectopic muscle differentiation (Christian and Moon, 1993). Conversely, inhibition of Wnt signaling by microinjection of a dominant-negative *Xwnt8* construct (Hoppler et al., 1996), dominant-negative *dishevelled* mRNA (Sokol, 1996) and of *Frzb1* mRNA (Leyns et al., 1997) lead to inhibition of muscle development in the trunk region.

There is variability among Frzb1-treated mouse embryos, ranging from modest morphological alterations and slightly delayed development, to severe malformations of caudal structures. This probably correlates with the number of Frzb1-producing cells that are retained in the placenta, although this is difficult to test. However, there is a correlation between number of cells injected and the severity of the resulting phenotype.

Differentiation is only partly affected in the least severe phenotypes, in which muscle and neural markers are retained, albeit at a reduced level. In the most severe phenotypes, myosin heavy chain and nestin expression is greatly reduced or absent, probably reflecting developmental retardation. Remarkably, blood vessels appear to be unaffected as shown by maintained expression of the endothelial marker PECAM, even in the most severe phenotypes.

A preferential effect on mesoderm suggests that Frzb1 acts, mainly on this tissue, in the strict temporal window of the placenta injection. However, it is possible that redundancy of different Wnts (some of which may not be bound by Frzb1) may explain a less severe effect, much as is observed in the single *Wnt1* or *Wnt3*a knock-out when compared to the double mutation (Ikeya et al., 1997). Gene inactivation of *Wnt5a* (Yamaguchi et al., 1999) results in reduced extension of the A-P axis due to reduced divisions of mesenchymal progenitors; *Wnt3a* knock-out embryos (Takada et al., 1994) have defects in the somites and in tailbud formation. Frzb1-injected embryos show a stronger phenotype than *Wnt3a* null embryos but they still express *Mox1* suggesting that Frzb1 affects already determined mesodermal cells and not their progenitors.

The expression of *Noggin* offers a clear example of the differential effect of Frzb1 on different structures since axial expression is preserved whereas expression in somites is greatly reduced cranially and abolished caudally. In contrast, *BMP4* expression is unchanged in the lateral mesoderm (data not shown). This may result in several consequences: if the balance between Noggin and BMP4 is perturbed, an increased concentration of the latter may contribute to the inhibition of myogenesis (Hirsinger et al., 1997; Marcelle et al., 1997). The persistence of BMP4 compared to a reduced concentration of Wnts, due to the antagonism of Frzb1, may also contribute to increased apoptosis (Schmidt et al., 1998). Indeed preliminary experiments, using TUNEL analysis, have shown that apoptosis is detected in most severely affected embryos (unpublished observations). It is becoming evident that the effect of most signaling molecules on the differentiation of a given tissue is the combined result of different activities on

gene activation, cell proliferation and survival. In the case of the Wnts, their activity on gene activation and on proliferation is well documented: it is therefore not surprising that increased concentration of Frzb1 may cause a marked effect in those tissues that already express high levels of the molecule. It is possible that a fine balance between Frzb1 and Wnts may be required to regulate myogenic determination as well as more generalized effects on growth. This is suggested by the morphology of the somites in Frzb1-treated embryos, where the dermomyotomal cell population appears to be still present and to express *Pax3*.

In conclusion, the novel method described here has allowed us to study the effect of a Wnt antagonist on mouse development in vivo: Frzb1 caused a marked effect on posterior mesoderm, consistent with the effects observed in amphibia with different methodologies. The inhibition of myogenesis by Frzb1, in vitro and in vivo, suggests that Wnts may regulate this process by a direct effect on gene activation. While we will apply this method to the study of other molecules active in the signaling of *Wnt* genes, inactivation studies for Wnt antagonists should confirm and extend the current scenario of Wnt action on early steps of vertebrate myogenesis.

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