Wnt4 activates the canonical β-catenin pathway and regulates negatively myostatin: functional implication in myogenesis

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Bernardi H, Gay S, Fedon Y, Vernus B, Bonnieu A, Bacou F. Wnt4 activates the canonical β-catenin pathway and regulates negatively myostatin: functional implication in myogenesis. Am J Physiol Cell Physiol 300: C1122–C1138, 2011. First published January 19, 2011; doi:10.1152/ajpcell.00214.2010.—Expression of Wnt proteins is known to be important for developmental processes such as embryonic pattern formation and determination of cell fate. Previous studies have shown that Wnt4 was involved in the myogenic fate of somites, in the myogenic proliferation, and differentiation of skeletal muscle. However, the function of this factor in adult muscle homeostasis remains not well understood. Here, we focus on the roles of Wnt4 during C2C12 myoblasts and satellite cells differentiation. We analyzed its myogenic activity, its mechanism of action, and its interaction with the anti-myogenic factor myostatin during differentiation. Established expression profiles indicate clearly that both types of cells express a few Wnts, and among these, only Wnt4 was not or barely detected during proliferation and was strongly induced during differentiation. As attested by myogenic factors expression pattern analysis and fusion index determination, overexpression of Wnt4 protein caused a strong increase in satellite cells and C2C12 myoblast differentiation leading to hypertrophic myotubes. By contrast, exposure of satellite and C2C12 cells to small interfering RNA against Wnt4 strongly diminished this process, confirming the myogenic activity of Wnt4. Moreover, we reported that Wnt4, which is usually described as a noncanonical Wnt, activates the canonical β-catenin pathway during myogenic differentiation in both cell types and that this factor regulates negatively the expression of myostatin and the regulating pathways associated with myostatin. Interestingly, we found that recombinant myostatin was sufficient to antagonize the differentiation-promoting activities of Wnt4. Reciprocally, we also found that the genetic deletion of myostatin renders the satellite cells refractory to the hypertrophic effect of Wnt4. These results suggest that the Wnt4-induced decrease of myostatin plays a functional role during hypertrophy. We propose that Wnt4 protein may be a key factor that regulates the extent of differentiation in satellite and C2C12 cells.

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of the transforming growth factor-β (TGF-β) superfamily, negatively regulates muscle mass via noncanonical Wnt signaling pathways (71). Mstn is an important regulator of skeletal muscle development and adult homeostasis. Naturally occurring mutations, as well as experimental knockout of the Mstn gene, lead to hypermuscular phenotype (30, 50, 83). Later studies have subsequently established that Mstn regulates the size and the number of muscle fibers by inhibiting myoblast proliferation and differentiation (29, 41, 66, 77). In this context, it has been shown that, among the Wnt genes, Wnt4 was the most responsive to Mstn, both on differentiation of human bone marrow-derived mesenchymal stem cells (25, 26) and on postnatal skeletal muscle growth and hypertrophy (71). Despite these advances, the details on the interactions between the Mstn and Wnt4 pathways remained to be specified.

In this study, by expression profiling we showed that satellite and C2C12 cells express only a few Wnts at proliferative stage, and among these, only Wnt4 was strongly induced at the early step of differentiation. Next, we examined the myogenic effects of Wnt4 by modulating its expression levels during C2C12 and satellite cell differentiation. Our results, based on Wnt4 overexpression and Wnt4 small interfering RNA (siRNA)-mediated inhibition experiments, show that Wnt4 has a hypertrophic myogenic activity and that this effect is only observed when cells are treated while proliferating. Interestingly, we found that in the context of myogenesis, although it is classified usually as a noncanonical Wnt, Wnt4 modulates the activity of canonical signaling. In addition, we showed by semiquantitative RT-PCR (sqRT-PCR)-based Mstn expression and Mstn fusion promoter approaches that Wnt4 inhibited Mstn expression. Furthermore, experiments using gene reporter specific for the Mstn/Smad signaling indicate an inhibitory effect of this pathway by Wnt4. Finally, we found that recombinant Mstn was sufficient to antagonize the differentiation-promoting activities of Wnt4. Reciprocally, we also found that the genetic deletion of Mstn renders the satellite cells refractory to the hypertrophic effect of Wnt4. These results suggest that the Wnt4-induced decrease of Mstn plays a functional role during hypertrophy.

Taken together, our results indicate that Wnt4 is a strong positive modulator of myogenesis that could interact with Mstn.

MATERIALS AND METHODS

Cell culture products. Dulbecco’s modified Eagle’s medium (DMEM), nutrient mixture F-12 (Ham), nutrient mixture F-10 (Ham), and horse serum were purchased from Sigma. Fetal calf serum (FCS) was from Hyclone. Recombinant Mstn was purchased from R&D Systems.

Cell cultures. The murine skeletal muscle cell line C2C12 was obtained from the American Type Culture Collection (ATCC CRL1772). C2C12 myoblasts were cultured according to Navarro et al. (53). To achieve differentiation, myoblasts grown for 4 days in growth medium (GM) were switched at 80% confluence to low serum differentiation medium (DMEM) containing 0.5% FCS. Cells were obtained by transfection with pcDNA3.1 expression vector (kindly provided by A. Kispert). Cells were transfected using DreamfectGold transfection reagent (Promega). C2C12 myoblasts were used for subcloning into pcDNA3.1 expression vector (Invitrogen). A Kozak consensus sequence (GCCACCATG) was inserted in the 5′ untranslated cDNA region. C2C12 myoblasts were transfected using JetPEI transfection reagent (Polyplus Transfection) with 1 μg of mouse Wnt4-pcDNA3.1. C2C12 cells were selected in the presence of 1 mg/ml G418 (Life Technologies) for 2 wk and used as polyclonal cell lines. Control cells were obtained by transfection with pcDNA3.1 expression vector.

Wnt4 inhibition. For the RNA interference experiment, the target region for Wnt4 (5′-aatacttcagctgatcagc-3′) was chosen according to Davies et al. (18). As a negative control, siRNA luciferase (5′-aatcgaagtattccgcgtacg-3′) was generated. siRNAs were synthesized by in vitro transcription using T7 Ribomax RNA interference system (Promega). C2C12 myoblasts were plated at a density of 2 × 10^4 cells/cm^2 and transfected 24 h later using HiPerfect transfection reagent (Qiagen) with 450 ng siRNA. Satellite cells were transfected using HiPerfect transfection reagent with 150 ng of siRNA at D3, D2, and D4.

Reporter assays. TOPflash reporter system was used to measure Tcf/LEF-dependent transcriptional activity. C2 myoblasts and satellite cells were transfected using DreamfectGold transfection reagent as described by the supplier (OZBiosciences) with a total

PCR analysis of tail DNA. In vitro studies were performed on 4- to 6-wk-old male mice.

Primary cultures were prepared from mice (our own breeding colony). All animals were treated in accordance with institutional and national guidelines. Satellite cells were isolated from the whole muscles of the paw according to Descamps et al. (19). Cells were plated at a density of 2 × 10^6 cells/cm^2 on Matrigel-coated Petri dishes (BD Biosciences) in 80% Ham’s F10 and 20% horse serum containing glutamine, penicillin, and amphotericin B (Invitrogen). They were maintained at 37°C in a water-saturated atmosphere containing 5% CO2 in air. After 2 days (D2), cells were washed with Ham’s F10 and placed in complete medium supplemented with 5 ng/ml basic fibroblast growth factor [bFGF]; GM). To achieve differentiation, satellite cells grown for 4 days in GM and then switched to DM consisting of Ham’s F10 and 20% horse serum without bFGF. RNAs were collected at various time intervals after differentiation.

sqRT-PCR. Total RNA was prepared by using RNeasy (Qiagen), according to the manufacturer’s instructions. Genomic DNA from all samples was removed with the DNA-free DNase digestion kit (Ambion) according to the manufacturer’s protocol. Ribosomal 18S and 28S from purified RNA were analyzed by electrophoresis on agarose gels, visualized under ultraviolet illumination, and quantified with ImageJ software. This analytical step associated to the measurement of optical density at 260 nm allowed an accurate determination of the RNA concentration.

First-strand cDNA was synthesized from 1 μg total RNA by AMV reverse transcriptase (Promega) in the presence of 500 ng oligo dT15 primer. A negative control without reverse transcriptase was included in each first-strand cDNA synthesis (19). Estimation of the yield of first-strand cDNA synthesis was made by PCR using GAPDH primers on a Bio-Rad Mini-Opticon thermocycler.

Briefly, one-tenth of the cDNA was used in each PCR using Promega GoTaq polymerase. Ethidium bromide intensity of agarose electrophoretic bands was quantified with ImageJ software in the linear amplification range determined from 30 to 35 PCR cycles amplification. Primers sequences and PCR conditions are described in Tables 1 and 2.

Wnt4 overexpression. Mouse Wnt4 cDNA was used for subcloning into pcDNA3.1 expression vector (Invitrogen). A Kozak consensus sequence (GCCACCATG) was inserted in the 5′ untranslated cDNA region. C2C12 myoblasts were transfected using JetPEI transfection reagent (Polyplus Transfection) with 1 μg of mouse Wnt4-pcDNA3.1. C2C12 cells were selected in the presence of 1 mg/ml G418 (Life Technologies) for 2 wk and used as polyclonal cell lines. Control cells were obtained by transfection with pcDNA3.1 expression vector.

Satellite cells were transfected using DreamfectGold transfection reagent (OZ Biosciences) with 1 μg of psp64-Wnt4-HA-pcDNA3 (p645) expression vector (kindly provided by A. Kispert). Cells were transfected at day 3 (D3) for 8 h, medium was renewed, and transfected cells were switched to DM at day 4 (D4). Control cells were obtained by transfection with pcDNA3.1 expression vector.

Wnt4 inhibition. For the RNA interference experiment, the target region for Wnt4 (5′-aatacttcagctgatcagc-3′) was chosen according to Davies et al. (18). As a negative control, siRNA luciferase (5′-aatcgaagtattccgcgtacg-3′) was generated. siRNAs were synthesized by in vitro transcription using T7 Ribomax RNA interference system (Promega). C2C12 myoblasts were plated at a density of 2 × 10^4 cells/cm^2 and transfected 24 h later using HiPerfect transfection reagent (Qiagen) with 450 ng siRNA. Satellite cells were transfected using HiPerfect transfection reagent with 150 ng of siRNA at D3, D2, and D4.

Reporter assays. TOPflash reporter system was used to measure Tcf/LEF-dependent transcriptional activity. C2 myoblasts and satellite cells were plated out in 24-well plates at a density of 1 × 10^4 cells/cm^2 in GM. After 24 h for C2 myoblasts and 72 h for satellite cells, cells were transfected using DreamfectGold transfection reagent as described by the supplier (OZBiosciences) with a total
Table 1. Wnts primer sequences and PCR conditions

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F, forward primer; R, reverse primer. All primers were purchased from Eurofins, MWG Operon, Germany. See text for more details.

of 1 μg total DNA composed from 100 ng Super8x TOPFlash or Super8x FOPFlash representing the background transcriptional activity (courtesy of R. Moon). PRL-TK (125 ng) (thymidine kinase promoter driving Renilla luciferase, as a control for transfection efficiency) and p645 (775 ng) (+Wnt4); or PRL-TK (125 ng) and pcDNA3 (775 ng) (−Wnt4) vectors were used. Cells were reincubated for 20 h in GM and then switched to DM supplemented or not with recombinant Mstn. Cells were harvested 72 h posttransfection in Passive Lysis Buffer (Promega), and activities of firefly and renilla luciferases were measured sequentially using the Dual-Luciferase Reporter Assay system (Promega) in a Synergy2 BioTek luminometer. Activities were expressed in relative light units that were normalized to the transfection efficiency by using pRL-TK vector. LiCl (Sigma) or 6-bromo-indirubine-3'-oxime (BIO, Sigma) were added directly 1 day before induction of differentiation and again 24 h later when the cells were provided with DM.

Mstn-induced transcription was measured by a luciferase reporter gene assay using the GDF-8-responsive, SMAD2/3-dependent pGL3-CAGA construct (CAGA reporter). Experimental procedure was the same as described for TOPflash activity, except that cells were transfected with 1 μg of total DNA composed of 100 ng CAGA, 400 ng Wnt4 expressing vector (p645), and 100 ng of pRL-TK. The total amount of DNA used for each plate was normalized with the empty expression vector pcDNA3.

Activity of Mstn promoter was measured with MS-1177wt-luciferase construct, which contains 1177 bp of the murine Mstn gene assay using the GDF-8-responsive, SMAD2/3-dependent pGL3-CAGA construct (CAGA reporter). Experimental procedure was the same as described for TOPflash activity, except that cells were transfected with 1 μg of total DNA composed of 100 ng CAGA, 400 ng Wnt4 expressing vector (p645), and 100 ng of pRL-TK. The total amount of DNA used for each plate was normalized with the empty expression vector pcDNA3.

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(ClaraVision, Orsay, France) using a Kappa DX20 digital CCD camera connected to a Zeiss Axiosphol microscope.

Fusion index was determined by counting the number of nuclei in differentiated myotubes and expressed as a percentage of the total number of nuclei (mononucleated and multinucleated cells). For each experimental situation, 1,000 nuclei per dish were counted in three independent cultures. To estimate myotube size, the diameter of at least 150 myotubes was measured using PerfectImage software. The average diameter per myotube was calculated as the mean of three measurements taken along the length of the myotube.

**Western blot analyses.** Hundred micrograms of total protein was separated by reducing SDS-PAGE and transferred onto nitrocellulose membrane. The membrane was blocked for 2 h at 20°C with SuperBlock T20 (Pierce), followed by overnight incubation with primary antibody at 4°C, and finally incubated for 1 h with secondary antibody conjugated with horseradish peroxidase (1:10,000). Antibody dilutions were as follows: myogenin (1:500; 556358, BD Biosciences), MyoD (1:500; IMG-132, IMgenex), tubulin (1:1,500, Sigma), or MyoD (1:500; 85). Detection was done using enhanced chemiluminescence system (Super signal West, Pierce). The density of the bands was quantified by densitometric analysis using ImageJ System.

**Statistical analysis.** Results are expressed as means ± SE. Experimental conditions were compared with controls by the Mann-Whitney test. When experimental designs included one factor and more than two experimental conditions, one-way ANOVA was conducted after testing standard assumptions. Statistical significance was set at P values <0.05.

**RESULTS**

**Expression profiles of Wnt4 during C2C12 and satellite cells differentiation.** Expression of the 19 mouse Wnt genes was studied by sqRT-PCR throughout the differentiation process of C2C12 and satellite cells. We found that only a few Wnts were expressed in proliferative and differentiated cells (Fig. 1).

Among the induced Wnts, Wnt4 showed the most striking expression during myogenesis. Wnt4 was barely detected during proliferation and was strongly induced during differentiation in both C2C12 myoblasts (5-fold increase) and satellite cells (39-fold increase). Under our culture conditions, Wnt4 expression peaked 96 h after switch to DM for C2C12 myoblasts and 48 h for satellite cells. However, Wnt4 expression did increase at D0, the day of switching from GM to DM (C2C12: 1.6-fold induction; satellite cells: 7-fold induction). This can be explained by the fact that at this stage, cells start to be aligned and to express myogenin (85).

Taken together, these results showed that during differentiation, Wnt family can be divided into three classes. The first class corresponding to an increase in Wnt expression (C2C12: Wnt4 and Wnt9a; satellite cells: Wnt4, Wnt5a, and Wnt9a), the second class corresponding to a stable expression (C2C12: Wnt5b, Wnt10b, and Wnt11; satellite cells: Wnt11), and the third class corresponding to a decrease in Wnt expression (Wnt10b for satellite cells). The fact that Wnt4 expression was induced in satellite and C2C12 cells during early myoblast differentiation and strongly increased thereafter suggested that this factor was closely related to the engagement to the myogenic differentiation.

**Effects of Wnt4 overexpression on myogenic differentiation.** C2C12 cells were chemically transfected with Wnt4 plasmid containing the neo-resistant gene. The cells were cultured in a medium containing G418 to expand the G418-resistant clones. During the selection period in GM, myoblasts differentiated spontaneously and formed large myotubes in some spotted

**Table 2. Primer sequences and PCR conditions**

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F, forward primer; R, reverse primer. All primers were purchased from Eurofins, MWG Operon, Germany. See text for more details.
areas. SqRT-PCR analysis of RNA extracted from these zones showed a fivefold Wnt4 expression increase compared with control (Fig. 2A). After being switched to DM, Wnt4 expression increased in control cells (threefold) and further increased by threefold in the areas where Wnt4-transfected cells exhibit a spontaneous differentiation pattern. However, because of this Wnt4-related strong spontaneous differentiation effect, we were not able to isolate and further maintain these cellular clones and, except when specifically notified, further studies were restricted to spontaneously differentiated areas of G418-resistant polyclonal C2C12 cell population.

Satellite cells were transiently transfected with a plasmid containing Wnt4. When compared with controls (Fig. 2B), Wnt4-transfected cells showed an increase in Wnt4 transcript expression in proliferative (9-fold) and differentiated (3-fold) myoblasts. Western blot analysis of control and Wnt4-transfected satellite cells 2 days after differentiation further shows that control cells expressed low level of Wnt4 protein and that Wnt4-transfected satellite cells expressed increased Wnt4 protein level (6- to 10-fold increase), as also shown by immunocytochemical staining (Fig. 2B). However, the paucity of useful antibodies to vertebrate Wnt proteins (55) associated with a Wnt4 production localized in cluster in polyclonal transfected C2C12 cells did not allow us to detect unambiguously Wnt4 in Western blot analysis on these cells.

Size and fusion index analysis showed clearly that myotubes issued from C2C12 myoblasts and satellite cells overexpressing Wnt4 were hypertrophied (Fig. 3, A and E). Mean myotube area of C2C12 (Fig. 3C) and transfected satellite cells (Fig. 3F) were 90% and 100% larger than control cells, respectively. Similarly, fusion index increases in both types of cells, from 5% to 32% (GM) and from 40% to 65% (DM) in C2C12 cells (Fig. 3C) and from 43% to 72% in differentiated satellite cells (Fig. 3F). Furthermore, as shown on Fig. 3C, the size and the fusion index of myotubes spontaneously differentiated in GM after stable Wnt4 transfection of C2C12 myoblasts were similar to control myotubes differentiated in DM.

Fig. 1. Semiquantitative RT-PCR (sqRT-PCR) expression profiles of 19 Wnts during C2C12 myoblast and satellite cell differentiation. A: sqRT-PCR was performed on total RNA extracted from C2C12 myoblasts and satellite cells at proliferative (Prol) or differentiated states [day 4 (D4) for C2C12 cells, day 2 (D2) for satellite cells]. B: quantification of the expression of activated Wnt genes was made by sqRT-PCR at Prol, day 0 (D0), day 2 (D2), day 4 (D4), and day 6 (D6) after switch to DM for C2C12 myoblasts and at Prol, D0, D2, and D4 after switch to DM for satellite cells. Expression levels were normalized to the level of GAPDH expression. Positive control corresponding to amplified fragment from Wnt plasmids (Ctrl+) and negative control without reverse transcriptase (Ctrl-) were included in each experiment. Histograms are presented as means ± SE from 5 independent experiments.
To investigate the possibility that the increased myonuclear number was due to an effect of Wnt4 on cell proliferation, the number of nuclei was assessed after 48 h in DM by nuclei counting. We found that Wnt4 overexpression leads to a moderate increase in the number of nuclei both in satellite and C2C12 cells (8 ± 2%). Furthermore, proliferation rate of satellite and C2C12 cells, as determined by cell counting, showed that Wnt4 overexpression led to a moderate increase of proliferation rate (data not shown). To rule out completely the possibility that the observed hypertrophic effect of Wnt4 could be due to an increase in cell density consequent to a rise in cell proliferation, cells were plated to give the same density the day of differentiation medium shift. In these conditions, we again observed hypertrophied myotubes in Wnt4-expressing cells excluding the possibility that the hypertrophic activity of Wnt4 results from its modest proliferative effect.

The MRFs Myf5, MyoD, myogenin, and MRF4 were studied 2 and 4 days after differentiation of Wnt4-transfected satellite and C2C12 cells, respectively. Analysis of transcripts by sqRT-PCR in C2C12 myoblasts indicated a 1.3-, 2-, and 1.5-fold increase for Myf5, myogenin, and MRF4, respectively (Fig. 3D), and in satellite cells an increase of a factor 2.8, 2.6, and 3 for Myf5, myogenin, and MRF4, respectively (Fig. 3G). MyoD and myogenin proteins expression showed a 3.6- and 2.8-fold increase in C2C12 myoblasts (Fig. 3D) and a 2- and 3-fold increase in satellite cells (Fig. 3G). The discrepancy observed between RNA and protein levels for MyoD indicates that this factor is submitted to a posttranscriptional regulation (21). Thus our results expand to satellite cells and C2C12 myoblasts, the differential activation by Wnt4 of Myf5 and MyoD described by Tajbaksh et al. in explants of mouse paraxial mesoderm (73).

Pax3 and Pax7 are paired-box transcription factors required for myogenic differentiation. As previously described (38), we found that the expression of Pax3 and Pax7 was rapidly downregulated when C2C12 (Pax3: −25%; Pax7: −15%) and satellite cells (Pax3: −52%; Pax7: −30%) were induced to differentiate into myotubes (data not shown). An analysis of the effect of Wnt4 overexpression on the expression of both factors showed that Wnt4 regulates negatively only the expression of Pax7. Thus, 2 days after differentiation of Wnt4-transfected cells, Pax7 expression in transfected C2C12 and satellite cells was reduced by 17% and 39%, respectively (see supplemental Fig. S1, A and B online at the AJP-Cell Physiol website).

DNA-binding protein inhibitors (Ids) are dominant negative helix-loop-helix transcription factors that promote cell cycle progression and inhibit differentiation of many cell types including myoblasts (57). Muscle cell differentiation is a process usually accompanied by downregulation of Ids genes expression (5). We analyzed Id1, Id2, Id3, and Id4 expressions by sqRT-PCR on Wnt4-transfected C2C12 and satellite cells. We were not able to detect Id4 expression in both cell types. As expected, we found a decrease of the expression of Id1, 2, and 3 during C2C12 and satellite cells differentiation. Under our experimental conditions, we failed to detect any significant changes of Ids gene expression in Wnt4-transfected C2C12 and satellite cells compared with mock-transfected cells (Supplement Fig. S1, A and B).

Taken together, our data indicate that Wnt4 exhibits a strong effect on myogenic activity leading to a hypertrophic phenotype as shown by 1) the increases in size and fusion index of myotubes; 2) the increase of the expression of myogenic regulatory genes Myf5, MyoD, myogenin, MRF4; and 3) the inhibition of the Pax7 expression.

Effects of knocking down the expression of Wnt4 on myogenic differentiation. Efficiency of Wnt4 silencing on C2C12 and satellite cells was measured by sqRT-PCR. We showed that siRNA specific to Wnt4 (siWnt4) reduced the expression level of Wnt4 compared with control siRNA specific to Luciferase (siLuc) -transfected cells to the same extent (roughly 65%) in both cell types (Fig. 4A). As reported previously, because of the paucity of Wnt4 antibody associated to the low level of endogenous Wnt4 expression in satellite and C2C12 cells, we were not able to unequivocally detect Wnt4 protein.
As shown in Fig. 4B, treatments of cells with Wnt4 siRNA induced an important inhibition of myoblastic differentiation in both C2C12 and satellite cells. Both types of cells are characterized by a 75% decrease in the mean myotubes areas (Fig. 4C). Similarly, fusion index of Wnt4 siRNA-treated cells represented 40% and 55% of siLuc values in C2C12 and satellite cells, respectively (Fig. 4C). These data clearly demonstrate that knockdown of Wnt4 impairs the differentiation of C2C12 and satellite cells and further confirm the myogenic properties of endogenous Wnt4. Analysis of myogenic factors transcription showed that Wnt4 silencing blocked the expression of these factors induced during differentiation. The most important inhibiting effect was observed on the Myf5 expression level, which decreased by 60% in C2C12 and satellite cells (Fig. 4D). Wnt4 silencing did not modify expression levels of Pax3, Id1, Id2, and Id3 but induced the expression of Pax7 in differentiated C2C12 myoblasts and satellite cells (+19% and +17%, respectively) (supplemental Fig. S1, A and B). As expected, this pattern was opposite to the effect of Wnt4 overexpression.

Concerning the myogenic activity of Wnt4, it is also possible that this factor induces other Wnts expression and, conse-
quently, stimulates indirectly myogenesis. Previous studies revealed that Wnts could be modulators of the canonical Wnt pathway (1, 31, 32, 39, 46, 47, 54, 69, 70). Thus we examined, by sqRT-PCR, expression levels of all 18 Wnts in Wnt4-overexpressed and siWnt4-treated C2C12 and satellite cells during differentiation. With the exception of Wnt10b, expression of all other Wnts was not modified. Interestingly, we found that 1) overexpression of Wnt4 induced Wnt10b expression in proliferative and differentiated C2C12 and satellite cells (supplemental Fig. S1, C and D) and that 2) Wnt4 silencing causes Wnt10b to be downregulated in proliferative and differentiated C2C12 myoblasts (supplemental Fig. S1, C). In satellite cells, this effect was measurable only during proliferation due to the decrease of Wnt10b expression during differentiation (Fig. 1 and supplemental Fig. S1, D).

Wnt4 activates the canonical β-catenin-mediated Wnt pathway. As canonical Wnt/β-catenin induces the transcription of Axin2 and Tcf4 (27, 68), we first examined the expression levels of these two factors during C2C12 and satellite cells differentiation (Fig. 5A). For both types of cells cultured in growth medium, Axin2 showed a very faint signal, whereas the expression of Tcf4 was higher. After being switched to DM, expression level of both factors increased rapidly in C2C12 myoblasts and reached a peak at D4 for Axin2 (9-fold activation) and Tcf4 (1.6-fold activation). Similarly for satellite cells, maximal activations of Axin2 and Tcf4 were reached at D3 (7-fold activation) and at D2 (2-fold activation), respectively. Interestingly, these kinetics were parallel to the activation of Wnt4 and myogenin expressions (Fig. 5A).

The results of in vitro TOPflash assay, a robust and well-established quantitative technique to determine β-catenin-Tcf/LEF-mediated gene transcription, provide direct evidence of the Wnt canonical activation during C2C12 and satellite cell differentiation (Fig. 5B). In agreement with the Axin2 and Tcf4
activation profile, we observed a strong induction of reporter activity at the beginning of the differentiation, which peak at D4 for C2C12 myoblasts and at D3 for satellite cells. The parallel time course between the activation of Wnt canonical signaling and myogenin expression suggests an implication of this pathway in myogenesis.

To further investigate Wnt4 signaling in a myoblastic differentiation context, Wnt4 was overexpressed by transient transfection in satellite and C2C12 cells or inhibited by si-Wnt4. Axin2 and Tcf4 expression as well as TOPflash activity (Fig. 5, C and D) were measured as described above. Four and two days after being switched to DM for C2C2 and satellite cells, respectively, Wnt4 overexpression activated transcription of the reporter (5- and 8-fold compared with the control for C2C12 and satellite cells, respectively; Fig. 5D). Results obtained with TOPflash were not represented after siWnt4 treatments because of a very low reporter activity in these conditions. These data indicate clearly that Wnt4 is a positive effector of the canonical pathway in C2C12 and satellite cells.

Our results showed that Wnt4 activates the canonical pathway, suggesting an implication of this pathway in myogenic differentiation. To further assess this point, we used LiCl and the more specific GSK3 inhibitor BIO to activate the canonical pathway. So far, most studies on the effect of LiCl on Wnt signaling were focused on established cell lines such as 3T3-L1 or C2C12 (60, 67, 79, 82) or on the proliferation of satellite cells (61, 63). There are only few reports about the effects of LiCl on differentiation of satellite cells (40, 84). We showed here that treatment with 10 mM LiCl led to an extended differentiation of satellite cells (Fig. 5D). Fusion index reached 71% for LiCl-treated cells against 40% for controls (P < 0.05).
Moreover, mean myotubes area was 2.5-fold larger in LiCl-treated cells (P < 0.05). TOPflash reporter analyses confirmed that LiCl treatment activates the canonical Wnt signaling (Fig. 5D). However, LiCl induced a fourfold more intense response than cells overexpressing Wnt4, suggesting that the canonical pathway is not completely activated by the overexpressed Wnt4 under our experimental conditions. As reported by Abiola et al. (1), BIO treatment was able to induce the formation of terminally differentiated myotubes from satellite cells. Here, we reported that in the presence of 1 μM BIO in DM, fusion index reached 60% against 40% for controls (P < 0.05), and mean myotubes area was 1.6-fold larger than control cells (P < 0.05). However, this myogenic activity was less important than that observed by using LiCl and was well correlated to the moderate Topflash reporter activity induced by 1 μM BIO (Fig. 5D). To obtain stronger β-catenin activation, we tested higher concentrations of BIO. However, such concentrations of BIO were toxic for satellite cells under our experimental conditions. Concerning C2C12 myoblasts, 1 μM BIO activates β-catenin (Fig. 5D) but has a moderate myogenic effect (Fusion index reached 50% and mean area was 1.2-fold larger than control. P < 0.05). Like satellite cells, myoblasts treated with concentrations higher than 1 μM BIO exhibited morphological changes revealing a deleterious side effect of BIO thus limiting the use of this compound.

β-Catenin signaling activation results in the specific regulation of multiple critical target genes. Here, we analyzed the effect of β-catenin activation by LiCl and BIO on Wnt4 expression. We found, on C2C12 and satellite cells, that both β-catenin pathway activators do not modify the expression profile of Wnt4 (data not shown). Taken together, our results show that 1) canonical pathway is induced during differentiation of C2C12 myoblasts and satellite cells, and 2) Wnt4 acts, at least partly, by inducing canonical signaling.

Wnt4 overexpression decreases both Mstn expression and activity in myogenic cells. We and others (29, 48) have showed that Mstn is involved in the inhibition of myoblast proliferation and differentiation. Here, we reported that activation of canonical Wnt signaling by Wnt4 induces myotube hypertrophy in both C2C12 mouse myoblasts and primary murine myoblasts. Since these myogenic effects are contrary, we asked whether Wnt4 could target Mstn signaling. To this aim, we examined the role of Wnt4 in modulating both the activity of Mstn signaling and its expression in myoblasts.

We investigated the consequences of altering Wnt4 signal upon Mstn activity using the CAGA reporter assay. This reporter gene assay has been shown to reflect Mstn activity (65, 76, 87). Luciferase reporter gene assays were performed in the presence or absence of recombinant Mstn in muscle cells overexpressing Wnt4 during proliferation (Fig. 6A) and differentiation (Fig. 6B). Under our culture conditions, we were unable to keep satellite cells in proliferation for more than 2 days. Even with the addition in the culture medium of the anti-differentiation factor bFGF, a fraction of satellite cells underwent differentiation. For this reason, results of the transfection experiments with reporter on satellite cells could be recorded only after differentiation of satellite cells.

In proliferating C2 myoblasts, CAGA reporter was activated by Mstn, and this response was significantly reduced in myoblasts overexpressing Wnt4 (−48%). We noticed that addition of Mstn on Wnt4 overexpressing cells did not reverse the inhibitory effect of Wnt4 (Fig. 6A). During differentiation, Mstn activated CAGA reporter in both myoblasts and satellite cells, and this activation was inhibited by Wnt4 overexpression (−38% and −48%, respectively). As reported for proliferating myoblasts, we observed that the addition of Mstn did not reverse the inhibitory effect of Wnt4 in both cell types.

Reciprocally, we investigated whether Mstn could modulate Wnt4 transcriptional activity. We have shown that Wnt4 in myoblasts led to an induction of β-catenin/Tcf signaling. We therefore evaluated the transcriptional activity of a transiently cotransfected β-catenin/Tcf luciferase (TOPflash) reporter in myoblasts with a Wnt4 expression vector in the presence or absence of recombinant Mstn in proliferative and differentiation conditions. We found that the transcriptional activity of the β-catenin/Tcf complex was increased by Wnt4 and that this response was not modulated by Mstn (Fig. 6, C and D). These results were similar for satellite cells in differentiation conditions (Fig. 6D).

Interestingly, we observed a dramatic decrease in the expression of Mstn mRNA in the myoblasts overexpressing Wnt4 relative to control cells. This inhibition reached 35% in proliferative myoblasts and 75% in differentiated myoblasts (Fig. 6E). We next examined whether this decrease was due to an inhibition of the activity of the Mstn promoter. For this purpose, myoblasts were transiently cotransfected with the pMstn-luc vector having 1177 bp of the murine Mstn promoter fused with luciferase and with Wnt4 expression vector. Under these conditions, the activity of the Mstn promoter was significantly and reproducibly repressed by 40% (Fig. 6F) compared with the vector control.

Our results showed that Wnt4 activates the canonical signaling in myogenic cells and inhibits the expression of Mstn. We studied the implication of this pathway in the regulation of Mstn expression by using 10 mM LiCl or 1 μM BIO. Myoblasts in proliferation were treated with the activators of canonical pathway and then shifted to DM 24 h later. RNAs were extracted for qRT-PCR analysis of Mstn expression. One day after treatment, cells being in GM, Mstn expression was reduced of 36% and 40% with LiCl or BIO treatment, respectively. At D1, Mstn expression remained downregulated (−22% LiCl, −25% BIO) (Fig. 6E). Surprisingly, we found that at D3, inhibitory activity of LiCl and BIO on Mstn expression was relaxed, whereas Wnt4 still downregulated Mstn expression (Fig. 6E). In summary, we showed that Wnt4 decreases Mstn transcriptional activity and that this effect was associated to an inhibition of Mstn expression in part via canonical β-catenin pathway activation.

Mstn is necessary to mediate the differentiation-promoting activities of Wnt4 in murine muscle cells. We then explored the role of Mstn in the differentiation-promoting activities of Wnt4 in murine muscle cells. First, we asked whether addition of recombinant Mstn was able to antagonize the Wnt4-induced myogenesis. We performed transient transfection assay in primary muscle myoblasts from wild-type mice muscles with Wnt4 expression vector. At 24 h posttransfection, cells were shifted and grown for 48 h in DM. They were supplemented or not with increasing amounts of recombinant Mstn and examined for myotube size and fusion index by immunofluorescence analysis (Fig. 7A, left). As expected, culture of myotubes issued from satellite cells overexpressing Wnt4 displayed larger myotubes than those issued from control satellite cells.
Interestingly, we observed that addition of Mstn prevented this Wnt4-induced differentiation. The decrease in fusion index of Wnt4-transfected satellite cell cultures by Mstn was detectable at a concentration of 1 μg/ml (21% decrease) and reached a maximum (28% decrease) at 2 μg/ml (Fig. 7B). Similarly, culture from Wnt4-transfected cells treated with Mstn at 1 or 2 μg/ml decreased by 10% and 40%, respectively, in the mean myotube diameter compared with controls (Fig. 7C). These results confirm clearly that Mstn treatment resulted in significant decreases of both the size and fusion index of Wnt4 murine myotubes.

Second, we investigated whether Mstn deletion was sufficient to impair the regulation of hypertrophy by Wnt4. Primary cultures of myoblasts from wild-type and Mstn−/− mice muscles were transfected with Wnt4. A–D: C2 myoblasts and satellite cells were transiently transfected with the CAGA and pRL-TK reporters (A and B) and Tcf (TOPflash) and pRL-TK (C and D). Cells were cotransfected with either empty (Ctrl) or Wnt4-containing (Wnt4) expression vector or treated with recombinant Mstn. Luciferase activity was measured and normalized to the pRL-TK. C2 myoblasts were analyzed in proliferation medium (A and C) or 2 days after switch to DM (B and D). Satellite cells were analyzed only in DM (B and D). Results are representative of experiments performed in triplicate and error bars represent SE. *Significantly different from the untreated control (P < 0.05); †significantly different from treated with Mstn alone (P < 0.05).

E: mRNA expression of Mstn in proliferative (Prol) and differentiated C2C12 myoblasts (D1 and D3) transfected with empty (Ctrl) or Wnt4 vectors; or treated with 10 mM LiCl or 1 μM BIO. *P < 0.05.

F: C2 myoblasts were transiently transfected with the MS1177 Mstn promoter construct and either empty (Ctrl) or Wnt4-containing (Wnt4) expression vector. Fold induction of MS1177-luc promoter reporter was calculated by normalizing firefly luciferase activity to Renilla luciferase 3 days after switch to DM. The average of three experiments is shown, and error bars represent SE *P < 0.05.
DISCUSSION

Wnt signaling has been shown to play a role in muscle development and in muscle homeostasis. Thus it was recently demonstrated that Wnts participate in the temporal control of satellite cell expansion versus differentiation during adult muscle regeneration (9) and that ectopic Wnt induces premature muscle differentiation, whereas inhibition of Wnt signaling interferes with muscle differentiation (37). The role of Wnt signaling in myoblast differentiation is further supported by the fact that Mstn, a member of the TGF-β superfamily, negatively regulates muscle mass via noncanonical Wnt signaling pathways (71).

Wnt gene expression profiles showed that in C2C12 and satellite cells, only the expression of Wnt4 (among the 19 existing Wnts) was strongly activated from the early steps of differentiation. As shown by Wnt4 overexpression or inhibition approaches, the present study shows the important role of Wnt4 on myoblast differentiation. After Wnt4 overexpression, the relative increases in myotube size were comparable 48, 72, and 96 h after switching to DM, indicating that Wnt4 signaling controls myotube size at the early steps of differentiation during myotube formation. The lack of Wnt4-mediated hypertrophic effect observed when satellite cells were transfected at day 0 (D0) or later indicates that this factor has to be expressed during the proliferative stage to be effective. Fusion consists of two distinct phases: myoblast/myoblast fusion to form nascent myotubes and subsequent myoblast/myotubes fusion, resulting in a rapid accretion of size. The efficiency of the first phase can

Fig. 7. Effect of Mstn on the differentiation-promoting activities of Wnt4 in murine muscle cells. A–C: primary cultured muscle cells from wild-type Mstn+/+ and Mstn−/− mice were transiently transfected with either empty (Ctrl) or Wnt4-containing (Wnt4) expression vector. At 24 h posttransfection, cells were shifted and grown for 48 h in DM. When shifted in DM, wild-type cells transfected with Wnt4 were daily treated or not with recombinant Mstn (increasing concentrations: 0.5; 1 and 2 µg/ml) for 48 h before harvesting. Independent cell cultures were obtained from at least five mice of the indicated genotype. Three assays were performed. A: representative images of myotube cell cultures from the wild-type Mstn+/+ (left) and Mstn−/− (right) mice are shown. Immunofluorescence was performed at D2 of differentiation with anti-MHC monoclonal antibody. Nuclei were revealed by Hoechst staining. Myotube cell cultures from wild-type mice transfected with Wnt4 were exposed to recombinant Mstn (2 µg/ml) for 48 h in DM. Fusion index and myotube diameter were evaluated at D2. Scale bar, 20 µm. B: quantification of fusion index of cell cultures from Mstn+/+ and Mstn−/− mice cultured as described in A. Fusion index was determined by counting 1,000 nuclei as described in MATERIALS AND METHODS. Results represent means ± SE for at least three assays. Statistical significance was assessed using ANOVA-1 analysis followed by the Tukey’s post test. *P < 0.05 versus control wild-type (Ctrl); ‡P < 0.05 and ‡‡P < 0.01 versus Wnt4 wild-type (Wnt4). C: quantification of myotube diameter of cell cultures from Mstn+/+ and Mstn−/− mice cultured as described in A. Histograms are means ± SE for at least three assays. ***P < 0.001 versus control wild-type (Ctrl); ‡‡‡P < 0.01 versus Wnt4 wild-type (Wnt4). At least 150 myotubes were analysed for each culture.
be evaluated by measuring the fusion index, which represents the proportion of the total cell population that has fused. After being switched to DM, Wnt4 overexpression in C2C12 and satellite cells gave a 62% and a 67% fusion index increase, respectively, suggesting that Wnt4 signaling control nascent myotube formation. When C2C12 myoblasts were transiently transfected, Wnt4 expression level was highly variable and rather low, leading to a moderate and variable myotube hypertrophy. Stable transfections gave us a more robust expression level and allowed us to highlight the strong hypertrophic activity of Wnt4 even when cells were in GM. These observations suggest that the myogenic activity of Wnt4 is highly correlated to its expression level.

Under our conditions, we found that Wnt4 exhibits a weak proliferative activity on both cell types. In agreement, Takata et al. (74) reported that Wnt4 exhibited a low but significant myogenic activity with an increase of proliferation rate in C2C12 cells. Conversely, Otto and co-workers (61) found, using coculture of Wnt4-producing NIH-3T3 cell line on single muscle fiber culture, a 24% reduction of satellite-cell proliferation. These conflicting results could be attributed either to the different specificities of the cellular models used or to the fact that effective concentration and physiological activity of ectopic Wnt4 can be eminently variable, depending on the nature and activity of produced Wnt4 (transfection of a tagged or untagged Wnt4, overexpression or coculture with cells producing tagged or untagged Wnt4). Taken together, these data are in agreement with a modest effect of Wnt4 on proliferation, suggesting a role of this factor in enhancing cell fusion rather than controlling the number of nuclei available for fusion.

Knockdown of Wnt4 expression in C2C12 and satellite cells inhibits their differentiation and further confirms that Wnt4 function acts as an effector of myogenesis. Under our conditions, the most important inhibitory differentiation effect was observed after multiple siRNA transfections (2 to 3 spaced from 24 h). This approach can improve the inhibiting potential of siRNA by 1) increasing its cellular concentration and/or 2) by maintaining its cellular concentration in long-lasting experiments. The fact that Wnt4 silencing peaked 12 to 20 h after transfection and then declined rapidly, associated with the fact that multiple transfections did not raise the potency of Wnt4 silencing, which reached maximally 65%, suggest rather that multiple transfections help to keep a sufficient siWnt4 level all along the proliferation/differentiation transition. However, addition of siWnt4 after switching to differentiation medium or 24 h before did not decrease significantly the differentiation rate. This confirms that Wnt4 acts as a positive regulator of myogenic differentiation only if it is expressed in the proliferative phase. As previously reported after Wnt4 overexpression, we failed to observe a modification of proliferation rate in siWnt4-treated C2C12 and satellite cells, confirming that the action of this factor on the myoblastic differentiation was not due to a variation in cell density.

Differentiation of myoblasts involves two major steps, the irreversible withdrawal from cell cycle and the subsequent expression and activation of myogenic factors. Myf5 and MyoD are expressed in early myogenesis. As cells progress toward a differentiated phenotype, myogenin and MRF4 are induced and cooperatively establish the irreversible commitment to terminal differentiation. Myf5 is a factor reported to be high in the Go phase, where cells were arrested to differentiate (42). Borello et al. (8) have previously showed a direct regulation of Myf5 expression by canonical Wnt signaling during somitogenesis. Here, we found that the expression of Myf5 was activated by Wnt4 overexpression and strongly inhibited by Wnt4 silencing during C2C12 myoblast and satellite cells differentiation, suggesting an implication of canonical pathway in the myogenic properties of Wnt4.

Myogenesis is regulated by transcription factors such as Pax3 and Pax7. They function to activate and maintain expression of MRFs and promote cell expansion (16, 49). Moreover, associated to their myogenic promoting activity, ectopic Pax3 or Pax7 expression restricts myoblast accretion and myotube growth (16). Both factors are expressed by proliferating C2C12 and satellite cells but are downregulated during myogenic differentiation. The fact that changes in Wnt4 expression affects only the expression of Pax7 suggests that Wnt4 exerts its myogenic activity mainly by modulating MRFs expression. Indeed, in our cellular models, Wnt4 is able to downregulate the expression of Pax7 and to upregulate the expression of MRFs. The reciprocal inhibition between Pax7 and MRFs reported by Olguin et al. (58, 59) in C2C12 and satellite cells explains probably the observed downregulation of Pax7 by Wnt4.

Although Wnt4 was originally described as a noncanonical Wnt, it has also been implicated in the activation or inhibition of the canonical Wnt pathway (6, 28, 44, 46, 72, 74, 75). In agreement with our results, Takata et al. (74) found that Wnt4 has an activity to transduce signals trough the β-catenin pathway. Likewise, Armstrong et al. (3, 4) have reported that the expression of β-catenin is necessary for physiological growth of skeletal muscle and that Wnt signaling pathway induces β-catenin activation of growth-control genes during overload-induced skeletal muscle hypertrophy. Our results showing that the hypertrophic activity of Wnt4 in C2C12 and satellite cells was linked to the Tcf/LEF promoter activation confirm the implication of β-catenin canonical pathway in the control of myogenesis. The implication of the canonical pathway in this process was confirmed by the hypertrophic activity of LiCl or BIO treatment. When LiCl was added to the culture medium, the area of myotubes and index fusion were increased in both C2C12 and satellite cells, reaching values similar to those observed after Wnt4-induced hypertrophy. Effects of BIO were more difficult to explain. Indeed, 1 μM BIO activated β-catenin signaling less efficiently than 10 mM LiCl and induced a moderate hypertrophic phenotype in myoblasts and satellite cells. This difference with LiCl treatment could be related to the fact that BIO exhibits, at higher concentrations, deleterious morphological changes in both cell types and thus can induce undesirable side effects even at the employed concentration.

On the other hand, the fact that LiCl exerts a higher Tcf/LEF activation than Wnt4 treatment, whereas the extent of differentiation is the same suggests that this process is dependent on other signaling pathways. In this respect, work of Otto et al. (61) on isolated muscle fibers showed that Wnt4 inhibited satellite cell proliferation but that canonical Wnts such as Wnt1, 3a, and 5a induced a greater degree of proliferation than control cells, confirming the implication of other pathways in Wnt4 signaling.

The stronger activity in terms of Tcf/LEF activation of LiCl compared with Wnt4 (+100% on C2C12 myoblasts, +250%
on satellite cells) treatments could explain the discrepancy between proliferative activities of Wnt4 and LiCl. Indeed, as described above, Wnt4 shows a weak effect on mitogenicity, whereas 10 mM LiCl has a strong anti-proliferative activity (−15% on satellite cells, −48% on satellite cells), suggesting that low levels of Tcf/LEF activation regulate myogenic differentiation, whereas higher levels regulate the proliferation. A study reported by Anakwe et al. (2) is also in agreement with a relation between the level of Tcf/LEF activity and the type of cellular response elicited. They reported an increase in the number of terminally differentiated cells in Wnt4-transfected myogenic cells, whereas the overexpression of Wnt3a (a typical activator of the β-catenin pathway) decreases the number of terminally differentiated myogenic cells. Nevertheless, this does not exclude that other signaling pathways like Wnt/calcium signaling are implicated in the growth and maintenance of skeletal muscle.

Concerning the myogenic activity of Wnt4, it is also possible that this factor induces other canonical Wnt expression and consequently stimulates indirectly myogenesis. Previous studies revealed that all Wnts could be modulators of the canonical Wnt pathway (1, 31, 32, 39, 46, 47, 54, 69, 70). Interestingly, we found that only the expression of Wnt10b is affected by Wnt4. Wnt10b is classified as a canonical Wnt, which activates the β-catenin-Tcf pathway. It plays an important role in the negative regulation of adipocytes differentiation and in the control of adipogenic potential of myoblasts and regenerating muscle (81). In addition, Wnt10b overexpression results in a sustained or enhanced myogenic differentiation (1, 81). Our unpublished data concerning the overexpression of Wnt10b in C2C12 and satellite cells did not show such hypertrophic activity as reported with Wnt4. Furthermore, contrary to Wnt4, Wnt10b expression during satellite cells differentiation is not induced but downregulated (Fig. 1). Despite the fact that both factors induce canonical β-catenin, the mechanisms underlying their specificities are different, and we cannot rule out the possibility that Wnt4 and Wnt10b differentially affect noncanonical signaling pathways. The fact that the expression of Wnt4 regulates in a similar manner the expression of Wnt10b suggests that these two factors have complementary functions and that the subsequent stabilization of β-catenin brought by both Wnts promoted myogenic differentiation. Nevertheless, Wnt4 and Wnt10b are not strictly redundant and we suggested that Wnt10b specifically controls adipogenic potential, whereas that Wnt4 influences myogenic potential.

Taken together, the differences in activity between Wnt4/ Wnt10b and Wnt3a/Wnt4 and the different ranges of Tcf/LEF stimulation by LiCl or BIO suggest equally that the level of β-catenin activation is not the only parameter to consider. Further study is needed to address the involvement of noncanonical pathways in Wnt4 signaling. Our future studies will address in detail the molecular mechanisms by which Wnt4 canonical and noncanonical pathways control the process of myogenic differentiation.

Here, we showed that activation of Wnt signaling by Wnt4 induces myotube hypertrophy in both C2C12 myoblasts and primary murine myoblasts. Since this phenocopies the effect of Mstn absence, we wanted to know if Wnt4 could target Mstn signaling. To this end, we examined the role of Wnt4 in modulating both the expression and signaling activity of Mstn. Takata et al. (74) have reported that Wnt4 had no effect on Smad2 phosphorylation, but it antagonized Smad2 phosphorylation induced by Mstn in differentiated C2C12 myoblasts. By using CAGA reporter, we confirmed the inhibition of Mstn-activated Smad signaling pathway by Wnt4, and we extended this result to proliferating myoblasts. Contrary to differentiated myoblasts, Wnt4 decreased the CAGA reporter activity in proliferating myoblasts below the basal level meaning that Wnt4 has a stronger anti-Mstn activity during proliferation than during differentiation. Furthermore, the fact that the addition of Mstn did not reverse the Wnt4-induced CAGA reporter inhibition in myoblasts and satellite cells suggests that Mstn acts upstream of Wnt4 or that Wnt4 regulates the Mstn/Smad pathway independently of Mstn.

The inhibition of the Mstn pathway by Wnt4 can be associated to the inhibition of Mstn expression and/or inhibition of the Mstn/Smad transduction pathway. By using sqRT-PCR and promoter reporter experiments, we showed that Wnt4 acted as an inhibitor of Mstn expression. Moreover, our study with LiCl and BIO, strongly suggests that Wnt4 regulates negatively Mstn expression through activation of the canonical β-catenin pathway. However, while Wnt4 downregulates Mstn expression in proliferative and differentiated cells, the activation of canonical signaling by LiCl or BIO inhibits Mstn expression only when cells are in proliferative or early differentiated stages. Thus, in function of the differentiation, various signaling pathways can be implicated in the inhibition of Mstn expression by Wnt4 in myogenic cells. A biphasic mode of action of Wnt4 can be proposed: in proliferative and early differentiated stages, Wnt4 acts by inducing canonical signaling, and later in the differentiation, canonical pathways is relaxed and another pathway maintains Mstn at low level.

However, the fact that Wnt4 displayed the strongest CAGA reporter inhibiting activity in proliferative myoblasts, whereas Wnt4 had the strongest Mstn expression inhibiting activity in differentiated myoblasts, implies that Wnt4 regulates both Mstn expression and Mstn/Smad signaling pathway. To corroborate possible interaction between Mstn/Smad and Wnt/β-catenin pathways, we performed in silico analysis to identify putative Tcf/LEF binding elements in the Mstn promoter. We found three putative sites within a 2-kb sequence upstream of the Mstn gene at position −1889, −1028, and −743 confirming the observed regulation of the Mstn expression by Wnt/β-catenin pathway.

As reported by Steelman et al. (71), we observed a slight repression of Wnt4 expression by Mstn (data not shown). To go further in the reciprocal regulation of the both signaling pathways, we analyzed the effects of Mstn on the activity of the TOPflash reporter. Surprisingly, whereas Mstn inhibits the accumulation of nuclear β-catenin in differentiated C2C12 (25), we found that Mstn was not able to inhibit Tcf/LEF activity induced by Wnt4 overexpression in proliferative and differentiated C2C12 myoblasts. Thus, contrary to adipogenic differentiation of human bone marrow-derived mesenchymal stem cells in which there is a cross communication of the Mstn signal to Wnt/β-catenin pathway (25), we did not observe a regulation of Wnt4/β-catenin signaling pathway by Mstn.

Mstn has been described by us and several group as an inhibitor of myotube formation in C2C12 and satellite cells (29, 41, 66, 77). The reversion of Wnt4 activity by recombinant Mstn and the absence of hypertrophic activity of this factor in Mstn−/− mice demonstrated clearly that Wnt4 requires the
presence of Mstn to elicit its differentiation promoting activity. Thus the negative regulation of Mstn by Wnt4 is a crucial step for the myotube formation and hypertrophy. The fact that the addition of Mstn reverses the hypertrophic activity of Wnt4 but does not reverse the Wnt4-induced CAGA reporter inhibition implies that 1) Wnt4 acts upstream of Mstn and that 2) Wnt4 inhibits CAGA reporter by another pathway than Mstn/Smad signaling.

In summary, our results demonstrate that Wnt4 is a strong hypertrophic factor whose expression is induced during C2C12 and satellite cells differentiation. The overactivation of β-catenin pathway induced by ectopic expression of Wnt4 suggests activating properties of the canonical pathway in the muscular differentiation process. Furthermore, the demonstration that Wnt4 can dominantly overcome the expression and activity of Mstn opens possibilities of the manipulation of Wnt4 or β-catenin levels as a likely target for therapeutic design.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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