Androgens Up-regulate Transcription of the Notch Inhibitor Numb in C2C12 Myoblasts via Wnt/β-Catenin Signaling to T Cell Factor Elements in the Numb Promoter*

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Xin-Hua Liu1,2, Yong Wu3, Shen Yao3, Alice C. Levine3, Alexander Kirschbaum4, Lauren Collier5, William A. Bauman6,7, and Christopher P. Cardozo1,2,4

From the 1National Center of Excellence for the Medical Consequences of Spinal Cord Injury, James J. Peter Veterans Affairs Medical Center, Bronx, New York 10468 and the Departments of 2Medicine, 3Urology, and 4Rehabilitation Medicine, Mount Sinai School of Medicine, New York, New York 10029

Background: Androgenic, Wnt, and Notch pathways regulate progenitor development through unknown mechanisms.

Results: Up-regulation of Numb was blocked by Wnt inhibitors and required β-catenin. Nandrolone promoted binding of β-catenin to the Numb promoter and thereby increased its activity through Tcf bound at two consensus sites.

Conclusion: Wnt/β-catenin signaling mediates nandrolone-induced Numb transcription.

Significance: AR signaling regulates Wnt-Notch interaction via Numb, a newly identified Wnt target gene.

Androgen signaling via the androgen receptor is a key pathway that contributes to development, cell fate decisions, and differentiation, including that of myogenic progenitors. Androgens and synthetic steroids have well established anabolic actions on skeletal muscle. Wnt and Notch signaling pathways are also essential to myogenic cell fate decisions during development and tissue repair. However, the interactions among these pathways are largely unknown. Androgenic regulation of Wnt signaling has been reported. Nandrolone, an anabolic steroid, has been shown to inhibit Notch signaling and up-regulate Wnt signaling has been reported. Nandrolone, an anabolic steroid, has been shown to inhibit Notch signaling and up-regulate Numb, a Notch inhibitor. To elucidate the mechanisms of interaction between nandrolone and Wnt/Notch signaling, we investigated the effects of nandrolone on Numb expression and Wnt signaling and determined the roles of Wnt signaling in nandrolone-induced Numb expression in C2C12 myoblasts. Nandrolone increased Numb mRNA and protein levels and T cell factor (Tcf) transcriptional activity via inhibition of glycogen synthase kinase 3β. Up-regulation of Numb expression by nandrolone was blocked by the Wnt inhibitors, sFRP1 and DKK1, whereas Wnt3a increased Numb mRNA and protein expression. In addition, we observed that the proximal promoter of the Numb gene had functional Tcf binding elements to which β-catenin was recruited in a manner enhanced by both nandrolone and Wnt3a. Moreover, site-directed mutagenesis indicated that the Tcf binding sites in the Numb promoter are required for the nandrolone-induced Numb transcriptional activation in this cell line. These results reveal a novel molecular mechanism underlying up-regulation of Numb transcription with a critical role for increased canonical Wnt signaling. In addition, the data identify Numb as a novel target gene of the Wnt signaling pathway by which Wnts would be able to inhibit Notch signaling.

Wnt signaling is a key regulator of muscle formation during embryogenesis and is essential for myogenic differentiation as well as for determining myogenic stem cell fate in the adult (1–3). Wnt signaling acts directly on muscle stems to control myogenic lineage progression and to promote muscle development as well as tissue repair (1–3). Activation of Wnt signaling results in the transition of progenitors from the proliferation phase to the differentiation phase during postnatal myogenesis (4). Canonical Wnt signals are mediated by the transcriptional effector β-catenin (5). Upon binding to their receptors and co-receptors, Wnt proteins induce a cascade of intracellular signaling events that promote stabilization and nuclear import of β-catenin, which associates with DNA bound to the Tcf/Lef family of transcription factors, converting them from transcriptional repressors to activators. GSK3β negatively regulates Wnt signaling by phosphorylating β-catenin, leading to its ubiquitination and proteolysis (5, 6). Therefore, inhibition of GSK3β activity mimics Wnt stimulation (7). Wnt signaling is inhibited by several naturally occurring inhibitors, such as secreted Frizzled-related proteins (sFRPs) and Dickkopf proteins (Dkk) (5).

Notch signaling plays an important role in tissue morphogenesis both during development and postnatal repair of injured skeletal muscle where it is critical to satellite cell activation and proliferation (4, 8–10).

Wnt and Notch signaling configure an integrated molecular device whose main function is to regulate transitions between cell states in development and homeostasis (11–16). However, the factors mediating the cross-talk between Notch and Wnt are not fully understood. One proposed such factor is Numb...
(17, 18). Numb inhibits Notch activity by targeting Notch and Notch intracellular domain for proteolytic degradation (19). Numb has also been shown to promote differentiation of satellite cells of the myogenic lineage during myogenic development and repair (8, 20, 21).

Androgen signaling via the androgen receptor (AR) is a key pathway that contributes to regulation of body composition. Androgens and other synthetic analogs have well established anabolic actions on skeletal muscle. Administration of testosterone promotes the differentiation of mesenchymal stem cells into the myogenic lineage (22). Testosterone is able to increase skeletal muscle mass and strength in elderly men and in many conditions resulting in muscle atrophy, such as cancer and burn-induced cachexia (23), microgravity (24), immobilization (25), and spinal cord injury (26). The precise mechanisms underlying these actions of androgens, however, are poorly understood. Although androgens have been shown to be able to modulate the activities of Notch (27) and Wnt (28–31) signaling, the precise processes that control these molecular events are largely unknown.

Cross-talk between Wnt/β-catenin and the AR signaling pathways has been demonstrated. The AR, β-catenin, and Tcf are reported to co-localize in the nucleus (28, 29). β-Catenin preferentially binds AR over several other steroid hormone receptors, including the estrogen receptor, progesterone receptor, and glucocorticoid receptor (28). Moreover, forced overexpression of β-catenin augments AR-mediated transcription (29, 32), indicating a role for β-catenin as a co-activator of AR. Although increasing evidence has demonstrated an interaction between AR and Wnt signaling in several cell lines, the possible cross-talk between the two pathways in the process of myogenic differentiation has not been well delineated.

Signaling through the AR has also been suggested to interact with Notch signaling. Testosterone has been shown to increase Notch expression in skeletal muscle and suggested to increase Notch signaling (27). We found that nandrolone, an anabolic steroid, reduced Notch signaling in denervated muscle associated with up-regulation of Numb (33). We also demonstrated in C2C12 cells that nandrolone, via the AR, up-regulates Numb protein levels by a post-translational mechanism whereby Numb is stabilized against degradation, at least in part, through suppression of mδn2 expression (34). However, mechanisms by which nandrolone up-regulates Numb mRNA expression remain unclear. With these considerations in mind, we investigated the effects of nandrolone on Numb mRNA and Wnt signaling and determined the role of Wnt signaling in nandrolone-induced transcriptional regulation of Numb in mouse C2C12 myoblasts.

**EXPERIMENTAL PROCEDURES**

**Cell Line and Cell Culture**—Mouse C2C12 cells were obtained from ATCC and maintained in DMEM containing 10% FBS supplemented with 1% penicillin/streptomycin at 37 °C. All experiments were performed with C2C12 cells that had been incubated for 48 h in DMEM containing 2% horse serum (HS) to initiate differentiation.

**Preparation of Cell Lysates and Immunoblotting**—C2C12 cells cultured under the desired conditions were lysed, as described previously (31). Briefly, cells were rinsed twice with ice-cold PBS and scraped with 1.5 ml of PBS containing 4 mM iodoacetate. After centrifugation, the pellets were resuspended in CHAPS extraction solution (10 mM CHAPS, 2 mM EDTA, pH 8.0, and 4 mM iodoacetate in PBS) with protease inhibitors. The samples were incubated for 30 min on ice and centrifuged at 15,000 × g for 10 min. The supernatants were collected and stored at −70°C. Proteins from the cytosolic and nuclear fractions were isolated using a commercial kit from Pierce, according to the manufacturer’s instructions. For immunoblotting, cell lysates were electrophoresed on SDS-polyacrylamide gels, electrophoretically transferred to a polyvinylidene difluoride membrane (Bio-Rad), and incubated with targeting primary antibodies overnight at 4 °C. Secondary horseradish peroxidase-linked donkey anti-mouse IgG (GE Healthcare) was then applied to the membranes and visualized by enhanced chemiluminescence (GE Healthcare). Antibodies against Notch intracellular domain, endogenous GSK3β, phospho-GSK3βSer9, and Numb were purchased from Cell Signaling Technology. Monoclonal anti-β-catenin and anti-active-β-catenin antibodies were obtained from Upstate Biotech-Millipore). Hey1 antibody was purchased from Abcam. Recombinant proteins Wnt3a, Wnt5a, Dkk1, and sFRP1 were obtained from R&D Systems. SB261762 was purchased from Sigma. β-Tubulin (Abcam) and histone (Santa Cruz Biotechnology) antibodies were used as loading controls.

**Immunohistochemical Staining and Microscopy**—Cells were incubated on glass coverslips and treated with either vehicle or nandrolone. Immunofluorescence staining was done as reported previously (31). Briefly, cells were fixed for 8 min in 3.5% paraformaldehyde in PBS and blocked with 15% normal goat serum containing 0.3% Triton X-100. Cells were then probed with an anti-Numb antibody (1:400). Secondary antibodies conjugated to fluorophores (Vector Laboratories) were used at a 1:100 dilution and were incubated for 1 h at 37°C followed by three 10-min washes. DAPI counterstaining was used to localize the nucleus. Images were acquired with a Zeiss LSM 700 confocal laser scanning microscope using identical settings for each photomicrograph.

**Transient Transfection and Luciferase Reporter Assay**—Transient transfection was done using Lipofectamine 2000 reagent according to the manufacturer’s instructions (Invitrogen). The Tcf/Lef reporter was premixed with a plasmid constitutively expressing Renilla luciferase which served as an internal control for normalizing transfection efficiencies. Cells were cultured in 12-well cluster plates and transfected with either 1 μg of the reporter plasmid or control vector as mock controls. The transfected cells were lysed by scraping into reporter buffer (Promega). The firefly luciferase activity was assayed and quantitated using a luminometer. The results were normalized to Renilla activity.

**Quantitative Real-time (Rt) PCR**—Rt-PCR was performed as described previously (35) using a thermocycler (model 7500; Applied Biosystems). For each sample, the determinations were performed in triplicate, and the means for the crossing points of triplicates were used in subsequent calculations. mRNA levels were expressed as -fold change using the 2^(-ΔΔCt) method (36). Data were normalized relative to 18S RNA.
Wnt/β-Catenin Mediates Numb Induction by an Anabolic Steroid

Small Interfering RNA (siRNA) Transfection—siRNA against β-catenin and nonsilencing random siRNA (negative control) was purchased from Applied Biosystems. Cells were cultured in 6-well plates and transfected with either nonsilencing random siRNA or 20 nM β-catenin-siRNA in 100 μl of PepMute Plus siRNA transfection reagent (SignaGen Laboratories, Ijamsville, MD), following the manufacturer’s recommended procedures. Cells were then treated with either vehicle or nandrolone (500 nm) under differentiating conditions.

Chromatin Immunoprecipitation (ChIP) Assay—A ChIP assay kit was purchased from USB-Affymetrix and performed as described previously (35). Briefly, cells were washed once with PBS and then cross-linked with 1.5% formaldehyde at 37 °C for 10 min. After washing twice with ice-cold PBS, the cells were collected in lysis buffer and incubated for 30 min on ice. Cell lysates were sonicated using a Sonicator 3000 and then diluted 5-fold with dilution buffer. Diluted cell lysates (500 μl) were precleared with salmon sperm DNA/protein A-agarose (USB Co.) for 2 h at 4 °C. Monoclonal anti-β-catenin antibody (5 μg) or normal mouse IgG (control) was used to immunoprecipitate protein-DNA complexes from precleared supernatants containing 500 μg of protein. Immunoprecipitated DNA was amplified by PCR (30 cycles) using Taq polymerase (New England Biolabs), resolved by 1.2% agarose gel electrophoresis, and visualized with ethidium bromide staining. The following primer set was used: forward, 5′-CAG GCT CCC TCC TGT and reverse, 5′-CAG ATG CAT CTC CCT CCA GGA CTC CAG-3′. The sequence of the DNA pulled down was verified by DNA sequencing (DNA Sequencing Facility, Albert Einstein College of Medicine, Bronx, NY).

Plasmids—The plasmid, pGL3-Tcf4-Numb.F1-Luc, containing the cDNA amplified after pulldown of β-catenin and containing several Tcf binding sites within Numb promoter (ChIP assay) was generated as follows. The band containing the PCR product amplified by the above ChIP assay was amplified using the primers as described in the ChIP assay which was modified to contain KpnI and XhoI sites in the forward and the reverse primers, respectively. The resulting PCR product was cloned into pCR1-TOPO vector (Invitrogen), and the sequence of the inserted PCR product was also verified by DNA sequencing. The PCR product was then excised with KpnI and XhoI and gel-purified, then ligated into a firefly luciferase reporter vector, pGL3 promoter (Promega), pRL-TK (Promega), a Renilla luciferase internal control vector, was used for normalization of firefly luciferase signals. pcDNA-myc-ΔN-hTcf4 was obtained from AddGene, which was originally deposited by Dr. B. Vogelstein (Johns Hopkins University Oncology Center, Baltimore, MD) (37). The Cignal Tcf/Lef-luciferase reporter assay kit with control plasmid was purchased from Qiagen-SABiosciences (Frederick, MD).

Site-directed Mutagenesis—The consensus sequences of the Tcf binding sites at −640 bp and −545 bp on the Numb promoter were mutated from CCTTTGCT to either CTTTTACT (Mut-1) or CTTTTGTT (Mut-2), and from GTCTAAACC to either GGTATAATCC (Mut-3) or GGTATAACTC (Mut-4), respectively. Mutagenesis of individual Tcf sites within the pGL3 promoter-Luc reporter was conducted using the Phusion site-directed mutagenesis kit (New England Biolabs) following the manufacturer’s recommended procedures with the following PCR conditions: initial denaturation at 98 °C for 30 s; amplification cycles of 98 °C for 10 s, 65 °C for 20 s, 72 °C for 2.5 min. The final extension was for 10 min at 72 °C. The cDNA insert containing the mutated promoter region was excised using KpnI and XhoI and subcloned back into pGL3 promoter-Luc at these same restriction sites.

To generate double mutants at both TCF binding sites, the consensus sequences of these sites were mutated from CCTTTGCT to CTTTTATTT and from GTCTAAACC to GGTATAATTCC. The corresponding cDNA sequence flanked by restriction sites for KpnI and XhoI was synthesized by IDT Inc. (Coralville, IA). The cDNA was digested with KpnI and XhoI and cloned into corresponding sites of pGL3 promoter-Luc. All sequences were verified by DNA sequencing.

Statistics—The data are expressed as mean values ± S.E. The significance of differences among means was tested by one-way analysis of variance (ANOVA) followed by Dunnett’s test.
analysis of variance with a Bonferroni test post hoc. Statistical calculations were performed using Prism 4.0 (GraphPad Software).

RESULTS

Nandrolone Up-regulated Numb Expression in C2C12 Myoblasts—We initially investigated the effect of nandrolone on Numb mRNA and protein expression in differentiating C2C12 cells. As shown in Fig. 1A, Rtc-PCR revealed that treatment of cells with nandrolone induced a significant increase in Numb mRNA which started at 16 h and reached a peak at 48 h after treatment; Numb mRNA declined to basal levels thereafter. The effects of nandrolone on Numb protein expression and subcellular distribution were also evaluated. Western blot analysis showed a significant up-regulation of Numb protein levels induced by nandrolone in both the nuclear and cytosolic fractions (Fig. 1B and C). To confirm this finding, we performed immunofluorescence staining using an antibody against Numb. In the vehicle-treated cells, the intensity of positive stained cells was low and was located mainly in cytosol fraction with only a few cells showing staining in the nucleus. In contrast, treatment of the cells with nandrolone for 3 days increased the intensity of staining for Numb in both cytoplasm and nucleus (Fig. 1D). These results confirmed a role for nandrolone in up-regulation of Numb expression, at the levels of mRNA and protein.

Nandrolone-promoted Wnt Signaling Activity Was Associated with Increased GSK3β Phosphorylation at Ser-9—Nandrolone is an androgen and acts via the AR (34). The AR pathway is a critical regulator of Wnt signaling (28, 29, 31, 40–42).
We investigated the effect of this anabolic steroid on the Wnt signaling activity. Levels of total \(\beta\)-catenin and nuclear active \(\beta\)-catenin protein were examined using antibodies against total \(\beta\)-catenin and active \(\beta\)-catenin, which lack phosphorylation on the sites of Ser-37 or Thr-41, respectively (38). C2C12 cells were cultured under differentiating conditions and treated with either vehicle or nandrolone. Whereas nandrolone treatment had no effect on total \(\beta\)-catenin protein and mRNA levels (Fig. 2, A and C), it induced a significant increase in nuclear active \(\beta\)-catenin protein levels in a time-dependent manner. Nuclear \(\beta\)-catenin increased by 16 h and peaked at 72 h after initiating the treatment (Fig. 2, A and B).

To determine whether nandrolone-induced nuclear accumulation of \(\beta\)-catenin led to an increase in \(\beta\)-catenin transcriptional activity, the effect of nandrolone on the activation of Tcf transcription factors was tested using a Tcf luciferase reporter assay. As shown in Fig. 2D, cells treated with nandrolone demonstrated a significant enhancement of Tcf luciferase activity that occurred in a time-dependent manner, indicating an increased transcriptional activity of Wnt signaling. Because intracellular \(\beta\)-catenin fate is regulated by GSK3\(\beta\) (4–6, 39), we next tested the effect of nandrolone on GSK3\(\beta\) activity by measuring the protein levels of phospho-GSK3\(\beta\) at Ser-9, which is an inhibitory phosphorylation site (6). Although there was no
change in the levels of endogenous GSK3β, an increase in phospho-GSK3β⁵⁶⁹ levels was observed in the cells treated with nandrolone (Fig. 2, E and F), indicating an inhibitory effect of nandrolone on GSK3β activity. To further confirm that nandrolone activated Wnt/β-catenin signaling, we examined mRNA levels for several Wnt target genes. Nandrolone increased mRNA expression of cyclin D1, Pitx2, and Runx2 in a time-dependent manner.

Enhancement of Wnt Activity Mimicked the Effect of Nandrolone on Numb Expression—To further explore the relationship between nandrolone-induced Numb expression and Wnt signaling, we tested the effect of Wnts on the levels of Numb mRNA and protein. Cells were incubated under differentiating conditions and treated with vehicle, Wnt3a, Wnt5a, or nandrolone. Wnt3a-treated cells demonstrated a significant up-regulation in Numb mRNA expression (Fig. 3A) suggesting a transcriptional regulation of Numb by Wnt. Wnt3a also increased levels of Numb protein (Fig. 3, B and C). Thus, Wnt3a mimicked the effect of nandrolone. In contrast, Wnt5a had no effect on the expression of either Numb mRNA or protein. These results supported an involvement of the canonical Wnt pathway in up-regulation of Numb mRNA levels by nandrolone. Because inhibition of GSK3β is a widely used approach to mimic the effects of an activated Wnt/β-catenin pathway, we treated cells with SB261762, a GSK3β inhibitor. We observed the expected accumulation of β-catenin in the nucleus which was concomitant with a significant increase in Numb protein expression. However, nandrolone did not induce a further increase in Numb protein expression in the presence of SB261762 (Fig. 3, D and E).

Nandrolone-up-regulated Numb Expression Was Prevented by Inhibition of Wnt/β-Catenin Signaling—To further investigate whether enhanced Wnt activity is responsible for the increased expression of Numb, the effects of two naturally occurring inhibitors of Wnt signaling on the expression of Numb were tested. Although incubation of cells with Dkk1 or sFRP1 alone had no significant effect on the expression of Numb mRNA or protein, both Dkk1 and sFRP1 prevented the increase in Numb mRNA and protein induced by nandrolone (Fig. 4, A–C). To extend these findings, β-catenin siRNA was employed to inhibit the expression of β-catenin. Cells transfected with β-catenin-siRNA demonstrated significant reductions in β-catenin mRNA and protein. Gene knockdown with β-catenin siRNA did not alter basal levels of Numb mRNA or protein but prevented nandrolone-induced up-regulation of Numb mRNA (Fig. 4D) and protein (Fig. 4, E and F), compared with the cells transfected with nonsilencing random siRNA (negative control). Thus, canonical Wnt signaling was not necessary for expression of Numb in untreated cells, but increases in such signaling were required for up-regulation of Numb expression by nandrolone.

Nandrolone Promoted the Binding of β-Catenin to the Numb Promoter—The above findings indicate a role for Wnt signaling in regulating Numb transcription. We next tested whether Numb is a direct transcriptional target of Wnt signaling. We analyzed the first 2 kb of the proximal promoter of the Numb gene by computational methods to search for potential Tcf sites using Transcription Element Search System (TESS) (University of Pennsylvania). This analysis identified several potential Tcf sites, including three putative Tcf4 binding sites (Fig. 5A). To determine the physiological relevance of the Tcf binding sites found in the Numb promoter, we performed ChIP assays with a monoclonal anti-β-catenin antibody. PCR amplification of the DNA fragments immunoprecipitated with this antibody showed that the anti-β-catenin antibody pulled down genomic DNA containing the Tcf1/4 and Tcf1 1/2/4 sites located at −545 and −640 bases upstream of the transcriptional start site (Fig. 5B). Moreover, binding of β-catenin to this promoter region was increased when cells were incubated with nandrolone and Wnt3a (Fig. 5B). To obtain further evidence for a functional interaction of β-catenin with Tcf4 bound to this region of the Numb promoter, we generated a luciferase reporter construct, pGL3-Tcf4-Numb.F1-Luc, by cloning the fragment pulled down by ChIP into the pGL3 promoter vector. We observed that there were 2.5- and 2.1-fold stimulations of the promoter activity in the presence of Wnt3a and nandrolone, respectively. This induction was suppressed by co-transfection of a dominant negative Tcf4 (pcDNA-myc-ΔN-Tcf4)...
consistent with a role for a Tcf transcription factor in H9252-catenin-induced up-regulation of luciferase reporter gene activity.

Mutations in the Predicted Tcf Binding Sites Abolished Nandrolone-induced Increases in Numb Transcriptional Activity—To determine whether the predicted Tcf binding sites located in the Numb promoter mediate nandrolone-induced stimulation of Numb promoter activity, we performed site-directed mutagenesis of those sites individually or in combination (Fig. 6A), and determined the activity of the resulting mutants in luciferase reporter assays in this cell line. The effect of Wnt3a was also tested as a positive control. Single binding site mutations (Mut-1, -2, -3, and -4) partially inhibited nandrolone-mediated luciferase activity. In contrast, mutation of both Tcf binding sites (double mutation) abolished the elevated luciferase activity stimulated by nandrolone (Fig. 6B), suggesting that these Tcf binding sites are required for nandrolone-up-regulated Numb promoter activity.

DISCUSSION

Our data indicate that in this C2C12 myoblast cell culture system, canonical Wnt signaling increases levels of Numb mRNA and that up-regulation of Numb mRNA levels by nandrolone is mediated by increased canonical Wnt signaling. The most compelling evidence supporting this conclusion is that nandrolone-induced up-regulation of Numb mRNA levels was blocked by sFRP1, which binds Wnts, and by Dkk1, which interacts with Wnt co-receptors LRP5/6 to inhibit canonical Wnt signaling. In addition, up-regulation of Numb mRNA and protein levels was prevented by knockdown of β-catenin expression using a siRNA. Our findings that addition of factors that increase canonical Wnt signaling (Wnt 3a and GSK3β inhibitor) increased Numb mRNA and protein levels further support these conclusions. Given the fact that Numb and androgens/anabolic steroids are able to promote myogenic differentiation of precursors (20–26), our findings support the notion that this
androgen action results from up-regulation of Numb induced by Wnt signaling in C2C12 myoblasts. Nandrolone has been shown to stabilize Numb protein, apparently by diminishing mdm2 levels (34), and, possibly, through altering levels of Musashi protein. An interesting question is whether these multiple levels of control of Numb expression share a common mechanism, such as canonical Wnt signaling.

Our data also support the conclusion that Wnts directly increase transcription of the Numb gene through association of β-catenin with a region of the Numb promoter located at −545 and −640 bp upstream of the transcriptional start site. It appears likely that β-catenin co-ordinates with Tcf1, Tcf2, or Tcf4 bound at one or both consensus DNA sequences for these transcription factors found to be present in this DNA sequence by computational analysis and manual inspection. This conclusion is supported by the fact that β-catenin was recruited to the region of the Numb promoter containing these Tcf sites and that nandrolone increased β-catenin recruitment to this region of the Numb promoter. In addition, this region of the Numb gene conferred Wnt and nandrolone responsiveness to a minimal promoter of a reporter gene, and expression of a dominant negative Tcf4 blocked up-regulation of the reporter gene in response to Wnts or nandrolone. More supportive evidence was that mutations in those Tcf binding sites abolished the increase in Numb transcriptional activity induced by both nandrolone and Wnt3a. Thus, our data demonstrate a novel mechanism whereby Wnts up-regulate Numb expression through canonical Wnt signaling and binding of β-catenin to regulatory regions of the Numb promoter, most likely at Tcf sites. Therefore, the present study identifies Numb as a novel downstream target gene of the Wnt/β-catenin signaling pathway.

Accumulating evidence has underlined the importance of the mutually inhibitory cross-talk between Wnt and Notch pathways (11–16, 43–45). In injured skeletal muscle, Notch and Wnt exert opposing influences on proliferation and differentiation of progenitor cells, respectively, as illustrated in studies by Brack et al. using cultured muscle satellite cells (4). The molecular mechanisms that underlie these opposing actions are incompletely understood. Notch signaling activates GSK3β (4) and reduces β-catenin levels through post-translational mechanisms (43). Conversely, Wnts down-regulate Notch expression (44) and have been found through undefined mechanisms to support Numb expression and promote cell fate determination in hepatic progenitor cells (45). Moreover, we observed an inhibitory effect of nandrolone on Notch activity in denervated skeletal muscle (33) and C2C12 cells, associated with increased levels of Numb. Given the fact that Numb is a critical inhibitor of Notch, our data demonstrate a novel mechanism by which Wnt and Notch signaling pathways interact, specifically, by up-regulation of Numb transcription through canonical Wnt signaling and, most likely, inhibition of Notch signaling.

Our data also suggest that this novel mechanism for interaction between Wnt and Notch signaling through Wnt-dependent up-regulation of Numb is activated by nandrolone, suggesting an involvement of the AR signaling in the regulation of Wnt/Notch cross-talk. This is because our data also indicate that nandrolone up-regulates Numb gene expression through canonical Wnt signaling and demonstrate that androgens stimulate Wnt signaling in cells of the myoblast lineage. Because both Numb and Wnts promote myogenic differentiation (1–3, 20, 21), our findings suggest a mechanism whereby androgens may promote myogenic differentiation of less differentiated progenitor cells through increased Wnt signaling and Numb expression and resultant reduced Notch signaling. Moreover, the finding that nandrolone up-regulates Numb expression in C2C12 cells raises the possibility that nandrolone or other androgens may regulate Numb expression and cell fate decisions in other populations of progenitor cells, such as cancer stem cells. In addition to inhibiting signaling through Notch, Numb also regulates signaling through p53 and Hedgehog (46, 47), and there are likely to be other biochemical and biological consequences of elevated Numb levels beyond reduced Notch signaling induced by the AR and Wnt signaling pathways. Further studies are warranted to explore these possibilities.

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