Notch3 and Mef2c Proteins Are Mutually Antagonistic via Mkp1 Protein and miR-1/206 MicroRNAs in Differentiating Myoblasts*

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**Background:** Notch3 is expressed in myogenic precursors, but its function is not well known.

**Results:** Notch3 represses the activity of Mef2c and is in turn inhibited by the microRNAs-1 and -206.

**Conclusion:** Notch3 serves as a regulator for preventing premature myogenic differentiation.

**Significance:** Understanding how precocious differentiation is prevented is critical for designing therapy for skeletal muscle regeneration.

The Notch signaling pathway is a well known regulator of skeletal muscle stem cells known as satellite cells. Loss of Notch1 signaling leads to spontaneous myogenic differentiation. Notch1, normally expressed in satellite cells, is targeted for proteosomal degradation by Numb during differentiation. A homolog of Notch1, Notch3, is also expressed in these cells but is not inhibited by Numb. We find that Notch3 is paradoxically up-regulated during the early stages of differentiation by an enhancer that requires both MyoD and activated Notch1. Notch3 itself strongly inhibits the myogenic transcription factor Mef2c, most likely by increasing the p38 phosphatase Mkp1, which inhibits the Mef2c activator p38 MAP kinase. Active Notch3 decreases differentiation. Mef2c, however, induces microRNAs miR-1 and miR-206, which directly down-regulate Notch3 and allow differentiation to proceed. Thus, the myogenic differentiation-induced microRNAs miR-1 and -206 are important for differentiation at least partly because they turn off Notch3. We suggest that the transient expression of Notch3 early in differentiation generates a temporal gap between myoblast activation by MyoD and terminal differentiation into myotubes directed by Mef2c.

Notch signaling has been shown to be critical for both skeletal muscle development and response to injury in adults. Classically, Notch signaling involves the interaction of a ligand on one cell and a receptor on its neighbor (for review, see Ref. 1). The receptor is proteolytically cleaved, releasing the intracellular domain (ICD), which then shuttles into the nucleus. The DNA-binding protein for Notch is Rbpj, which is bound to DNA even in the absence of Notch ICD. Without the ICD, Rbpj is associated with transcriptional repressors (2, 3). When ICD interacts with Rbpj along with the co-activator MamL (4), it displaces the repressors and initiates transcription. Genes promoted by the ICD are generally inhibitory to terminal differentiation of skeletal muscle (5). If Rbpj is deleted in skeletal muscle by tissue-specific knock-out, the result is uncontrolled differentiation of myogenic progenitors resulting in severe hypotrophy (6).

After embryonic development, adult postmitotic skeletal muscle retains a reservoir of progenitor cells that are referred to as satellite cells due to their anatomical location outside of the fiber but underneath the basement membrane (7, 8). Deletion of Rbpj in satellite cells by inducible recombination results in spontaneous differentiation of quiescent satellite cells (9–11), indicating that Notch signaling is continually required to maintain quiescence. Notch has been shown to antagonize the activity of the basic helix-loop-helix transcription factors collectively called muscle regulatory factors (MRFs) (12, 13). These are four genes (Myf5, MyoD, Myog, Myf6) that promote myogenic progression and transdifferentiation. They can work in concert with transcription factor Mef2c (14), which is also known to be antagonized by Notch (15).

The activity of MRFs and Mef2c can be regulated via microRNAs (for review, see Ref. 16). MicroRNAs are genes that are initially transcribed as a long transcript by Pol II or Pol III, which is referred to as the pri-miRNA. The RNA then folds into a hairpin and is cut by Drosha/DGCR8 into a hairpin shaped pre-miRNA and exported to the cytoplasm. Most miRNAs are then processed by Dicer into a 19–24-bp single-stranded mature miRNA. One strand of the hairpin is then preferentially loaded into the RNA-induced silencing complex. Silencing is achieved through destabilizing target mRNAs and blocking translation. Among the first microRNAs to be identified in skeletal muscle are miR-1 and miR-206 (17, 18). They are highly

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4 The abbreviations used are: ICD, intracellular domain; ChIP-seq, chromatin immunoprecipitation and high throughput sequencing; DM(1–5), differentiation medium (days in DM); FF, firefly luciferase; GM, growth medium; MCK, muscle creatine kinase; MRF, muscle regulatory factor; qRT-PCR, quantitative RT-PCR; RL, Renilla luciferase; shSCR, scrambled hairpin; TK, thymidine kinase.
up-regulated in differentiation, and their expression is much higher in striated muscle than all other tissue.

Satellite cells, like all stem cells, undergo asymmetric cell divisions; one daughter cell begins to differentiate; the other returns to quiescence as a progenitor. Notch1 is normally expressed in progenitor cells, whereas the differentiating cell turns off Notch1 by targeting it for degradation by Numb (19) via the E3 ubiquitin ligase Itch (20). A subset of satellite cells has never expressed the myogenic transcription factor Myf5, and these stem-like cells express Notch3 at a higher level than those that have expressed Myf5 (21). After a cell division, the differentiating daughter cell begins to express Myf5 as well as the Notch ligand Dll1. Aside from high expression in Myf5-negative satellite cells, the role of Notch3 in skeletal muscle is not well known. The expression of Notch3 was shown to be higher in quiescent than actively dividing satellite cells (22). Notch3 (23) has been shown to be targeted by miR-206, but in the context of HeLa cells. We set out to investigate whether miRNA regulation of Notch3 has important effects on myogenic differentiation in skeletal muscle. To our surprise, we discovered that Notch3 is initially induced during differentiation even though activated Notch3 normally inhibits differentiation. The subsequent inhibition of Notch3 by miR-1 and miR-206 is therefore critical for differentiation. Intriguingly, Notch3 and the myogenic transcription factor Mef2c set up a mutually antagonistic network that is dependent on the actions of the microRNAs and that acts as a bistable switch. In one position the switch is antagonistic to differentiation, whereas in the opposite position it promotes differentiation.

MATERIALS AND METHODS

Cell Culture—C2C12 myoblasts were cultured in DMEM with 20% fetal bovine serum (FBS) and 1% penicillin/streptomycin (growth medium or GM). 10T1/2 fibroblasts were cultured similarly but with 10% FBS. Cells were induced to differentiate by replacing 20% FBS with DMEM containing 2% horse serum (differentiation medium or DM). Transfection with miRNA mimics or inhibitors was performed as described before (24). Retroviruses for the creation of stable cells were generated in 293T cells. All transfections were performed with Lipofectamine 2000 (Invitrogen). Cells were transfected with the viral vector, as well as a vesicular stomatitis virus G and a gag/pol encoding plasmid. After 48 h, the supernatant was collected, centrifuged to pellet any 293T cells, and passed through a 45-μm filter. This viral supernatant was then added to the target cells in the presence of Polybrene. Stable cell lines were selected by puromycin (1.5 mg/ml) for 48 h.

Plasmids—Mir-1/206 encoding oligonucleotides were cloned into a plasmid based on the miR-30 conformation as described previously (25). The short hairpin for Notch3 knockdown was designed by The RNAi Consortium (clone TRCN0000075569) cloned into pLKO.1 plasmid. Scrambled hairpin (shSCR) was a predesigned control (Addgene plasmids 10878 and 10879). The target sequence of shNotch3 is CGTGTGTTAGACCGGTGCAATA. The target sequence of synthetic siRNA to Notch3 is GTACAGAGATGGTGGATGA. Notch3-ICD expression plasmids were cloned by PCR from C2C12 cDNA and ligated into pBabe-puro vectors. Mutagenesis was performed by PCR amplification and DpnI digestion to remove parental DNA. Notch3 3′-UTR sequence was PCR-amplified from C2C12 genomic DNA and cloned into pRL-CMV vector. Enhancer activity was tested by cloning a ~500-bp region around the ChIP-seq binding site and ligating it into a pGL3 promoter plasmid. MCK luciferase was a kind gift from Dr. Stephen Tapscott. The 4RE and 3′XMe2 plasmids were ordered from Addgene (plasmid 16057/32967).

RT-PCR—Cells were lysed and total RNA was extracted using TRIzol reagent (Invitrogen) following the manufacturer’s instructions. Ncode miRNA first-strand cDNA synthesis and a qRT-PCR kit (Invitrogen) were used to perform qRT-PCR for microRNA detection. For mRNA detection, cDNA synthesis was carried out using the Superscript III first-strand synthesis system for RT-PCR (Invitrogen). Then, qPCR was carried out using SYBR Green PCR master mix in an ABI cycler. ABI 7300 software was used for quantification (Applied Biosystem).

Luciferase Assays—For the assays measuring enhancer activity (see Figs. 2, 6, C and D, and 8D), the negative control reporter is the firefly luciferase (FF) driven by a minimal SV40 promoter (pGL3 promoter), and the test reporters are pGL3 into which the test enhancer (Notch3 enhancer in Fig. 2, 3′XME2 binding sites in Fig. 6D) has been cloned. For MCK activity (see Figs. 6C and 8D), the MCK enhancer-promoter drives the FF. In all experiments we co-transfected a Renilla luciferase (RL) reporter driven by the herpes simplex virus thymidine kinase (TK) promoter-enhancer as a transfection control and where indicated additional pcDNA3 based plasmid expressing transcription factors. All transfections in an experiment contained equal amounts of DNA through the addition of pcDNA3 plasmid when necessary. The transfections were performed in NIH3T3, 10T1/2 fibroblasts, or C2C12 cells as indicated. Experiments were analyzed with the dual-luciferase reporter assay system (Promega) following the manufacturer’s instructions. The luminescent signal was quantified by luminometer (Monolight 3020; BD) Luciferase activity expressed as the FF/RL ratio measures the strength of the test enhancer-promoter, which is plotted either as a raw value (see Figs. 2D and 6C and D) or after normalization to the ratio seen under basal conditions (see Figs. 2B and 6C, and 8D). p values were calculated by Student’s t test.

For the assays measuring the repression of the Notch3 3′-UTR (see Fig. 3B), a miRNA expression plasmid, a RL test reporter containing the Notch3 3′-UTR (2 ng), and a FF control with a vector-derived 3′-UTR (5 ng) were co-transfected into NIH3T3 cells. The luciferase activity expressed as the RL/FF ratio is plotted in the figure after normalization to the ratio in the shCON-transfected cells. In Fig. 3C the RL (test) and FF (control) plasmids were transfected into C2C12 cells held in GM or in DM for 3 days. The RL/FF ratio is plotted as luciferase activity.

ChIP-seq Mapping—We used Novoalign (version 2.05.04) to align reads from the Sequence Read Archive to the mouse reference genome (NCBI v37, mm9). PeakSeq (version 1.1) was used to identify enriched areas of Notch1 (SRR243559 and SRR243551) over control (SRR243561). From the PeakSeq results, we created BedGraph files containing peaks with a p
value of at most 0.0001. MyoD ChIP-seq mapping was described previously (26).

Western Blotting and Antibodies—For Western blotting cells were lysed in radioimmune precipitation assay buffer supplemented with protease inhibitor mix (Sigma). Proteins were resolved by SDS-PAGE, transferred, and immunoblotted with various antibodies. The antibodies used anti-myosin heavy chain (mouse; Sigma), anti-GAPDH (mouse; Sigma), anti-Mef2c (5030; Cell Signaling), anti-Mkp1 (M-18; Santa Cruz Biotechnology), and anti-Notch3 (M-134; Santa Cruz Biotechnology).

Animal Experiments—Animal experiments were performed as described previously (26).

RESULTS

Notch3 Levels Increase Early during Differentiation—Based on previous reports we had expected Notch3 to have higher expression in quiescent cells. We used a mouse model of muscle injury where the tibialis anterior is injected with snake venom cardiotoxin. Following extensive degeneration of myofibers during the first 2 days, there is rapid proliferation of myogenic precursors followed by differentiation so that the muscle heals and resembles uninjured tissue again by day 14 after injection. In this context we surprisingly found that the expression of Notch3 increases very early in the differentiation process, on days 1–3 (Fig. 1A). The pattern of expression mimicked, albeit with lower fold change, the pattern observed for MyoD (Fig. 1B). It is worth noting, that in regenerating skeletal muscle, the miR-206 microRNA decreased on day 1, but rapidly increases on day 3 to a level above base line after day 4 as differentiation supersedes stem cell proliferation (27).

In another model of differentiation, C2C12 myoblasts are induced to differentiate by transfer from serum-rich GM to serum-poor DM. In this model, there was also a transient increase of Notch3 mRNA (Fig. 1C) and protein (see Fig. 7A) early in the differentiation program. miR-206 begins to rise on day 1 after transfer to DM (24).

Notch3 Is Induced by an Enhancer That Responds to the Combined Presence of MyoD and Activated Notch—The mRNA expression pattern of Notch3 peaking at day 3 before declining was not consistent with previously described miR-206 targets DNA polymerase α (18) and Pax7 (24). Those peaking on DM3 decreased precipitously from day 1 in DM. Notch3 has previously been shown to have increased expression in C2C12 co-culture with NIH3T3 fibroblasts that stably express Notch ligand (28). Consistent with the established regulation of Notch3 by canonical Notch signaling, we found that overexpression of the Notch3 intracellular domain (N3-ICD) induces endogenous Notch3 expression at all time points in C2C12 differentiation. However, other canonical Notch targets, such as Hes1, have minimal changes in mRNA expression during C2C12 differentiation (29). Therefore, we hypothesized that Notch3 is induced via myogenic transcription factors. Analysis of previously published ChIP-seq data (30) showed the presence of a MyoD binding site near the 3′ end of intron 1 of the Notch3 gene that is close to several E boxes (Fig. 2A).

We thus tested whether the 3′ end of intron 1 of Notch3 has a MyoD-responsive enhancer by cloning this site into an enhancerless luciferase reporter. When we found that the putative enhancer was not activated by co-transfection of MyoD in fibroblasts (Fig. 2B, MyoD alone), we therefore considered the possibility that Notch and MyoD were both necessary for induction. We examined Notch1 and Rbpj ChIP-seq data (31) for evidence of canonical Notch binding within the Notch3 locus. Surprisingly, there was a Notch1-bound peak at the same locus as MyoD (Fig. 2A) in published ChIP-seq data (31). This
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FIGURE 2. Transcription of Notch3 is stimulated by an enhancer with both MyoD and Notch1 binding. A, top, ChIP-seq shows MyoD binding relative to the end of intron 1 of the Notch3 locus in C2C12 myoblasts. The y axis represents the number of independent sequence reads that overlap within a given 10-base window in the MyoD ChIP. The primary data are from Ref. 30. The locations of canonical E-boxes are represented by black bars at the top of the graph. A, bottom, ChIP-seq shows Notch1 binding to the same locus. The window size has been reduced to 1 bp to show two distinct binding sites, corresponding with two canonical Rbpj binding sequences (TGGGAA). B, the Notch3 enhancer is activated only when Notch3-ICD and MyoD are present together. FF driven by no enhancer or the Notch3 enhancer and RL driven by the HSV TK promoter were co-transfected with plasmid indicated on the x axis into NIH3T3 (details under “Materials and Methods”). The luciferase activities (FF/RL) were normalized to that from pGL3 in the absence of co-transfected transcription factors and plotted as mean ± S.D. (error bars) of three measurements. C, the Notch3 enhancer requires both E-boxes and Rbpj sites. Mutations were made in the Notch3 enhancer plasmid in either the three E-boxes (Ebox mut) or the two Rbpj sites (Rbpj mut). Co-transfection of the FF plasmid with RL driven by HSV TK promoter and cDNA3 plasmid expressing nothing, MyoD or Notch3-ICD was repeated as in B. The -fold stimulation of luciferase activity (FF/RL) by MyoD + N3-ICD relative to a pcDNA3 empty vector is plotted after normalization to the -fold stimulation of pGL3 as mean ± S.D. of three measurements. D, activation of the Notch3 enhancer during C2C12 differentiation requires both E-boxes and Rbpj sites. Indicated Notch3 enhancer reporters (FF) and HSV TK-driven RL were transfected into C2C12 and assayed after 24 h in GM or after 48 additional h in DM. FF/RL ratios on the y axis are mean ± S.D. of three measurements.

site is also marked by H3K27ac in ENCODE project dataset in human skeletal muscle myoblasts (data not shown). Furthermore, there seems to be some specificity as only the myoblasts and lymphoblasts have H3K27ac enrichment at this locus. The Notch1 binding peak is composed of two local maxima corresponding with two canonical Rbpj binding sequences (TGGGAA) with the tails facing the E-box pair (Fig. 2A).

All of these ChIP-seq data are consistent with the possibility that the enhancer at the 3’ end of intron 1 of Notch3 responds to the combined action of Notch and MyoD. Indeed, in subconfluent fibroblasts, co-transfection of the enhancer-driven luciferase reporter with either Notch1-ICD (data not shown) or Notch3-ICD (Fig. 2B) failed to activate the enhancer. However, when either Notch1-ICD or Notch3-ICD was co-transfected with MyoD, there was a significant activation of the Notch3 enhancer (Fig. 2B). We confirmed that the E-boxes (for attracting MyoD) and the Rbpj boxes (for attracting Rbpj and Notch) were independently required for this stimulation by site-directed mutagenesis. Either set of mutations was sufficient to decrease the activation of the enhancer by MyoD or N1-ICD (Fig. 2C). In C2C12, the WT reporter was not active at all in undifferentiated myoblasts, but each mutant had weak activity (Fig. 2D). After 48 h in differentiation media, the WT enhancer was the most active, whereas each mutant had significantly less activity. However, in C2C12, the E-box mutant displayed lower activity than the Rbpj mutant. Together, this suggests that the Notch3 enhancer is a specialized response element that responds to the combined presence of MyoD and activated Notch, accounting for the specific induction of Notch3 early during the differentiation process.

Notch3 Is Targeted by miR-1/206—The induction of Notch3 in both the in vivo and in vitro differentiation models was transient. Because Notch3 can activate itself, we reasoned that the repression of Notch3 by muscle differentiation-induced microRNAs could switch the equilibrium in the direction of Notch3 repression. The earlier report in HeLa cell showed one miR-206 binding site in the Notch3 3’-UTR (23) (Fig. 3A, site 2). In addition, we found that there is a second binding sites for miR-1/206 in the Notch3 3’-UTR (Fig. 3A, site 1). This new site uses central base pairing with the microRNA (32) in contrast to the traditional seed pairing found in site 2 (Fig. 3A). Because recent reports have shown a difference in activity for miR-1 and miR-206 in certain cases (33), we tested whether both miR-1 and miR-206 repress the 3’-UTR of Notch3 by co-transfecting the luciferase-Notch3 3’-UTR constructs with plasmids expressing short hairpins that were control, miR-1, or miR-206 (Fig. 3B). Both microRNAs effectively repressed the 3’-UTR, and the repression was mediated by both sites 1 and 2 in the 3’-UTR. The mutation of both sites prevented any repression by either microRNA. To show that the repression occurs with endogenous microRNA levels, the 3’-UTR constructs were transfected into differentiating C2C12. The wild type 3’-UTR was repressed on day 3 of differentiation, but the 3’-UTR with the miR-1/206 sites mutated was not (Fig. 3C). We created a C2C12 cell line that stably expresses an inhibitor (34) of miR-1/206. In both myoblasts and differentiated cultures, the levels of Notch3 mRNA
were higher in the presence of the miR-1/206 inhibitor (Fig. 3D).

**Notch3 Represses miR-1 and miR-206**—In multiple instances, we have found that a muscle differentiation-induced microRNA represses an inhibitor of differentiation, and we also discovered that the inhibitor of differentiation directly or indirectly represses the microRNA (16). We therefore tested whether in addition to promoting its own transcription by transactivation, Notch3 could reduce the expression of miR-1/206. We first tested this by making C2C12 cell lines that stably expressed the ICD of Notch3 (N3-ICD). On the 4th day of differentiation (DM4) both miR-1 and miR-206 were significantly repressed in the C2C12 cells expressing the N3-ICD (Fig. 4A).

**Notch3 Prevents Premature Differentiation via Inhibition of Me2c**—We next tested whether Notch3 interacts with and inhibits the myogenic transcription factors that induce the myogenic differentiation-induced microRNAs. Activated Notch3 delayed but did not block myoblast differentiation, as evidenced by the consistently reduced expression of the myosin heavy chain Myh3 on all days during differentiation (Fig. 5A). In contrast, the basic helix-loop-helix myogenic transcription factors, MyoD and myogenin were lower initially on DM1 in the C2C12 cells stably expressing N3-ICD compared with empty vector controls, but then recovered and were higher at later time points (Fig. 5, B and C). Me2c mRNA, on the other hand, was consistently repressed at all time points of differentiation (Fig. 6A). Me2c protein was also significantly repressed in differentiating C2C12 by N3-ICD, whereas myogenin was not (Fig. 6B). These results suggested that Notch3 may delay differ-

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> FIGURE 3. **The Notch3 3′-UTR is repressed by miR-1/206.** A, miR-1 and miR-206 binding sites 1 and 2 within the Notch3 3′-UTR are shown. Line indicates complementary base pairing, and colon (:) represents G-U base pairing. B, miR-1 or -206 represses the Notch3 3′-UTR through their binding sites. RL test reporter containing wild type Notch3 3′-UTR or mutants of binding sites 1, 2, or both (as indicated on the x axis) and a negative control FF reporter containing vector-derived 3′-UTR were transfected into NIH3T3 cells along with plasmids expressing hairpin precursors for miR-1 (sh1), miR-206 (sh206), or scrambled control (shCON). Values on y axis represent mean ± S.D. (error bars) of three measurements of RL/FF after normalization to the ratio seen with shCON. C, repression of Notch3 3′-UTR in differentiating C2C12 requires the miR-1/206 binding sites. The RL reporter containing indicated Notch3 3′-UTR were transfected as in B with FF control into C2C12 cells in GM, which were then allowed to differentiate for 72 h (DM3). Values represent the average of three biological replicates of RL/FF in DM3 divided by the RL/FF in GM. D, inhibition of miR-206 induces Notch3 mRNA in C2C12 cells. Stable expression of an inhibitor (34) of miR-206 (TuD NC) or a negative control (TuD 1) in C2C12 cells in GM or DM4 is shown. Notch3 mRNA was measured by qRT-PCR, normalized to GAPDH mRNA, and expressed relative to the value in C2C12 cells transfected with TuD NC and held in GM. Results are mean ± S.D. of three measurements.

> FIGURE 4. **Notch3 ICD has a negative feedback on miR-1/206 levels.** A, expression of miR-1/206 was measured by qRT-PCR (and normalized to snu6) is blocked by overexpression of Notch3 ICD in C2C12 cells. y axis represents the stimulation of miRNA on DM4 versus GM. Values are the mean ± S.D. (error bars) of three measurements. B, knockdown of Notch3 leads to precocious expression of miR-1/206 on DM1 in C2C12 cells. Cells were infected with a retrovirus expressing either a short hairpin with scrambled sequence (shSCR) or a hairpin targeting Notch3 (shN3). y axis represents the increase in miRNA (normalized to snu6) on DM1 versus GM. Values are the mean ± S.D. of three measurements. C, knockdown of Notch3 with synthetic siRNA leads to decreased Notch3 mRNA level. Cells were transfected with either GL2 control siRNA targeting Notch3 (siN3). D and E, knockdown of Notch3 with synthetic siRNA to Notch3 had any sequence homology to Notch1. Therefore Notch3 represses miR-1 and -206.
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The activity of Mef2c is the more likely target of inhibition rather than transcription. The decrease in Mef2c mRNA is most likely explained by Mef2c autoactivating its own promoter (36).

Because Mef2c has been reported to induce miR-1 (37) and because the 3′-UTR of Notch3 is repressed by miR-1/206, we hypothesized that whereas Notch3 can repress Mef2c, the latter can itself negatively feed back and repress Notch3 via miR-1/206. We transfected equal amounts of constructs expressing just the N3-ICD, the ICD and the 3′-UTR, or the ICD and UTR with the miR-1/206 sites mutated. All three N3-ICD expressing vectors repressed Mef2 transcriptional activity, but the N3-ICD with the wild type 3′-UTR was significantly weaker at repressing Mef2 than the other two (Fig. 6C). This result is consistent with the hypothesis that (i) Notch3 suppresses Mef2c activity and (ii) Mef2c can suppress Notch3 through the miR-1/206 sites in the 3′-UTR of Notch3.

Notch3 Delays Differentiation—If Notch3 inhibits Mef2c, it is expected to inhibit differentiation. To test this hypothesis, we created stable C2C12 cell lines infected with lentiviral vectors that express shRNA to Notch3 or a shSCR. These cells suppressed the transient induction of Notch3 early in differentiation (DM1 and 2, Fig. 7A). The knockdown of Notch3 induced earlier differentiation as seen from the earlier and higher expression of myosin heavy chain and troponin T compared with controls (Fig. 7A). The troponin began to decrease later in differentiation consistent with earlier findings (38). Whereas the expression of MyoD was somewhat higher in the shNotch3, myogenin was not. Knockdown of Notch3 promoted the for-

entiation through the inhibition of Mef2c mRNA and protein expression.

The mutual exclusivity of Notch3 and Mef2c expression is also seen from examining published microarray data of gene expression (Table 1). In C2C12 culture, it is possible to separate the differentiated myotubes from the undifferentiated reserve cells by brief treatment with diluted trypsin (35). On DM4, Notch3 mRNA was only about one-tenth the level in myotubes compared with the reserve cells, whereas Mef2c mRNA was nearly 10 times more abundant in the myotubes compared with reserve cells (Table 1, last column). Notch1, in contrast was nearly equally abundant in the two populations of cells.

To ascertain whether active Notch3 inhibited the activity of Mef2c, we co-transfected exogenous Mef2c with N3-ICD. The myogenic activity was read by MCK luciferase activity on DM2. By itself, the exogenous N3-ICD had a small but not statistically significant reduction on MCK activity (Fig. 6C). Addition of exogenous Mef2c greatly increased MCK activity, but was completely blocked by the addition of N3-ICD. This indicates that
low the transdifferentiation of 10T1/2 cells into muscle by transfection of MyoD, we measured the activation of a luciferase reporter-driven by MCK. N3-ICD repressed the induction of MCK luciferase (Fig. 8a). Co-transfection of increasing doses of MKK6, the activator of p38 MAPK, overcame the inhibition of p38 MAPK, which presumably leads to inhibition of Mef2c.

**DISCUSSION**

The results of this study and previously published papers from other groups that are discussed below lead to a model where Notch3 and Mef2c are antagonistic with each other in a closed circle that requires the action of the microRNAs miR-1 and -206 (Fig. 9). In differentiating cells the equilibrium switches in favor of Mef2c and differentiation. This is uncannily similar to the bistable switches that we have described involving other inhibitors of differentiation such as Pax7 or MyoR and the myogenic activators such as MyoD working through microRNAs such as miR-206 and miR-378 (24, 26). There, too, the inhibitors prevent the activity of MyoD, but once the latter gains ascendance, the resulting induction of the microRNAs represses the inhibitors to switch the equilibrium in favor of MyoD and differentiation.

We describe a novel enhancer at the end of intron 1 of Notch3 that requires the concurrent activity of MyoD and activated Notch. This enhancer induces Notch3 when MyoD is present in cells that have not yet completely degraded Notch1. The Notch3 can autoactivate its own enhancer (with MyoD) and sustain Notch3 expression even when Notch1 has been degraded completely. Notch3 inhibits differentiation of myoblasts through the inhibition of p38 MAPK, so that we can experimentally reverse the inhibition by overexpressing an acti-

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**TABLE 1**

Expression of Notch3 and Mef2c is mutually exclusive in reserve cells and myotubes

| Probe label                  | Accession     | GM       | DM 24 HR | MT       | Reserve cells | MT/Reserve |
|-----------------------------|---------------|----------|----------|----------|---------------|------------|
| Notch gene homolog 3        | NM_008716     | 391.117  | 1,188.15 | 147.341  | 1,159.81      | 0.13       |
| Notch gene homolog 3        | NM_008716     | 800.627  | 2,245.24 | 342.916  | 2,194.22      | 0.16       |
| MAPK phosphatase 1          | NM_013642     | 1,055.5  | 1,360.32 | 750.649  | 2,013.29      | 0.37       |
| Myocyte enhancer factor 2C  | NM_025282     | 132.746  | 178.134  | 2,753.1  | 281.752       | 9.77       |
| Myocyte enhancer factor 2C  | NM_025282     | 87.4229  | 154.299  | 3,473.1  | 309.379       | 11.23      |
| Notch gene homolog 1        | NM_008714     | 331.796  | 395.991  | 394.185  | 309.379       | 1.11       |
vator of MAPK, MKK6 (Fig. 8D). MAPK is known to be important for myogenesis via phosphorylation and activation of Mef2c (41, 42). Notch3, therefore, represses Mef2c activity and because of the autoactivation of Mef2c, suppresses Mef2c mRNA accumulation.

Although Notch3 may repress differentiation through weak activation of canonical Notch target genes (39), few of these genes showed any change in the muscle of Notch3 knock-out mice (40). We chose to look at p38 due to recent findings that in rhabdomyosarcoma, Notch3 overexpression leads to a decrease in the phosphorylation of p38 (44). We chose to investigate Mkp1 because it is the only other gene to our knowledge whose expression in myoblasts is induced by both Notch1 (43) and MyoD (45). Indeed, C2C12 cells expressing constitutively active Notch3 ICD showed an increase in Mkp1 mRNA and protein (Fig. 8, A and B). Therefore, we propose that active Notch3 increases Mkp1 mRNA and protein and thus inhibits p38 MAPK. Mkp1 has already been shown to be important for proper skeletal muscle regeneration (45). Although p38 MAPK has several effects in myogenesis, we believe that in the context of Notch3, its major role is as an activator of Mef2c. Thus, Notch3 inhibits Mef2c (and differentiation) by stabilizing Mkp1, inhibiting p38 MAPK, and thus inhibiting Mef2c.

Because Notch3 is not inhibited by Numb and because Notch3 clearly inhibits Mef2c and differentiation, there must be another mechanism by which Notch3 is turned off as cells proceed to differentiate. We suggest that this is one of many important roles of miR-1/206: repression of Notch3 in differentiating myoblasts. Despite the activity of the Notch3 enhancer on DM1, Notch3 levels do not continue to rise on
DM2, presumably due to the appearance of the muscle differentiation-induced miRNAs miR-1/206. Beginning on day 3, Notch3 level begins to decrease due to the continued increase of miR-1 and 206 and the consequent inactivation of the enhancer because of the disappearance of active Notch. Thus, the microRNAs are very important for switching the equilibrium toward loss of Notch3 and differentiation. There are multiple pathways by which Mef2c can induce microRNAs to feedback and inhibit Notch3. Mef2c is known to induce miR-1 (37). The MRFs induce miR-206. Because Mef2c can increase the activity of the MRFs independent of its own DNA binding (46), Mef2c is also involved in the indirect stimulation of miR-206.

miR-1/206 could be induced during differentiation through additional pathways, such as the activation of p38 MAPK, the very same kinase that activates Mef2c. p38 MAPK is known to activate Akt in myogenesis (47), and Akt has a significant role in promoting the processing of miR-1/206 from the primary transcript (48).

Once induced, the two microRNAs inhibit Notch3 by acting on its 3′-UTR and thus switch the balance in favor of Mef2c and differentiation. Besides stimulating Mef2c by inhibiting Notch3, miR-1/206 can also stimulate Mef2c by inhibiting the histone deacetylase, HDAC4 (17, 49). Thus there are multiple pathways by which miR-1/206, once induced, can stimulate the Mef2c activity and promote differentiation.
Unlike MyoD or myogenin, which are widely expressed in differentiating myoblasts, Mef2c is detected exclusively in myotubes. In myotubes the Mef2c-mediated stimulation of miR-1 (37) and miR-206 (described above) could force Notch3 levels below the threshold necessary to act on the Notch3 enhancer so that Notch3 mRNA expression is shut off. On the other hand, reserve cells not expressing Mef2c express high levels of Notch3 mRNA. The combination of the Notch- and MyoD-responsive Notch3 enhancer and the repression of Notch3 by muscle differentiation-induced microRNAs like miR-1/206 explains the expression pattern seen with Notch3 and Mef2c being mutually exclusive during muscle differentiation.

We suggest that the slower inhibition of Notch3 through the induction of miRNAs is better suited than fast degradation of Notch1 by Numb for the express purpose of allowing Notch3 to persist long enough even after Notch1 degradation. The transient induction and delayed repression of Notch3 produce a lag in the induction and activation of Mef2c after the activation of MRFs like MyoD and myogenin. Because differentiation of myoblasts to myotubes requires the co-ordination of changes in multiple pathways, we suspect that such a lag is important for optimal differentiation because it gives time to complete changes in other pathways before Mef2c is turned on.

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