Disruption of Meox or Gli Activity Ablates Skeletal Myogenesis in P19 Cells*

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Gli2 and Meox1 are transcription factors that are expressed in the developing somite and play roles in the commitment of cells to the skeletal muscle lineage. To further define their roles in regulating myogenesis, the function of wild type and dominant-negative forms of Gli2 and Meox1 were examined in the context of differentiating P19 stem cells. We found that Gli2 overexpression up-regulated transcript levels of Meox1 and, conversely, Meox1 overexpression resulted in the up-regulation of Gli2 transcripts. Furthermore, dominant-negative forms of either Meox1 or Gli2 disrupted the ability of P19 cells to commit to the muscle lineage and to properly express either Gli2 or Meox1, respectively. Finally, Pax3 transcripts were induced by Gli2 overexpression and lost in the presence of either mutants Meox1 or Gli2. Taken together, these results support the existence of a regulatory loop between Gli2, Meox1, and Pax3 that is essential for specification of mesodermal cells into the muscle lineage.

Skeletal muscle development occurs in the somite of the developing embryo. Somites give rise to the sclerotome, dermomyotome, and myotome. Differentiation of epaxial muscle in the myotome is controlled by the myogenic regulatory factor family of muscle-specific transcription factors, including MyoD, Myf-5, myogenin, and MRF4 (1, 2). Signaling factors from tissues surrounding the somite, including Wnts and Sonic hedgehog (Shh),3 direct the processes of somite patterning and tissues surrounding the somite, including Wnts and Sonic hedgehog (Shh),3 direct the processes of somite patterning and subsequent skeletal muscle development in the myotome (3–6). Several transcription factors appear to mediate the response to signals from surrounding tissues and regulate the commitment of cells into the muscle lineage. These include Gli2 (7, 8), Pax3 (9–11), and Meox1/2 (12, 13).

Wnt and Shh cooperate in the activation of the Gli genes in avian somite formation (8). The Drosophila homolog of the Gli family of zinc finger transcription factors, cubitus interruptus, is a downstream effector of Shh signaling in the fly (14). The Gli family, including Gli1, Gli2, and Gli3, are all expressed in the developing somite (15). Further analysis in avian embryos indicate that Gli1 is expressed predominantly in the ventromedial somite, whereas Gli2 and Gli3 become restricted to the dorsomedial somite, which gives rise to the epaxial myotome (8). Their expression pattern suggests that the Gli factors may play a role in the commitment of cells to the skeletal muscle pathway. Indeed, the Gli factors regulate the maintenance of Myf-5 expression in the early somite, although initial activation is independent of Shh function and an intact Gli binding site (7, 16, 17). The Gli also play a role in MRF expression and skeletal muscle development in Zebrafish (18, 19) and Xenopus (20). Together, these studies suggest a conserved role for the Gli factors in regulating the expression of the myogenic regulatory factors, leading to skeletal muscle development.

Pax3 and Pax7 are functionally similar members of a family of paired box transcription factors (21). Pax3 was found to be initially expressed throughout the somite before becoming restricted to the dermomyotome and the cells that migrate to the limb bud (22, 23). The splotch mouse carries a mutant allele of the Pax3 gene (24). Splotch mutant embryos were devoid of muscle precursors that migrate from the dermomyotome to populate the limb bud, whereas epaxial muscle appeared unaffected (22, 25). Because Pax7 expression was shown to be expanded into regions that normally express only Pax3 in Splotch mutant embryos (26), it is likely that Pax7 compensated for the loss of Pax3 in epaxial muscle.

A cross between the Splotch mouse and the Myf-5-null mouse implied a role for Pax3 in epaxial muscle, because both epaxial and hypaxial muscle precursors were lost in double mutant embryos (11). The lack of MyoD in the double mutant indicated that MyoD is regulated by both Pax3 and Myf-5. Overexpression of Pax3 in avian paraxial mesoderm induced MyoD, Myf-5, and myogenin expression (10). Furthermore, overexpression of a dominant-negative Pax3 in P19 embryonal carcinoma cells resulted in a loss of MyoD and myogenin expression and subsequent myogenesis (9). Together these studies indicate a crucial role for Pax3 in activating the expression of the myogenic regulatory factors in the myotome.

Mecox1 and Mecox2 are closely related homeobox proteins expressed in the early developing somite (27). At later stages, Mecox1 was found in the dermomyotome, whereas Mecox2 was found in the developing limb bud (27, 28), suggesting Mecox1 and Mecox2 may regulate epaxial and hypaxial muscle development, respectively. Mecox2-null mice exhibited down-regulation in Pax3 and Myf-5 expression and a decrease in differentiated

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1 The abbreviations used are: Shh, sonic hedgehog; Gli/EnR, replacement of the Gli2 activation domain with the engrailed repressor domain; P19(Gli2), P19 cells overexpressing Gli2; Meox/EnR, fusion of the engrailed repressor domain to the C terminus of Meox1; MRF, myogenic regulatory factor; MHC, myosin heavy chain; RT, reverse transcriptase; CMV, cytomegalovirus; PGK, phosphoglycerate kinase.
GATATC amplified by PCR utilizing oligonucleotides 5'/H11032GAGGAG- utilizing SacI/KpnI sites. The engrailed repression domain was cut out using SacI/KpnI and subcloned into the PGK-vector by Easy (Promega) vector (the start codon is underlined). The Meox1 cDNA was subcloned into the PGK vector using SacI/KpnI. To construct the Meox/EnR chimera, the C-terminal domain of Gli2, which includes the activation domain, was removed by utilizing ApaI. This deletion corresponds to the ΔC4 mutant that could not activate a multimerized Gli-site promoter in transfection assays (30). The remaining N terminus of Gli2, containing the zinc finger domain, was fused to the 198-amino acid N-terminal repression domain of the mouse EN-2 protein. The engrafted repression domain was amplified utilizing oligonucleotides 5’-AAAGGCCCGGAGGAGAGGATCTCAAACCAGC and 3’-AAAGCAGCAGCTGTCGCTGCTTCG. The 5’ and 3’ oligonucleotides contained ApaI and NotI sites, respectively (bold), to facilitate cloning and the 3’-oligonucleotide contained a stop codon (underlined). Gli/EnR was subcloned into the PGK vector, which contains the phosphoglycerate kinase (pgk-1) promoter (31). The PGK-Puro and PGK-LacZ constructs were described previously (32). Meox1 cDNA was kindly provided by B. Mankoo. The Meox1 cDNA was subcloned into the PGK vector using SacI/KpnI. To construct the Meox/EnR chimera, Meox1 cDNA was amplified utilizing oligonucleotides 5'-AAGAAGCTCAGAGATGACCAGTCG and 3’-AAGGTACCCG-ATATCCCTGAACTTGAGGCTGC and subcloned into pGEM-T-Easy (Promega) vector (the start codon is underlined). The Meox1 cDNA was cut out using SacI/KpnI and subcloned into the PGK-vector by utilizing SacI/KpnI sites. The engrafted repression domain was amplified by PCR utilizing oligonucleotides 5’-AAGATATCCGAGAAGGATCTGAGTCCAG and 3’-AAGGTACCCG-ATATCCCTGAACTTGAGGCTGC. The 5’- and 3’-oligonucleotides contained EcoRV sites (bold) to facilitate cloning and the 3’-oligonucleotide contained a stop codon (underlined). The 198-amino acid N-terminal repression domain of the mouse EN-2 protein was then fused to the C terminus of the Meox1 cDNA by subcloning the amplified engrafted repression domain into the PGK-Meox1 vector by utilizing EcoRV. The activation domain of Meox1 is currently unknown and thus was not removed prior to construction of the Meox/EnR chimera.

**Cell Culture and Transfections**—P19 cells were cultured as described (33) in α-minimum essential media supplemented with 5% Cosmic calf serum (HyClone, Logan, UT) and 5% fetal bovine serum (CanSera, Red Dale, Ontario, Canada). Stable cell lines were isolated as described previously (9, 32, 34–37). Briefly, CMV-Gli2 and CMV-Gli2/engrailed were stably transduced utilizing the FuGENETM 6 transfection reagent as per the manufacturer’s instructions (Roche Diagnostics, Laval, QC, Canada). A mixture containing 2.04 μg of CMV-Gli2, CMV-Gli2/engrailed, or PGK vector alone for controls, and 0.09 μg of PGK-puro, 0.17 μg of PGK-LacZ, 0.77 μg of B17 was used to create the Gli2 cell lines. A mixture containing 6 μg of PGK-Meox1, PGK-Meox1/engrailed, or PGK vector alone for controls, and 0.25 μg of PGK-puro, 0.50 μg of PGK-LacZ, and 2.25 μg of B17 was used to create the Meox cell lines. For all stable transfections, a mixture of DNA with FuGENE 6 reagent was added to 2.5 × 10^5 cells in 35-mm tissue culture dishes. Transfection efficiency for each experiment was determined by [γ-galactosidase] assays as described previously (38). Two selected for puromycin resistance (2 μg/ml) for 7–9 days. Resulting colonies were tested for expression by slot blot analysis and stable cell lines were isolated and termed P19(Gli2), P19(Gli/EnR), P19(Meox1), P19(Meox/EnR), or P19(control) cells. P19(control), P19(Gli2), and P19(Meox1) cell lines were differentiated in the absence of MeSO and P19(control), P19(Gli/EnR), and P19(Meox/EnR) cell lines were differentiated in the presence of 0.8% MeSO, as described (9, 32, 34–37). Differentiation involved aggregation of cells for 4 days in Petri dishes (in the absence or presence of MeSO) followed by plating into tissue culture dishes on day 4. Cells were harvested for total RNA on day 9. At least three clonal populations, which behaved similarly, were isolated for each cell line.

**Immunofluorescence**—Cells were plated on gelatin-coated coverslips on day 4 and fixed on day 9 in −20°C methanol. Cells were rehydrated in phosphate-buffered saline and myosin heavy chain expression (MHC) was detected utilizing monoclonal MP20 antibody supernatant (40) and nuclei were detected by Hoechst stain as described previously (9). In brief, 40 μl of MP20 supernatant in 40 μl of phosphate-buffered saline was incubated on coverslips for 1 h at room temperature. Coverslips were then washed 3× 5 min in phosphate-buffered saline, and incubated with 80 μl of 1:100 dilution of goat anti-mouse Cy3-linked antibody (Jackson ImmunoResearch) for 1 h at room temperature. After phosphate-buffered saline washes, coverslips were counterstained and imaged using fluorescence microscopy. Images were captured on a Sony 3CCD camera and processed utilizing Axiosview, Adobe Photoshop 7, and Canvass software.

**Northern Blot Analysis**—Total RNA was isolated from each differentiation utilizing the LiCl method, as described (32). Twelve μg of total RNA were separated on a 1% agarose, formaldehyde gel, transferred onto Hybond-N (Amersham Biosciences) by capillary action and cross-linked by UV irradiation. Blots were then hybridized to DNA probes labeled with [α-32P]dNTP by multiprime labeling (Amersham Biosciences) for 16 h at 42°C. Probes for Pax3, Meox1, Six1, MyoD, myogenin, and Six1, and for Pax3, Meox1, Six1, MyoD, myogenin, and Six1.5 (for standardization of loading) have been described previously (9). In brief, 40 μl of MP20 supernatant in 40 μl of phosphate-buffered saline was incubated on coverslips for 1 h at room temperature. Coverslips were then washed 3× 5 min in phosphate-buffered saline, and incubated with 80 μl of 1:100 dilution of goat anti-mouse Cy3-linked antibody (Jackson ImmunoResearch) for 1 h at room temperature. After phosphate-buffered saline washes, coverslips were counterstained and imaged using fluorescence microscopy. Images were captured on a Sony 3CCD camera and processed utilizing Axiosview, Adobe Photoshop 7, and Canvass software.

**Reverse Transcription-Polymerase Chain Reaction**—Total RNA was harvested on day 9 utilizing TRIzol reagent, as per the manufacturer’s protocol (Invitrogen), or the LiCl method, followed by purification with RNeasy® Mini Kit (Qiagen, Mississauga, Ontario, Canada). Approximately 1 μg of DNAse I-treated RNA was used to synthesize first strand DNA, utilizing the SuperScript First-strand Synthesis Kit (Invitrogen). Reverse transcriptase and PCR conditions for each primer set are provided in Table 1. First-strand cDNA was then amplified utilizing oligonucleotides 5’-AAAGAAGCTCAGAGATGACCAGTCG and 3’-AAGGTACCGCGCATCGTCGCGCTTCG. The 5’ and 3’ oligonucleotides contained ApaI and NotI sites, respectively (bold), to facilitate cloning and the 3’-oligonucleotide contained a stop codon (underlined). Gli2 cDNA was subcloned into the PGK vector, which contains the phosphoglycerate kinase (pgk-1) promoter (31). The PGK-Puro and PGK-LacZ constructs were described previously (32). Two μl of first-strand DNA was then used for PCR. Oligonucleotides to amplify Myf-5 were 5’-GAGCTGCTGAGGGAACAGGTG and 3’-GCTCAGGTACGCCGCTCGTCGTT. The 5’ and 3’ oligonucleotides contained EcoRV sites (bold) to facilitate cloning and the 3’-oligonucleotide contained a stop codon (underlined). The 198-amino acid N-terminal repression domain of the mouse EN-2 protein was then fused to the C terminus of the Meox1 cDNA by subcloning the amplified engrafted repression domain into the PGK-Meox1 vector by utilizing EcoRV. The activation domain of Meox1 is currently unknown and thus was not removed prior to construction of the Meox/EnR chimera.
Gli2 could induce myogenic conversion, 10T1/2 cells were transfected with 1.4–3 μg of CMV-Gli2, PGK-Meox1, or PGK-Pax3. All transfections included 0.5 μg of pEGFP-N1 (Clontech Laboratories, Inc., Palo Alto, CA) for transfection efficiency. Total DNA in each transfection was brought up to 4 μg with PGK vector plasmid. Twenty-four hours after transfection, cells were transferred to differentiation media containing 2% horse serum. Transfection efficiency was calculated by counting green fluorescent protein-positive cells 2–3 days after transfection. Six days after transfection, cells were fixed and stained for the presence of myosin heavy chain by immunofluorescence with MF20. Myogenic conversion was quantitated by counting cells expressing myosin heavy chain.

RESULTS

Gli2 and Meox1 Activate the Expression of Each Other in Aggregated P19 Cells—To examine the ability of Gli2 to induce skeletal myogenesis, cell lines stably expressing Gli2 (termed P19(Gli2) cells) were isolated. P19(Gli2) cells and control cells were aggregated in the absence of Me2SO. Under these conditions, control cells do not differentiate into skeletal muscle, as shown by the lack of MHC-expressing cells in these cultures on day 9 (Fig. 1B). In contrast, P19(Gli2) cells did differentiate into skeletal muscle, as shown by the presence of bipolar MHC-expressing myocytes (Fig. 1D), representing <4% of these cultures. Therefore, Gli2 can induce low levels of skeletal myogenesis in aggregated P19 cells.

To identify the factors activated by Gli2 expression, total RNA was harvested on days 0 and 9 of differentiation. By Northern blot analysis, Gli2 transcript levels were high in P19(Gli2) cell lines, but not in control cell lines (Fig. 2, panel I, A). Transcription factors expressed in the dermomyotome of the developing somite, such as Pax3 and Meox1, were activated by day 9 of differentiation in P19(Gli2) cell lines but not control cell lines (Fig. 2, panel I, B and C). Interestingly, Meox1 transcripts were also present in monolayer cultures, suggesting that Gli2 can activate Meox1 expression in the absence of additional factors induced by aggregation (Fig. 2, panel I, B). Furthermore, expression of Myf-5 in aggregated P19 cells induced skeletal myogenesis as well as Meox1 and Pax3 transcript levels (data not shown), similar to the Gli2-induced skeletal muscle program. Therefore, Gli factors can induce the expression of Pax3 and Meox1, which are factors thought to be involved in the specification of mesodermal precursors to the myogenic lineage.

To determine whether Gli2 overexpression can activate MRF expression in P19 cells, transcripts were analyzed on day 9 of differentiation in P19(Gli2) and control cell lines. MyoD and myogenin transcripts were not observed by Northern blot analysis, indicating that MRFs were not induced to high levels in P19(Gli2) cells (data not shown). By RT-PCR analysis, both Myf-5 and myogenin transcripts were detected in P19(Gli2) cell lines, confirming that commitment into the skeletal muscle lineage occurred in these cultures and not control cells (Fig. 2, panel II, E and F). Very low levels of MRF transcripts were present in the absence of muscle in the control cultures on days 0 (data not shown) and 9, in agreement with studies in other systems (41, 42). Although exogenous Gli2 was expressed at high levels on day 0 in P19(Gli2) cells, Myf-5 was not activated on day 0 (data not shown). Together, these data indicate that Gli2 can up-regulate the expression of the myogenic factors, and that the activation is dependent on cellular aggregation in P19 cells.

In contrast to the results in P19 cells, Gli2 was not able to convert 10T1/2 fibroblasts into skeletal muscle using a myogenic conversion assay (data not shown). This is similar to findings with Pax3, which can induce myogenesis in P19 cells (9) as well as paraxial and lateral plate mesoderm explants (10), but not in 10T1/2 fibroblasts (10). It is likely that Gli2 function requires factors present in aggregated P19 cells but not in fibroblasts.
Meox1 and Gli2 Function in Skeletal Myogenesis

Because Meox1 transcripts were up-regulated by Gli2 expression and during Me2SO-induced myogenesis (9), we examined the ability of Meox1 to induce skeletal myogenesis. Stable cell lines overexpressing Meox1, termed P19(Meox1) cells, were created and aggregated in the absence of Me2SO, along with control cell lines. Cultures were examined by immunofluorescence (data not shown), Northern blot analysis, and RT-PCR (Fig. 3). Bipolar skeletal myocytes were not observed after staining with an anti-MHC antibody (data not shown). Therefore, in contrast to results with Gli2, and previous results with activated β-catenin (13) and Pax3 (9), Meox1 overexpression was not sufficient to induce skeletal myogenesis in aggregated P19 cells.

By Northern blot analysis, Meox1 transcripts were present at high levels in P19(Meox1) cells on day 9 and lower levels on day 0 (Fig. 3, panel I, A). This variability in PGK-driven transcript expression is because of the enhancer/silencer effects at the site of insertion and is seen in many P19 cell lines (35, 37). Interestingly, Gli2 transcripts were up-regulated in P19(Meox1) cells, compared with control cells, whereas Pax3 transcripts were not significantly increased over background (Fig. 3, panel I, B and C). As a positive control, both Gli2 and Pax3 transcripts were detected in control cells treated with Me2SO to induce the endogenous skeletal muscle program (Fig. 3, panel I, B and C, lane 1). Therefore, Meox1 can activate Gli2 but not Pax3 expression in aggregated P19 cells.

MyoD and myogenin transcripts were not detectable by Northern blot analysis (data not shown). By RT-PCR analysis, very low levels of myogenin mRNA were detected in some cell lines (Fig. 3, panel II). However, Myf-5 transcripts were not observed to be significantly over background. Therefore, while Meox1 expression can up-regulate Gli2 expression, it cannot up-regulate Pax3 or MRF expression, leading to skeletal myogenesis. Taken together, it appears that Meox1 and Gli2 can activate the expression of each other, implying the presence of a regulatory loop.

Gli/Engrailed Expression Inhibits Muscle Specification in P19 Cells—Because we have shown that Gli2 is sufficient to induce the skeletal muscle program in P19 cells, we tested if Gli activity was also necessary for myogenesis by construction of a Gli/engrailed chimera (termed Gli/EnR). The activation domain of Gli2 was replaced with the engrailed repression domain that actively blocks transcription by interacting with transcriptional repressor proteins (43, 44). P19(Gli/EnR) and control cells were aggregated in the presence of 0.8% Me2SO and examined on day 9 of differentiation. Under these conditions, bipolar skeletal myocytes were detected by immunofluorescence in control cells but not P19(Gli/EnR) cells (data not shown). P19 cells expressing the engrailed repressor domain alone differentiated inefficiently into skeletal muscle (data not shown). Analysis by RT-PCR showed a lack of Myf-5 mRNA in P19(Gli/EnR) cells when compared with
Meox1 and Gli2 Function in Skeletal Myogenesis

P19(control) cells (Fig. 4, panel I), indicating that Gli factors, or genes containing Gli binding sites, are essential for Myf-5 expression and skeletal myogenesis.

Northern blot analysis was performed on a time course of differentiation to determine at what stage the myogenic program was disrupted (Fig. 4, panel II). The P19(Gli/EnR) cell line expressed high levels of Gli/EnR transcripts, as shown by hybridization to a cDNA fragment of the engrailed repressor domain (Fig. 4, panel II, A). The lower band provides the expected size for the fusion transcript and the upper band is not seen in all clonal populations isolated. It may occur due to the use of alternative polyadenylation signals at the site of insertion. Brachyury T, a protein expressed in the primitive streak during gastrulation, was expressed in both P19(control) and P19(Gli/EnR) cell lines, indicating that mesoderm induction still occurred (Fig. 4, panel II, B).

Factors found in the differentiating somite, including Gli2, Meox1, Pax3, and Six1 were all expressed during MeSO-induced differentiation of control cell lines by days 3–5 (Fig. 4, panel II, C–F). Expression of the Gli/EnR chimera did not down-regulate the transcript levels of endogenous Gli2 compared with control cells (Fig. 4, panel II, C). However, Meox1, Pax3, and Six1 expression was down-regulated in P19(Gli/EnR) cell lines (Fig. 4, panel II, D–F). Meox1 transcripts were first detected on day 5 in P19(Gli/EnR) cells, 2 days after expression was initiated in the control cell lines. Subsequently,
Moe1 transcripts were lost on days 6–9 in P19(Gli/EnR) cells (Fig. 4, panel II, D). Similar to Moe1, Pax3 transcripts were initially detectable at very low levels on day 5 in P19(Gli/EnR) cells, 1 day after expression was initiated in control cell lines. Then, Pax3 transcripts were absent on days 6–9 in P19(Gli/EnR) cells (Fig. 4, panel II, E). Finally, SIX1 transcript levels were not present from days 6 to 9 in P19(Gli/EnR) cells compared with control cell lines (Fig. 4, panel II, F). Therefore, the earliest disruption in the myogenic program in P19(Gli/EnR) cultures was the down-regulation of Pax3 and Moe1 expression on days 3–4 of differentiation.

MyoD transcripts were present on day 7 in the control cells but not in P19(Gli/EnR) cultures (Fig. 4, panel II, G). Myf-5 expression was also initiated on day 7 in control cells but not in P19(Gli/EnR) cultures (data not shown). Therefore, the disruption of wild type Gli2 activity resulted in the loss of the expression of somite patterning factors, such as Moe1 and Pax3, leading to the absence of MRF expression and commitment into the muscle lineage.

Moe1/Engrailed Expression Inhibits Muscle Specification in P19 Cells—Although Moe1 expression was not sufficient to induce skeletal myogenesis, it was still important to test whether it is essential. A dominant-negative Moe1 chimera was constructed by fusing the engrailed repressor domain to the C terminus of Moe1. Stable cell lines were created and termed P19(Meox/EnR) cells. P19(control) and P19(Meox/EnR) cells were differentiated in the presence of Me2SO and examined by immunofluorescence and Northern blot analysis. Immunofluorescence with an anti-MHC antibody indicated a loss of detectable bipolar skeletal myocytes in P19(Meox/EnR) cells, compared with control cells (data not shown).

Analysis of transcript levels at day 9 of differentiation showed overexpression of Moe1/EnR transcripts in the P19(Meox/EnR) cells, compared with control cells (Fig. 5, panel I, A). The expression of the MRFs, MyoD, and myogenin, as well as the factors found in the developing somite, such as Pax3, Pax7, Six1, Eya2, and Gli2 was down-regulated in P19(Meox/EnR) cells, compared with control cells (Fig. 5, panel I, B–H).

To determine the earliest stage that Moe1/EnR expression disrupts myogenesis, time courses of differentiation experiments were examined by Northern blot analysis. Endogenous Moe1 and MyoD transcripts were completely lost throughout the differentiation time course in P19(Meox/EnR) cells compared with control cells, in which Moe1 transcripts are detected on day 4 and MyoD by day 7 (Fig. 5, panel II, A and B). In contrast, Brachyury T transcripts were present in both P19(control) and P19(Meox/EnR) cells, indicating that mesoderm induction still occurred in P19(Meox/EnR) cells (Fig. 5, panel II, C). Pax3 expression was severely down-regulated in P19(Meox/EnR) cell lines from days 4 to 9, compared with control cell lines (Fig. 5, panel II, D). Gli2 expression was down-regulated but not lost in P19(Meox/EnR) cells from days 4 to 9, compared with control cell lines (Fig. 5, panel II, E). Therefore, disruption of myogenesis occurred as early as day 4 of differentiation, with the down-regulation of Moe1 and Pax3 expression. Taken together, Moe1 factors, or genes having Moe1 binding sites, are essential for the proper expression of factors involved in somitogenesis and for subsequent skeletal myogenesis.

**DISCUSSION**

We have shown that Gli2 and Moe1 can activate the expression of each other in aggregated P19 cells. In addition, Gli2 can up-regulate Pax3 and MRF transcript levels, leading to skeletal myogenesis. Finally, overexpression of either dominant-negative Gli2 or Moe1 inhibited the specification of mesodermal precursors into the muscle lineage, resulting in the loss of MRF expression. P19(Gli/EnR) cells, Moe1 levels were down-regulated and in P19(Meox/EnR) cells Gli2 levels were down-regulated. Therefore, these findings have led to a model in which Gli and Moe1 activate the expression of each other and are essential for the specification of cells into the skeletal muscle lineage (Fig. 6).

Expression of either Gli2/Meox1 or Gli1/Meox1 fusion proteins resulted in the down-regulation of Pax3 transcript levels, indicating that Meox1 or Gli2 factors, or genes containing Meox or Gli binding sites, are necessary for Pax3 expression (Fig. 6). Gli2 but not Meox1 overexpression was sufficient to induce transcription of Pax3. Previous results have shown that Pax3 overexpression could initiate transcription of Moe1 and that a Pax3/EnR fusion could down-regulate Moe1 transcript levels (9). Taken together, these results support the presence of a regulatory loop between Gli2, Moe1, and Pax3 that is important for the maintenance of their expression and subsequent commitment to the skeletal muscle lineage (Fig. 6).

The observation that Gli/EnR expression does not abolish the endogenous transcript levels of Gli2, indicates that Gli2 does not regulate its own expression. Conversely, Meox/EnR expression resulted in the loss of endogenous Moe1 transcripts and Pax3 was shown to be self-regulating in P19 cells by expression of a Pax3/engrailed chimera (9). Meox factors have been shown to bind Pax factors (29), although the effect of this association on the function of these factors remains unknown. Taken together, Moe1 and Pax3 can regulate their own expression, either directly or indirectly, and this self-regulation likely contributes to the stabilization of the Gli2-Moe1-Pax3 regulatory loop essential for proper muscle specification (Fig. 6).

Previous results have shown that β-catenin was essential and sufficient for the initiation of Gli2, Moe1, and Pax3 expression in aggregated P19 cells (13), implying an upstream role for Wnt signaling via β-catenin (Fig. 6). In addition, P19 cells grown in monolayer cultures expressing an activated form of β-catenin showed an up-regulation in Gli2 and Pax3 transcript levels, but not Moe1 (13). Consequently, it is possible to detect Pax3 transcripts in the absence of Moe1 transcripts, indicating that Moe1 is likely not the sole contributor regulating Pax3 expression. Moe1 was expressed strongly on day 5 of differentiation in P19(Gli/EnR) cultures (Fig. 4, panel II, D, lane 16). This indicates that a portion of Moe1 expression is controlled in a Gli-independent fashion. Gli2 expression was down-regulated but not lost in P19(Meox1/EnR) cultures, indicating that part of Gli2 expression is independent of Moe1 function. The extent to which β-catenin regulates Gli2, Moe1, and Pax3, and whether this regulation is direct or indirect remains to be determined.

Gene ablation experiments in the mouse have shown that the
loss of functional Meox2, which is expressed in the developing limb bud (27, 28), resulted in down-regulation of Pax3 and Myf-5 expression and a decrease in skeletal muscle in the limb (12). Furthermore, mice carrying null mutations for both Meox genes displayed a loss of Pax3, Myf-5, and myogenin expression, leading to severe deficiencies in somitogenesis and skeletal myogenesis (45). Consequently, our experiments in P19 cells, using an EnR fusion approach, have provided similar results to those in mice using gene ablation technology. Furthermore, mice lacking Meox genes display a similar phenotype to mice lacking hedgehog signaling (46), suggesting that Meox may mediate the response to hedgehog signals (45). The results presented here implicate Meox as a mediator of hedgehog signaling by regulating Gli2 expression.

Gli2 was sufficient to induce MRF transcripts and low levels of skeletal myogenesis. However, given that Gli2 expression first induced Meox1 and Pax3 transcripts, it is unclear which of skeletal myogenesis. However, given that Gli2 expression can activate Meox1 and Pax3, it is consistent with our previous results, showing that a dominant-negative Meox1 and Pax3 transcripts, it is unclear which

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