Wnt Signaling Regulates the Function of MyoD and Myogenin*

Received for publication, May 19, 2000, and in revised form, July 20, 2000
Published, JBC Papers in Press, July 27, 2000, DOI 10.1074/jbc.M004349200

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The myogenic regulatory factors (MRFs), MyoD and myogenin, can induce myogenesis in a variety of cell lines but not efficiently in monolayer cultures of P19 embryonal carcinoma stem cells. Aggregation of cells expressing MRFs, termed P19[MRF] cells, results in an approximately 30-fold enhancement of myogenesis. Here we examine molecular events occurring during P19 cell aggregation to identify potential mechanisms regulating MRF activity. Although myogenin protein was continually present in the nuclei of >90% of P19[myogenin] cells, only a fraction of these cells differentiated. Consequently, it appears that post-translational regulation controls myogenin activity in a cell lineage-specific manner. A correlation was obtained between the expression of factors involved in somite patterning, including Wnt3a, Wnt5b, BMP-2/4, and Pax3, and the induction of myogenesis. Co-culturing P19[myogenin] cells with P19[MRF] cells in monolayer resulted in a 5- to 8-fold increase in myogenesis. Neither BMP-4 nor Pax3 was efficient in enhancing MRF activity in unaggregated P19 cultures. Furthermore, BMP-4 abrogated the enhanced myogenesis induced by Wnt signaling. Consequently, signaling events resulting from Wnt3a expression but not BMP-4 signaling or Pax3 expression, regulate MRF function. Therefore, the P19 cell culture system can be used to study the link between somite patterning events and myogenesis.

A family of myogenic basic helix-loop-helix transcription factors (MRFs)* plays a major role in controlling the events leading to skeletal muscle development (1, 2). These transcription factors, MyoD, myf-5, myogenin, and myf-6/MRF-4/herculin (3–9), heterodimerize with E-type basic helix-loop-helix transcription factors leading to regulation of MRF function (10–15). These heterodimers regulate transcription by binding to E box consensus sites (CANNTG) found in the promoters of many muscle-specific genes (16). In addition to regulating transcription on their own, these heterodimers are able to interact with other families of transcription factors, such as the MEF2 family, resulting in a cooperative activation of function (17–19). Ectopic expression of any one MRF in a wide variety of non-muscle cell types results in the conversion of these cells to the myogenic lineage (20).

During embryogenesis, cells become committed to the muscle lineage by expression of MRFs in the somites. Somites arise from the presegmental mesoderm adjacent to the neural tube. Extensive tissue interactions and signaling result in patterning of the somite to form the sclerotome, dermomyotome, and myotome (21–24). Signals from the surface ectoderm, axial structures, including the dorsal neural tube and the notochord, and the lateral mesoderm are involved in patterning the somite and regulating the differentiation of the myotome (25–28). Explant studies from avian embryos have shown that the inductive properties of the axial structures can be replaced by a combination of Sonic Hedgehog (SHH) and members of the Wnt family of signaling molecules (29, 30). However, SHH is not required in older explant cultures (29) but has been shown to be involved in the initial expression of myf-5 in the medial lip (31, 32).

The regulation of myf-5 expression by the dorsal neural tube during myotome formation can be replaced by cells expressing Wnt1 (33). Moreover, the double knock-out of Wnt1 and Wnt3a in mice ablates the dorsal medial region of the dermomyotome and results in the loss of normal early expression of myf-5 but not MyoD (34). This indicates an essential role for these signaling molecules in the regulation of myogenic gene expression within the dorsal medial dermomyotome. MyoD expression in the more lateral region of the myotome is thought to be controlled preferentially by signals from the dorsal ectoderm (21). Cells expressing Wnt7a are capable of replacing the dorsal ectoderm and regulating the expression of MyoD in the more lateral regions of the dermomyotome (33). The signaling initiated by Wnt1 and Wnt7a likely utilize different molecular pathways. Wnt1 signals by binding to its receptor, Frizzled 1 (Fz1) and signals through a classic Dishevelled (Dsh) → GSK3 → β-catenin → TCF pathway. In contrast, Wnt7a binds to Fz7 and signals through a β-catenin-independent pathway, utilizing protein kinase C (reviewed in Refs. 21, 35, 36). Both pathways result in the activation of gene expression. Although it is clear that Wnt signaling events result in the expression of MRFs during embryogenesis, a role for Wnt signaling in the regulation of MRF activity has not yet been studied.

Another family of signaling molecules involved in patterning the somite are the bone morphogenic proteins (BMPs) (23, 24, 37). BMP-4 expression in the lateral mesoderm inhibits muscle differentiation (38), whereas BMP signaling in the dorsal neural tube is important for Wnt expression leading to proper maturation of cells in the dorso-medial lip (39). Pax3, a member of the paired box family of transcription factors, is expressed in the maturing cells of the dorso-medial lip, marking the early stages of myogenic cell specification (40, 41). The level of BMPs

* This work was supported in part by a grant from the Medical Research Council of Canada. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by a Natural Sciences and Engineering Council of Canada studentship and an Ontario Graduate Scholarship.

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The abbreviations used are: MRF, myogenic regulatory factor; SHH, Sonic Hedgehog signaling molecule; Fz1, Frizzled 1 receptor; MEF2C, myocyte enhancer factor 2C; LEF1, lymphoid-enhancer factor 1; TCF, T-cell factor; BMP, bone morphogenic protein; kb, kilobase(s); PBS phosphate-buffered saline; MyHC, myosin heavy chain.
within the somite, combined with the presence or absence of its antagonist noggin, controls the ability of Pax3-positive cells to activate MyoD and myf-5 expression (42). For example, BMP signaling in the absence of noggin inhibits the ability of Pax3 to activate MyoD. Pax3 is necessary for the expression of MyoD in embryos lacking myf-5, indicating that Pax3 functions upstream of MyoD (43). Furthermore, the overexpression of Pax3 in paraxial mesoderm leads to activation of MyoD and myf-5 expression (44).

A tissue culture system capable of emulating early embryonic events that occur during somitogenesis would be valuable for further analysis of the mechanisms involved. The P19 cell culture system may be such a system, because the differentiation of these pluripotent stem cells simulates the biochemical and morphological processes that occur during early embryonic development (45–47). Aggregation of P19 cells induces the expression of the mesoderm marker, Brachyury T (48), but few of these cells continue to differentiate. Aggregates treated with dimethyl sulfoxide (Me2SO) differentiate into cardiac and skeletal muscle along with other mesodermal and endodermal cell types (49). Cardiocytes first appear on day 5 following Me2SO treatment, whereas skeletal muscle does not appear until day 9 following treatment. The appearance of cardiac and skeletal muscle is dependent both on the presence of Me2SO and on unknown factors in the fetal calf serum (50). In co-culture experiments, skeletal muscle development in P19 cells was regulated by factors secreted from the neural tube (51). Thus, P19 cells provide an easily manipulable system to examine early developmental events in tissue culture.

Previous studies examined how the ectopic expression of MyoD affects the developmental potential of P19 cells (52). P19 cells expressing MyoD (termed P19[MyoD]) cells), retained stem cell characteristics and did not differentiate into skeletal muscle until the cells were aggregated, either with or without Me2SO. These results suggested that mesoderm induction, via cellular aggregation, was essential for MyoD activity. Similar results were obtained by others in embryonic stem cells (53).

Studies of P19[MyoD] cells have shown that MyoD protein is present and capable of binding DNA both before and after aggregation (54). This finding suggests that cellular aggregation is responsible for initiating signaling cascades that regulate MyoD directly. Alternatively, aggregation may indirectly effect MyoD activity, possibly by inducing the expression of an essential cofactor or by altering chromatin structure at muscle-specific promoters. Previous studies have shown that myogenin, like MyoD, requires cellular aggregation to initiate myogenesis (55).

In the present study, we have examined potential mechanisms involved in regulating MRF activity during cellular aggregation. Here we show that myogenin activity appears to be regulated in a cell lineage-specific and post-translational manner. During aggregation, somite-patterning factors such as Wnt3a, BMP-2/4, and Pax3 are expressed. In monolayer cultures, Wnt3a, but not Pax3 or BMP-4, can activate MRF-induced myogenesis in P19 cells, bypassing the requirement for aggregation.

**Materials and Methods**

**Plasmid Constructs**—All cDNAs in expression vectors are driven by the phosphoglycerate kinase (pgk-1) promoter (56). The DNA construct PGK-MyoD contains a 1.7-kb EcoRI fragment containing the complete open reading frame of MyoD cDNA (3), as described (57). The construct PGK-myogenin contains a 1.4-kb EcoRI fragment containing the complete open reading frame of rat myogenin cDNA (7). The construct PGK-Pax3 contains a 2.3-kb EcoRI fragment containing the complete open reading frame of Pax3 cDNA (58). The construct PGK-Wnt3a contains a 1.4-kb EcoRI fragment containing the complete open reading frame of Wnt3a (59). The construct PGK-Puro contains the gene encoding puromycin resistance, as described (52). The construct PGK-LacZ contains the gene encoding β-galactosidase. PGK-vector DNA is a plasmid containing the pgh-1 promoter alone.

**Cell Culture and DNA Transfections**—P19 embryonal carcinoma cells were cultured as described (47, 50) in 5% COSmic calf serum (HyClone, Logan, UT) and 5% fetal bovine serum (Cambrex, Rexdale, Ontario). Cells were transfected by the calcium phosphate method (60) unless otherwise stated. Stable cell lines expressing myogenin, MyoD, or Wnt3a were generated as described previously (55, 61). Duplicate transfections were performed with 8 μg of PGK-myogenin or 5 μg of PGK-LacZ, 1 μg of PGK-Pax3, and 2.5 μg of PGK-B17 (62). To isolate P19 control cell lines, duplicate transfections were performed with 8 μg of PGK-vector, 1 μg of PGK-Puro, 1 μg of PGK-LacZ, and 2.5 μg of B17. To generate cells expressing both MyoD and myogenin, duplicate transfections were performed with 4.5 μg of PGK-MyoD, 4.5 μg of PGK-myogenin, 1 μg of PGK-Puro, 1 μg of PGK-LacZ, and 2.5 μg of B17. After 24 h, β-galactosidase assays were performed on one set to ensure high transfection efficiency, and 2 × 10⁶ cells were plated in a 150-mm dish and selected for puromycin resistance (2 μg/ml). After 7 days, colonies were isolated for further studies. Cells expressing MyoD, myogenin, and both MyoD and myogenin are termed P19[MyoD] (52), P19[Mgn] (55), and P19[MyoD+Mgn], respectively.

**Differentiation** was induced by plating 5 × 10⁴ P19 control, P19[MyoD], P19[Mgn], or P19[MyoD + Mgn] cells into 60-mm dishes containing either 0.8% Me2SO or no Me2SO. The presence or absence of Me2SO had no effect on the ability of the MRFs to induce myogenesis. However, only in the presence of Me2SO will control cells differentiate into cardiac muscle on day 5 and skeletal muscle on day 9. Cells were cultured as aggregates for 4 days and then plated in tissue culture dishes and harvested for RNA, protein, or fixed for immunofluorescence, at the time indicated. In the aggregation time course experiment, cells were aggregated for 1–4 days and harvested 1 day after transfer into tissue culture dishes.

To determine the effect of Pax3 expression on the activity of MyoD or myogenin, PGK-Pax3 was transiently transfected into 4 P19[Mgn] and 4 P19[MyoD] cell lines. 7 μg of PGK-Pax3 and 1 μg of PGK-LacZ were transfected into P19[Mgn] and P19[MyoD] cells using the FuGene 6 transfection system (Roche Molecular Biochemicals) according to the manufacturer’s protocol. After 24 h, cells were plated onto coverslips and allowed to grow in monolayer and fixed on day 6. To produce cells that stably expressed MyoD and Pax3, P19[MyoD] cells were transfected with 10 μg of PGK-Pax3, 1 μg of PGK-Puro, 1 μg of PGK-LacZ, and 2.5 μg of B17, using the CaPO₄ transfection method (60). Transfection efficiencies were confirmed to be high (as above), and clones were selected in puromycin (2 μg/ml) for 10 days. Clones were isolated, and those expressing both MyoD and Pax3 were differentiated as described above.

To determine the effects of BMP-4 on the ability of MyoD and myogenin to induce myogenesis, P19[MyoD] and P19[Mgn] cells were grown in monolayer and differentiated (described above) in the presence and absence of 5, 25, 100, and 200 ng/ml BMP-4 (Genetics Institute, Cambridge, MA) and fixed after 2, 4, or 6 days in monolayer culture or after 6 days of differentiation.

To determine the effects of Wnt3a on the ability of MyoD or myogenin to induce myogenesis, P19[MyoD] and P19[Mgn] cells were grown in monolayer and differentiated (described above) in the presence and absence of 5, 25, 100, and 200 ng/ml Wnt3a (Genetics Institute, Cambridge, MA) and fixed after 2, 4, or 6 days in monolayer culture or after 6 days of differentiation.

**Immunofluorescence**—Cells were fixed in either methanol at −20 °C for 5 min or Lane’s fixative (4% paraformaldehyde, 14% v/v saturated picric acid, 125 mM sodium phosphate) for 30 min, rehydrated in PBS for 30 min at room temperature, and then incubated with the appropriate antibody. For total muscle myosin staining, 50 μl of a mouse anti-MHC monoclonal antibody supernatant, MF20 (63), was incubated for 1 h at room temperature. For myogenin staining, 100 μl of the anti-myogenin monoclonal antibody supernatant, F5D (64), containing 0.03% Triton X-100 and 5% fetal calf serum, was incubated at 4 °C for 24 h. After three 5-min washes in PBS, cells were incubated for 1 h in 200 μl of PBS with 1 μl of goat anti-mouse IgG(14L) Cy3-linked antibody (Jackson Immunoresearch Laboratories, PA). Sections were mounted in a solution of 50% glycerol, 40% PBS, 9.9% p-phenylenediamine, and 0.1% Hoechst stain. Immunofluorescence was visualized with a Zeiss Axioskop microscope, and images were captured with a Sony 3CCD color video camera, processed using Northern Exposure, Adobe Photoshop, and Corel Draw software, and printed with a dye sublimation phaser 450 Tektronix printer. Immunofluorescence experi-
cardiac v1.11 software from Molecular Dynamics. Visualized by autoradiography and with a PhosphorImager SI from 0.2% SDS (0.13M SSC, 0.2% SDS) for Wnt5b blots. Hybridization was performed at 65 °C in 0.2× SSC, 0.2% SDS for Wnt5b blots. The corresponding Hoechst staining is shown in A, C, E, G, I, and K (magnification, ×16 for A–F and I–L; ×40 for G and H).

Results

Expression of Both MyoD and Myogenin Does Not Bypass the Requirement for Cellular Aggregation—Previous studies have shown that stable P19 cell lines expressing either MyoD or myogenin required aggregation to initiate myogenesis (52, 55). To examine whether the expression of both MyoD and myogenin could bypass the requirement for cellular aggregation, stable cell lines were isolated that expressed both MRFs. Similar to P19[MyoD] (52) and P19[MyoD+Mgn] cells (55), P19[MyoD+Mgn] cell lines did not express significant levels of MyHC when grown as a monolayer, as indicated by immunoreaction with the anti-MyHC antibody MF20 (data not shown). However, after 4 days of aggregation with (Fig. 1, A, C, and E) or without (Fig. 1, B, D, and F) Me2SO, P19[MyoD] (Fig. 1, C and D) and P19[MyoD+Mgn] (Fig. 1, E and F) cells appeared bipolar and expressed MyHC on day 6. P19 control cells did not differentiate into skeletal muscle either with (Fig. 1A) or without Me2SO (Fig. 1B) on day 6. P19 control cells differentiated into cardiac muscle in the presence of Me2SO (Fig. 1A), as described previously (47). Consequently, the activity of myogenin protein, alone or in combination with MyoD, was regulated by cellular aggregation.

Myogenin Is Post-translationally Regulated in a Cell Type-specific Manner—Because myogenin mRNA was present in P19 stem cells on day 0 before aggregation (55), the inability of myogenin to initiate differentiation in monolayer suggests that the myogenin protein was either not present or not functional. It is possible that post-transcriptional regulation prevented myogenin protein from being expressed. To examine this question, immunofluorescence with an anti-myogenin antibody was performed on P19[Mgn] and P19 cells before and after aggregation (Fig. 2). Myogenin protein (Fig. 2, F, H, and J) was present in the Hoechst stained nuclei (Fig. 2, E, G, and I) of P19[Mgn] cells before (Fig. 2, E–H) and after (Fig. 2, I and J) aggregation. In all cell lines examined, myogenin was found to be present in >90% of the nuclei (quantitated by counting cells on two coverslips from each of four cell lines). A higher magnification shows that some myogenin protein (Fig. 2H) was also present in the cytoplasm when compared with the Hoechst
staining of the nuclei (Fig. 2G), which is probably due to a saturation of the nuclear transport machinery by the high levels of exogenous myogenin expression. P19 control cells (Fig. 2, A–D) did not express myogenin protein (Fig. 2, B and D) before (Fig. 2, A and B) or after (Fig. 2, C and D) aggregation. Because myogenin protein is present in the nucleus of cells before aggregation, a form of post-translational regulation may modify the activity of the protein.

Not all of the myogenin-positive cells differentiated into skeletal muscle, because only 30–45% of the Hoechst-stained nuclei (Fig. 2K) expressed MyHC (Fig. 2L), quantitated by counting cells on two coverslips from each of four cell lines. This suggests that the potential post-translational regulation of myogenin during the differentiation of P19 cells could be cell lineage-specific. Consequently, only a subset of the P19[Mgn] cells have the proper cellular environment permissive for full myogenin activity. It seems likely, therefore, that there is a subset of cells that express factors involved in positively regulating MRF activity.

Expression of Factors Involved in Somite Patterning Correlates with MRF Activation—To determine the optimal length of time required for skeletal myogenesis, a time course of aggregation was performed for P19[MyoD] and P19[Mgn] cells. Cultures were aggregated for 1–4 days in the presence of Me$_2$SO, and harvested for RNA 1 day after transfer to tissue culture dishes (A). Northern blots containing 6 µg of total RNA were probed with cardiac α-actin. Expression levels were quantified by densitometry and shown in B.

To identify candidate molecules that may be involved in regulating MyoD and myogenin activity, a time course of skeletal muscle development was analyzed in P19, P19[MyoD], and P19[Mgn] cells aggregated for 4 days without Me$_2$SO. The expression of factors involved in somite patterning, such as Wnt1, -3a, -5b, and -7a, BMP-2 and -4, and Pax3, were examined by Northern blot analysis. The results obtained for P19[MyoD] (Fig. 4) and P19[Mgn] (data not shown) cells were found to be similar compared with P19 control cells (Fig. 4). MyoD was expressed throughout the time course from day 0 to day 6 in P19[MyoD] cells and not in the control cell line (Fig. 4A). The skeletal muscle-specific marker MLC 1/3 was expressed following aggregation on day 5 and increased on day 6 (Fig. 4B) in cells expressing MyoD. The mesoderm marker Brachyury T was expressed on days 2 through 4 in P19 and P19[MyoD] cells (Fig. 4C), indicating the induction of mesoderm, as previously reported (48). Expression of Brachyury T in P19 cells treated without Me$_2$SO did not lead to any further differentiation of these cells. Wnt5b was expressed from days 2 through 5, peaking on days 2 and 3, and then decreasing (Fig. 4D). Wnt5b was also expressed at lower levels in the control cells (Fig. 4D) indicating that aggregation alone up-regulates Wnt5b expression. Wnt3a was expressed from days 2 through 6, peaking on day 4 (Fig. 4E). Wnt1 and -7a expression was undetectable or at very low levels during the time course (data not shown). The expression of BMP-2 and BMP-4 appeared on day 3 (Fig. 4, F and G), and Pax3 expression first appeared on day 4 (Fig. 4H). Therefore, the expression of factors involved in somite patterning was activated by aggregation of P19, P19[MyoD], and P19[Mgn] cells at the appropriate time to make these factors candidates for regulating MRF activity.

Factors Involved in Somite Patterning Are Expressed During Me$_2$SO-induced Skeletal Myogenesis—A time course of Me$_2$SO-induced skeletal myogenesis was analyzed for the expression of factors shown to be present during MRF-induced myogenesis. P19 parental cells were aggregated in the presence of 0.8% Me$_2$SO for 4 days under serum conditions, which enhanced the population of skeletal myocytes and decreased the number of cardiomyocytes formed (50). Northern blots were performed on RNA harvested from each day during the differentiation. P19 cells aggregated in Me$_2$SO expressed Brachyury T at high levels on days 1 through 3 (Fig. 5A). Wnt5b was expressed from
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**Fig. 5.** The temporal pattern of expression of somite-patternning factors during Me₂SO-induced skeletal myogenesis in P19 cells. P19 parental cells were aggregated in the presence of 0.8% Me₂SO for 4 days and plated on tissue culture dishes. Total RNA was isolated from a time course of differentiation from days 0 through 9 during the differentiation. Identical Northern blots containing 6 μg of total RNA were probed with the cDNAs indicated on the right. The loading was standardized by hybridization to an 18 S probe (18).

Days 1 through 4, peaking on day 3 (Fig. 5B). Wnt3a was the next factor expressed from days 2 through 4 (Fig. 5C), and Wnt7a was not expressed at significant levels during the Me₂SO-induced differentiation program (data not shown). BMP-4 was expressed from days 3 through 9 (Fig. 5D), and Pax3 from days 4 through 9 (Fig. 5E). The timing of the expression of each of these factors is similar to their expression in the MRF-induced time course shown in Fig. 4. This indicates that the expression patterns of these early factors during myogenesis is ordered in a specific manner.

During the endogenous differentiation pathway, MEF2 and MRF family members were also expressed. MEF2C, previously shown to synergize with the MRF family of factors (18), was expressed during aggregation in both MRF-induced and Me₂SO-induced skeletal myogenesis (Figs. 4 and 5). Wnt3a and myogenin, were expressed from days 7 through 9 (Fig. 5, G and H).

**Wnt3a but Not BMP or Pax3 Can Activate MyoD and Myogenin**—Due to the findings that myogenin was regulated in a post-translational and cell type-specific manner in P19[Mgn] cells and that there was an ordered expression pattern for factors expressed during aggregation in both MRF-induced and Me₂SO-induced skeletal myogenesis (Figs. 4 and 5), we hypothesized that a factor(s) expressed during aggregation may be involved in regulating the activity of the MRFs. Expression of this factor(s) should consequently bypass the requirement for cellular aggregation. To test this hypothesis, mixing experiments were carried out without aggregation. Monolayers of P19[Mgn] cells were mixed with various combinations of P19[Wnt3a] cells and P19 control cells in the presence and absence of BMP-4 for 6 days. P19[Mgn] cells mixed with P19 control cells differentiated into a very low percentage of MyHC-positive cells (Fig. 6B). When the same mixture was grown in the presence of BMP-4 (5 ng/ml), no increase in the number of MyHC-positive cells was observed (Fig. 6D).

P19[Mgn] cultures mixed with P19[Wnt3a] cells, showed an increase in the number of MyHC-positive bipolar myocytes present (Fig. 6F).

The transcription factor Pax3 was also expressed during aggregation, before myogenesis (Figs. 4 and 5). The possibility that Pax3 could directly or indirectly regulate MRF activity was tested by transiently expressing Pax3 in P19[MyoD] and P19[Mgn] cell lines. After transfection these cells were plated onto coverslips and grown in monolayer for 6 days. No increase in the number of MyHC-positive cells occurred after transient Pax3 expression (data not shown). The involvement of Pax3 in MRF activation was further tested by stably expressing Pax3 in P19[MyoD] cells. Again, no increase in myogenesis occurred in these cell lines either grown in monolayer or aggregated to induce myogenesis (data not shown).

To quantitate results observed in Fig. 6, the number of MyHC-positive cells present on a coverslip were counted, and the results of these counts are shown in a bar graph (Fig. 7). The presence of Wnt3a-expressing cells increased the number of MyHC-positive cells in P19[MyoD] cultures 5-fold (±1, n = 9) and P19[Mgn] cultures 8-fold (±2, n = 10) (Fig. 7). The number of MyHC-positive cells decreased slightly in P19[MyoD] cells by 0.6-fold (±0.3, n = 4) and in P19[Mgn] cells by 0.3-fold (±0.01, n = 2) in the presence of 5 ng/ml BMP-4 (Fig. 7). Furthermore, the presence of BMP-4 in co-cultures of P19[MRF] cells and P19[Wnt3a] cells inhibited Wnt3a activation of MyoD and myogenin function (Fig. 7). In addition, P19[MyoD] and P19[Mgn] cell lines aggregated in the presence of various concentrations of BMP-4 (1, 5, 25, 100, and 200 ng/ml) did not show increases in the number of skeletal myocytes formed (data not shown). These findings indicate that Wnt3a expression but not Pax3 or BMP can lead to an activation of MRF function in P19 cells. Furthermore, BMP expression can antagonize the ability of Wnt to induce MRF function.

**DISCUSSION**

The mechanisms involved in the activation of MRF function during cellular aggregation of P19 cells were examined. Myogenin, alone or in combination with MyoD, was unable to induce significant levels of myogenesis in P19 cells in the absence of cellular aggregation. Myogenin protein was found in
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The temporal pattern of expression of factors in P19 cells supports the current embryonic model in which Wnt and BMP molecules, expressed in the dorsal neural tube and surface ectoderm, initiate a cascade of events that results in the proper cellular environment to activate the expression of the MRFs, possibly through a mechanism involving Pax3 (30, 33, 39, 42–44, 69). Our result also extends this model to suggest that the cascade of events initiated by Wnt signaling is important for regulating MRF activity in addition to regulating MRF expression. The possibility exists that Wnts expressed in the neural tube, surface ectoderm, as well as in the mesoderm itself, function to activate the MRFs during myogenesis in the embryo. Of those tested, Wnt3a and Wnt5b appear to be the most abundant Wnt family members expressed during P19 cell differentiation. Wnt3a is expressed in the dorsal neural tube (59). Wnt5b is expressed in the primitive streak and tail bud during gastrulation (70, 71) and in the segmental plate mesoderm just before myogenesis.2 Both of these factors could function in MRF activation in the embryo.

P19 cells originate from the inner cell mass of day 6 murine embryos (47). As such, these cells have been isolated before the occurrence of gastrulation and muscle specification and are therefore pluripotent in nature. Clearly, the differentiation of pluripotent stem cells into skeletal myocytes must involve the creation of a specific cellular environment in which only a subset of cells is permissive to MRF-induced myogenesis. This is reminiscent of the complexity of regulatory mechanisms that control multiple lineage determinations during embryogenesis. Identification of the factors expressed during aggregation should allow for the elucidation of pathways involved in regulating MRF activity. Analysis of the MRF and Me2SO-induced myogenesis in P19 cells revealed an ordered pattern of factor expression. Brachyury T is present early in the time course and indicates the induction of mesoderm (48), which is one of the first steps required for a pluripotent stem cell to develop into a mesoderm-derived cell type such as a myocyte. Wnt3a, Wnt5b, BMP-2/4, and Pax3 are also expressed during aggregation in a pattern consistent with their expression in the embryo. This ordered pattern of expression of factors in P19 cells further supports the hypothesis that P19 cells are a good model system for studying embryogenesis.

There are several mechanisms by which Wnt signaling may regulate MRF activity. Wnt has been shown to signal by at least two pathways. Wnt1 binds to its receptor, Fz1, and signals through the classic Dsh → GSK3 → β-catenin → LEF1/TCF pathway, whereas Wnt7a binds to Fz7 and signals through protein kinase C independent of β-catenin, both resulting in gene expression. It is possible, therefore, that Wnt signaling results in the expression of a co-activator of MRF activity.

Recent work has shown that, in addition to the classic linear pathways stimulated by Wnt molecules, networks likely exist whereby Wnt signaling is mediated by a number of alternative receptors and signaling pathways (35). For example Wnt signaling can activate c-Jun N-terminal kinases (72, 73). Studies in myoblasts have shown that activated p38 kinase is essential for myogenesis in myoblast cell lines (74–76). Activated p38 kinase can be detected in the nuclei of myotubules, and the p38 kinase-specific inhibitor, SB203580, can inhibit myogenesis. Therefore, it is possible that kinases activated in response to Wnt signaling are either directly or indirectly regulating the activity of the MRFs. Finally, LEF1/TCF transcription factors may not activate transcription independently but may create specific changes in chromatin conformation that are permissive for transcription (77). Therefore, Wnt signaling may be involved in changing chromatin structure such that MRF activity is enhanced. Future experiments are required to examine the mechanism of Wnt signaling involved in MRF activation.

Pax3 is expressed after Wnt in Me2SO and MRF-induced myogenesis of P19 cells. Pax3 has been implicated in regulating the expression of MyoD during myogenesis in the embryo (43, 44). However, in the embryo (40, 41) and in P19 cells (78), Pax3 is not expressed in fully differentiated embryonic muscle cells. Pax3 is expressed in the proliferating cells of the dermomyotome before MRF expression. Limb muscle precursors ex-

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2 C. Marcelle, personal communication.
press Pax3 as they migrate from the dermomyotome, and Pax3 is essential for their proper migration and maturation into muscle (40, 79). Our finding that Pax3 is unable to activate MRF function in P19 cells does not preclude a role for Pax3 upstream of MRF expression. Indeed, Pax3 is expressed several days before the MRFs in MeSO- induced myogenesis in P19 cells and, therefore, may be regulating MRF expression in some way.

BMP-2/4 are also expressed in MeSO and MRF-induced myogenesis in P19 cells. BMP-4 inhibited myogenesis when present during the differentiation of P19 and P19[MRF] cells. Our results thus agree with previous studies that show inhibition of myogenesis by BMP in the embryo (38, 42) and in myoblast cell lines (80–82). Furthermore, we show that BMP signaling antagonizes the Wnt-induced activation of the MRFs. BMP is known to activate TAK1, a member of the mitogen-activated protein kinase family (83). Signaling through the TAK1-NLK pathway antagonizes the activity of β-catenin/TCF complexes and, therefore, Wnt signaling (84). Consequently, the BMP antagonism of the Wnt-induced activation of the MRFs may occur by the inactivation of β-catenin/TCF complexes. Recently, it has been shown that Smad4, a mediator of BMP signals, can interact with LEF1/TCF complexes and modulate their function (85). Future studies to discern the Wnt signaling pathway may clarify this issue.

Myogenesis in P19 cells induced by MeSO is marked by the expression of MyoD and myogenin late in the time course. MEF2C is expressed before the MRFs. The expression of MEF2C is the result of early events controlling early cellular differentiation. We thank Layla Katiraee for her excellent technical assistance. We thank Daniel MacPhee, Peter Merrifield, B. D. Edmondson, and D. G. Olson for their contributions and for providing critical comments on this manuscript. We thank Layla Katiraee for her excellent technical assistance. We thank Daniel MacPhee, Peter Merrifield, B. D. Edmondson, and D. G. Olson for their contributions and for providing critical comments on this manuscript.

Targets for SMAD4 Knockout Mice

The expression of MEF2C before the expression of the MRF family members may indicate that MEF2C initiates the expression of the MRFs in P19 cells. However, an alternative explanation is that cardiac muscle precursor cells are formed under the serum conditions used and that the expression of MEF2C is the result of early stages of cardiomyogenesis (47, 86). Due to the heterogeneity of the cultures after aggregation, distinguishing between the two possibilities is difficult.

In summary, mechanisms controlling myogenesis in P19[MRF] cells are similar to those present in the embryo. Furthermore, the temporal pattern of expression of somite patterning factors, Wnt3a, Wnt15, BMP-2/4, and Pax3, is correlated with the activation of MRF function. Wnt3a, but not BMP-4 or Pax3, is able to activate MyoD and myogenin in P19 stem cells and lead to differentiation in the absence of aggregation. By linking mesoderm induction with myogenesis, the P19 model system is valuable for analyzing molecular mechanisms controlling early cellular differentiation.
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