Developmental Biology **224,** 189–203 (2000)
doi:10.1006/dbio.2000.9784, available online at http://www.idealibrary.com on IDEAL®

metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

IGFs, Insulin, Shh, bFGF, and TGF-β1 Interact Synergistically to Promote Somite Myogenesis *in Vitro*

Asta Pirskanen,[1] Julie C. Kiefer, and Stephen D. Hauschka[2]

Department of Biochemistry, University of Washington, Box 357350, Seattle, Washington 98195

Studies from our group and others have shown that *in vitro* somite myogenesis is regulated by neural tube and notochord factors including Wnt, Sonic hedgehog (Shh), and basic fibroblast growth factor (bFGF) together with transforming growth factor-β1 (TGF-β1). In this study we report that insulin and insulin-like growth factors I and II (IGF-I and -II) also promote myogenesis in explant cultures containing single somites or somite-sized pieces of segmental plate mesoderm from 2-day (stage 10–14) chicken embryos and that the combination of insulin/IGFs with bFGF plus TGF-β1 promotes even higher levels of myogenesis. We also found that Shh promotes myogenesis in this *in vitro* system and that Shh interacts synergistically with insulin/IGFs to promote high levels of myogenesis. RT-PCR analysis detected insulin, IGF-II, insulin receptor, and IGF receptor mRNAs in both the neural tube and the somites, whereas IGF-I transcripts were detected in entire embryos but not in the neural tube or somites. Treatment of somite–neural tube cocultures with anti-insulin, anti-IGF-II, anti-insulin receptor, or anti-IGF receptor blocking antibodies caused a significant decrease in myogenesis. These results are consistent with the hypothesis that systemic IGF-I as well as insulin and IGF-II secreted by the neural tube act as additional early myogenic signals during embryogenesis. Further studies indicate that insulin, IGFs, bFGF, and Shh also stimulate somite cell proliferation and influence apoptosis. © 2000 Academic Press

Key Words: **somite; myogenesis; muscle; neural tube; insulin-like growth factors (IGFs); IGF receptors; Sonic hedgehog (Shh); fibroblast growth factor; TGF-β1; apoptosis.**

INTRODUCTION

Somite myogenesis is both positively and negatively regulated by factors produced by the neural tube, notochord, ectoderm, and lateral plate mesoderm. Positive signals include Wnt family members and Sonic hedgehog (Shh) (Christ *et al.,* 1992; Buffinger and Stockdale, 1994; Münsterberg *et al.,* 1995a,b; Stern *et al.,* 1995; Cossu *et al.,* 1996; Fan *et al.,* 1997; Capdevila *et al.,* 1998; Borycki *et al.,* 1998, 1999; Marcelle *et al.,* 1997, 1999). In contrast, bone morphogenetic protein (BMP4) inhibits myogenesis (Pourquié *et al.,* 1995, 1996) by repressing expression of the muscle determination factors MyoD and Myf5 (Reshef *et al.,* 1998), while noggin counteracts BMP4 repression (Hirsinger *et al.,* 1997). Interestingly, Shh and Wnt signals can also compete with each other in the specification of presomitic mesoderm to become sclerotome or dermamyotome (Lee *et al.,* 2000). Several other factors, when acting combinatorially or synergistically, can also induce/promote somite myogenesis *in vitro,* e.g., Stern *et al.* (1997) reported that basic fibroblast growth factor (bFGF) + transforming growth factor-β1 (TGF-β1) stimulated high levels of myogenesis in a single somite assay system; however, the full spectrum of factors and the mechanisms by which they affect somite myogenesis are unknown.

In addition to direct myogenic "inducing/promoting" effects, Marcelle *et al.* (1999) have reported that Shh is capable of maintaining but *not* inducing somite MyoD expression, Teillet *et al.* (1998) have shown that Shh enhances myogenic cell survival, and Cann *et al.* (1999) have reported that Shh mimics effects of the ventral neural tube by increasing myogenic progenitor cell proliferation, inhib-

[1] Current address: Department of Medical Biochemistry, University of Oulu, Box 5000, FIN-90014, Oulu, Finland.

[2] To whom correspondence should be addressed at the Department of Biochemistry, University of Washington, Box 357350, Seattle, Washington 98195. Fax: (206) 685-1792. E-mail: haus@u.washington.edu.

0012-1606/00 $35.00
Copyright © 2000 by Academic Press
All rights of reproduction in any form reserved.

iting apoptosis, and enhancing the number of primary muscle fibers expressing slow myosin heavy chain. In contrast, Borycki *et al.* (1999) reported that Shh is required for the survival of sclerotomal cells, but not for the survival or proliferation of epaxial myogenic progenitors. Finally, many studies indicate that the end results of inductive signals depend upon the sequence of exposure, concentrations of positive and negative signals, and somite stage (Hirsinger *et al.*, 1997; Reshef *et al.,* 1998; Williams and Ordahl, 1997). Taken together, these studies imply that the orderly induction of somite myogenesis *in vivo* entails a complex temporal and quantitative interplay between multiple positive and negative signals. This further implies that signaling molecules not yet considered "direct" components of early myogenic development could influence these processes.

Insulin and insulin-like growth factors I and II (IGF-I and IGF-II) are hormonal peptides with overlapping biological effects on muscle (Cohick and Clemmons, 1993; Florini *et al.,* 1996). Expression of insulin and IGFs and their receptors has been found in chicken embryos as early as day 1 (Perez-Villamil *et al.,* 1994; Holzenberger *et al.,* 1996; Morales *et al.,* 1997), and effects of these factors on neural development have been observed in day 2 embryos (de la Rosa *et al.,* 1994; De Pablo and de la Rosa, 1995). IGF-I is present in egg yolk (Scavo *et al.,* 1989) and it is thought that insulin and the IGFs are present systemically at these early stages. In addition to stimulating myoblast proliferation, these factors stimulate many anabolic processes in differentiated skeletal muscle, and all three members of the IGF family also potentiate muscle cell terminal differentiation *in vitro.* The importance of IGF-I for embryonal muscle development has also been demonstrated by IGF-I-knockout experiments (Baker *et al.,* 1993; Powell-Braxton *et al.,* 1993). In addition to their significantly smaller size, IGF-I-deficient mice exhibit a severe muscular dystrophy.

Insulin and IGF-I receptors are heterotetrameric transmembrane proteins containing an intracellular ATP-binding site and a tyrosine cluster which requires phosphorylation for activation of kinase activity (for review, see Florini *et al.,* 1996). After activation, the receptors can interact with intermediate signaling proteins, e.g., insulin receptor substrate-1 and Src homology containing protein, leading to activation of protein kinase pathways. The IGF-II receptor is identical to the cation-independent mannose 6-phosphate receptor (Florini *et al.,* 1996; Zhou *et al.,* 1995). It is *not* a signal transducer and has been found to serve primarily as a sink for excess IGF-II. Thus, the biological actions of both IGF-I and IGF-II are mediated mainly by IGF-I receptor.

To better understand the contribution of insulin and IGFs to early myogenesis we investigated their function using an *in vitro* single-somite assay (Stern and Hauschka, 1995). In this system, somites I–III/IV from stage 13/14 chick embryos exhibit no differentiated muscle cells when cultured alone and are thus operationally defined as "uncommitted" with respect to myogenesis. In contrast, somites V–VIII, while not containing myosin heavy chain (MyHC)-positive cells *in vivo,* are able to form such cells after 3 days of *in vitro* culture and are thus operationally defined as "committed" to the myogenic lineage. When uncommitted somites are cultured with neural tube, notochord, Wnt-1-producing fibroblasts, or FGF + TGF-β1, some cells become MyHC-positive and are thus operationally defined as having been "induced" to become myogenic (Stern and Hauschka, 1995; Stern *et al.,* 1995, 1997). It is critical to emphasize that it is not known how far along a hypothetical myogenic developmental pathway the cells in uncommitted somites have progressed at the time of somite removal from the embryo. When dissociated, some somite/segmental plate and even epiblast cells form muscle without needing exposure to the neural tube (George-Weinstein *et al.,* 1994, 1996), and RT-PCR assays detect both Myf5 and MyoD in somites I–III (Münsterberg and Lassar, 1995b; Kiefer and Hauschka, unpublished); but despite prior expression of these myogenic factors, cells in *intact* uncommitted somites are unable to proceed to terminal differentiation *in vitro* without exposure to additional "inductive" stimuli. These can be provided either by coculture with the neural tube/notochord or by addition of defined factors.

In the present study we used a single-somite assay system to determine whether insulin and IGFs induce/promote myogenesis in uncommitted somites and if they stimulate myogenesis in somites containing cells already committed to myogenesis. We demonstrate that, in the absence of other known inducing factors, insulin, IGF-I, and IGF-II induce as well as stimulate additional somite myogenesis in a dose-dependent manner. Furthermore, mRNAs for insulin, IGF-II, and insulin and IGF receptors are found in the neural tube and in the paraxial mesoderm, and IGF-I is expressed systemically. The hypothesis that insulin and IGFs are involved in the normal neural tube-mediated induction of somite myogenesis is further supported by the finding that anti-insulin and anti-IGF-II blocking antibodies inhibit neural tube-induced myogenesis. Thus insulin and the IGFs should be added to the growing list of soluble factors involved in early aspects of somite myogenesis. Our studies also show that insulin and IGFs function synergistically with other myogenic inducing factors including bFGF + TGF-β1 and Shh. Two aspects of IGFs' and Shh's positive effects on somite myogenesis *in vitro* may involve stimulating cell proliferation and inhibiting cell death.

MATERIALS AND METHODS

Dissections. White Leghorn chicken eggs (H and N International) were incubated in a 38°C forced-draft incubator at 100% humidity for 2 days. Stage 10–11 and 13–14 embryos (Hamburger and Hamilton, 1951) were used for dissections. Tissues were dissected as previously described (Stern and Hauschka, 1995). Somites I–III were obtained from both sets of stages, whereas somite IV was obtained only from embryos of stage 13 and younger. This was necessary because at stage 14 somite pair IV contains

Copyright © 2000 by Academic Press. All rights of reproduction in any form reserved.

some cells which are capable of undergoing myogenesis *in vitro* without further neural tube signals (Stern and Hauschka, 1995). In experiments employing somites from embryos as old as stage 14, the somite numbers and stages are thus indicated as "somites I–III/IV from stages 13–14." In experiments designed to analyze the behavior of somites containing many cells already committed to myogenesis, somites V–VIII were dissected from stage 10–11 and 13–14 embryos. In studies designed to analyze the behavior of presomitic cells, segmental plates were dissected from stage 10–11 embryos and cut into caudal and rostral halves and each half was then cut into 4 somite-sized pieces. From stage 13–14 embryos, segmental plates were cut into caudal, medial, and rostral regions. Caudal and rostral regions were then cut into 4 somite-sized pieces. Neural tube was dissected from between the rostral half of the segmental plate and somite VIII from embryos of the same stages. It was then cut transversely into 10 equal pieces. Previous studies had indicated no differences in the myogenic inducing activity between rostral and caudal neural tube segments from these embryonic stages (Stern *et al.,* 1995).

Tissue culture. The dissected explants were transferred into the centers of gelatin-coated 96-well tissue culture plates containing 200 μl of culture medium. Basal somite medium (BSM) consisted of 99% Ham's F10C nutrient medium (Ham's F10 with Ca^{2+} adjusted to 1.26 mM) plus 30 nM sodium selenate (Sigma), 100 μM putrescine (Calbiochem), 0.1 mg/ml chick transferrin (Sigma; conalbumin), and antibiotics (60 μg/ml gentamicin and 250 ng/ml Fungizone (Life Technologies)), plus 1% chick embryo extract (CEE) (Konigsberg, 1968). Since CEE contains many unknown components as well as some of the defined factors being tested, e.g., bFGF (Linkhart *et al.,* 1981; Seed *et al.,* 1988), certain studies were also performed in the absence of CEE (Fig. 4, Table 2) so that interpretations could be made without the ambiguity of unknown CEE components. Defined factors insulin, IGF-I, IGF-II, bFGF, TGF-β1, and Shh were added to BSM as specified in the text. Recombinant human IGF-I and IGF-II were purchased from Sigma, and bovine insulin was obtained from Collaborative Biomedical. Human recombinant bFGF and acid-activated human recombinant TGF-β1 were kindly provided by Bristol–Myers Squibb. Shh-conditioned medium (~100 nM) was produced in COS cells transfected with pShh (Roelink *et al.,* 1995). After 72 h, cultures were fixed and immunohistochemistry was performed as described below.

Neutralizing antibody studies. In antibody neutralization studies, neural tubes from the same stages as the tested somites were cut into somite-sized pieces, transferred to 96-well tissue culture plates, and cultured in contact with single somites. Neutralizing antisera were added to BSM as described in the text and figure legends: goat anti-IGF-I and anti-IGF-II antibodies (R&D Systems), rabbit anti-insulin receptor and sheep anti-insulin antibodies (Biodesign International), and anti-IGF receptor monoclonal antibody (Santa Cruz Biotechnology, Inc.). As controls, normal rabbit and normal goat IgGs (R&D Systems) were used in amounts equivalent to those of the neutralizing antibodies.

Cell number, cell proliferation (S phase), and cell death assays. Somites I–IV from stage 10–11 embryos were cultured in BSM with various growth factors for 3 days. Explants were then trypsinized in 25 μl and cells were counted in a hemacytometer. To study growth factor effects on proliferation, BrdU was added to the medium of 23-h cultures at a final concentration of 100 μM for 1 h. In other studies, cultures were labeled with BrdU (10 μM) for 24 h starting at 24 h in order to assess the total percentage of replicating cells. The cultures were then fixed in 4% (w/v) paraformaldehyde and BrdU-substituted DNA was detected as described below. The percentage BrdU-positive cells in the 1-h pulse study thus represents the percentage cells in S phase between the first and the second days of culture, while the 24-h labeling study indicates the total fraction of replicating cells.

Apoptotic cell death was studied via the TUNEL assay. Somites I–IV from stage 10–11 embryos were cultured in BSM plus various growth factors. After 24 h, cultures were fixed in 4% (w/v) paraformaldehyde and apoptotic cells were detected by the Dead End Colorimetric Apoptosis Detection System (TUNEL assay) from Promega.

Immunohistochemistry. As an indication of myogenic differentiation, fixed cultures were stained for MyHC using the MF20 monoclonal antibody (Bader *et al.,* 1982) as previously described (Stern *et al.,* 1995), except that 2% nonfat dry milk was used as a blocking agent. Cells were counted as MyHC-positive cells only if they stained intensely brown. Proliferating cells were detected via BrdU staining as described by Foster *et al.* (1987). After 4% (w/v) paraformaldehyde fixing, DNA was denatured by exposure to 2 N HCl for 40 min, rinsed in PBS, preincubated in 2% nonfat milk, and then incubated with mouse anti-BrdU antibody (1:2000) which was kindly provided by S. J. Kaufman (George-Weinstein *et al.,* 1993). The secondary staining process employed the Vectastain ABC-AP kit (Vector Laboratories).

RT-PCR assay. Neural tube, segmental plate, or single somites were dissected from stage 10–14 embryos and placed immediately in Trizol Reagent (Gibco BRL). Glycogen was added as a carrier (30 μg RNase-free glycogen; Boehringer Mannheim) and RNA was isolated according to the manufacturer's directions. RNA was subsequently primed with oligo(dT) followed by cDNA synthesis using Superscript II reverse transcriptase (Gibco BRL). One-tenth of the cDNA was amplified in a 50-μl reaction using Advantage cDNA Polymerase Mix (Clontech) with the addition of 0.1 μCi [α-^{32}P]dATP. After an initial denaturation step of 94°C for 4 min, the reactions were cycled between 94 (1 min), 64 (1 min), and 72°C (1 min) in a Stratagene Robocycler. cDNAs for the insulin and IGF-I receptors were amplified for 37 cycles, those for IGF-I, IGF-II, and insulin were amplified for 40 cycles, and that of S6 ribosomal protein was amplified for 30 cycles. S6 is ubiquitously expressed and served as a positive control. The following primers were used: for insulin, IGF-I, and IGF-I receptor, both primer pairs were exactly as described by Serna *et al.* (1996). For IGF-II, nucleotides 150–160 in exon 1 + 1–13 in exon 2, 5′-CTTCTACTTCAGTAGACCAGTGGG-3′, and nucleotides 922–945, 5′-GCGTGAGATGGCTTCTGGAAGCTC-3′; for insulin receptor, nucleotides 94–117, 5′-CTGCGGGAGCGCATTGAGTTCCTC-3′, and nucleotides 261–284, 5′-CGACCAGGGTTATTCTCAGCGTCG-3′; and for S6 ribosomal protein, nucleotides 299–317, 5′-CGCAAGTCTGTTCGGGGTT-3′, and nucleotides 678–696, 5′-TCCTGGCGTTTTTCCTTGG-3′, were used.

One-third of the PCR was run on a 6% polyacrylamide gel, dried, and exposed to X-ray film at −20°C for 2–16 h. The expected product sizes are as follows: insulin, 323 bp; IGF-I, 209 bp; IGF-I receptor, 197 bp; IGF-II, 203 bp; insulin receptor, 191 bp; and S6, 398 bp. In all cases, PCR amplification was shown to be dependent on reverse transcription of RNA template. The identity of each product was confirmed by restriction enzyme digests.

Statistics. Statistical analysis of differences was performed by the Student *t* test for two independent variables using Microsoft Excel 97.

Copyright © 2000 by Academic Press. All rights of reproduction in any form reserved.

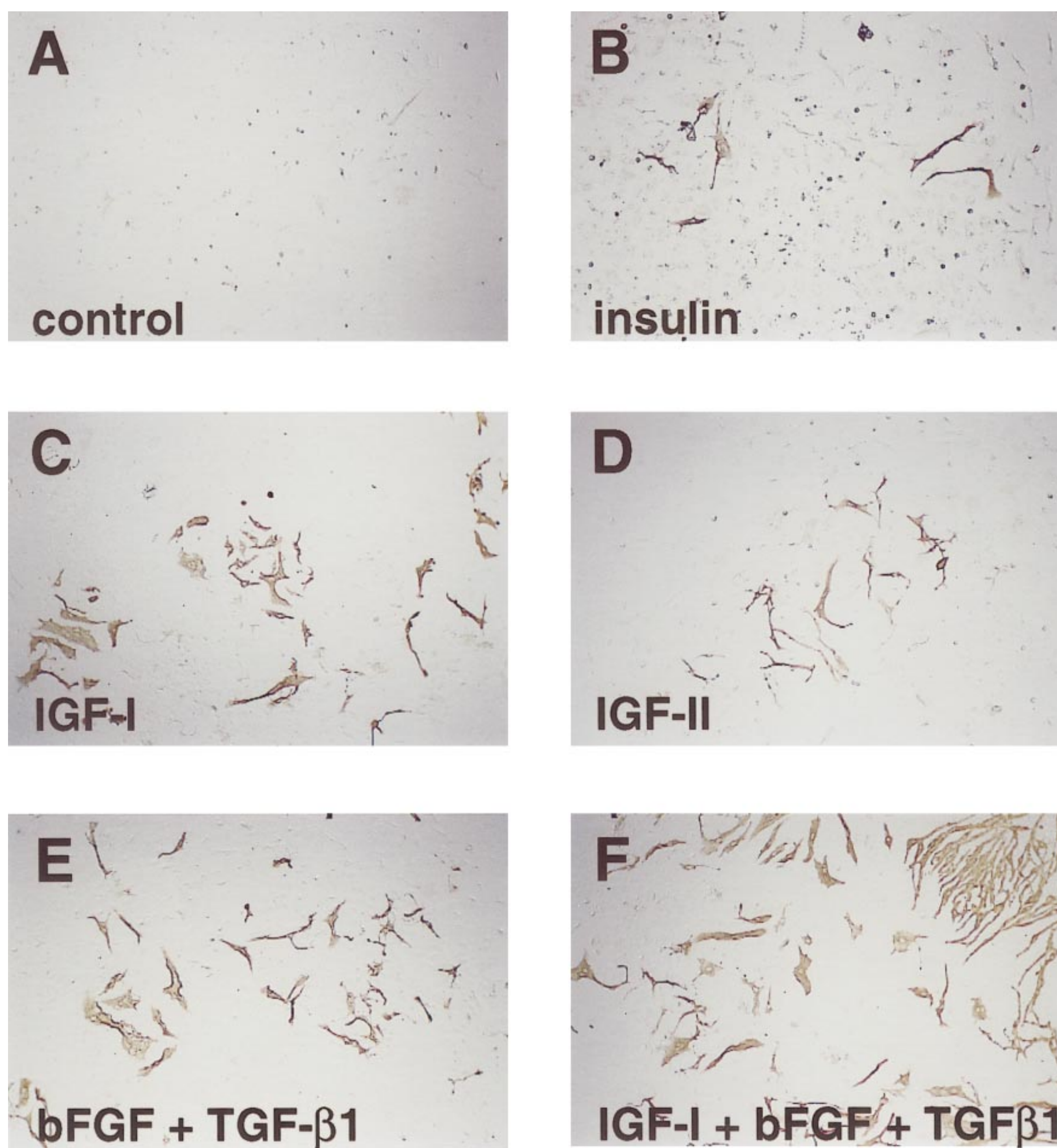

FIG. 1. *In vitro* somite myogenesis in response to defined factors. Somite III explants from stage 13 embryos were cultured for 3 days in BSM (A) or in BSM plus insulin (B), IGF-I (C), IGF-II (D), bFGF + TGF-β1 (E), or IGF-I +bFGF + TGF-β1 (F) and were then fixed and stained for MyHC. Concentrations of defined factors were 40 ng/ml insulin, IGF-I, and IGF-II; 30 ng/ml bFGF; and 10 ng/ml TGF-β1.

RESULTS

Induction of Somite Myogenesis by IGFs and Insulin

To study effects of insulin and IGFs on muscle development, uncommitted somites I–III/IV from stage 13–14 embryos were cultured for 3 days in BSM with varying concentrations of insulin, IGF-I, or IGF-II. Each factor promoted substantial myogenesis (MyHC-positive cells) compared to somites cultured in BSM alone (Fig. 1, compare BSM (A) to BSM + insulin (B), BSM + IGF-I (C), and BSM + IGF-II (D)). Dose–response curves for each factor were carried out with both somite and segmental plate explants prior to the present set of studies. Each factor promoted myogenesis in a dose-dependent manner; differentiation was induced by IGF-I and IGF-II levels as low as 0.1 ng/ml and by insulin levels as low as 1 ng/ml, while maximal muscle differentiation was induced by

Copyright © 2000 by Academic Press. All rights of reproduction in any form reserved.

TABLE 1
Stage-Dependent Differences in Somite Myogenesis in Response to IGF-I, bFGF, and TGF-β1

	Stage 10–11 embryos				Stage 13–14 embryos			
	Somite I–IV[b]		Somite V–VIII[c]		Somite I–III/IV[b]		Somite V–VIII[c]	
Treatment[a]	MyHC+ explants (%)[d]	MyHC+ cells[e]	MyHC+ explants (%)[d]	MyHC+ cells[e]	MyHC+ explants (%)[d]	MyHC+ cells[e]	MyHC+ explants (%)[d]	MyHC+ cells[e]
BSM, control	0	—	27	9 ± 5	0	—	73	13 ± 2
IGF-I	4	12*	76**	39 ± 10*	50***	28 ± 9*	100**	47 ± 10**
bFGF	0	—	29	2 ± 1	15*	3 ± 1*	79	23 ± 6
TGF-β1	4	1	42	7 ± 2	10	1 ± 0*	58	14 ± 4
bFGF + TGF-β1	0	—	27	31 ± 17	54***	14 ± 4**	77	21 ± 6
IGF-I + bFGF	8	20 ± 12*	46	44 ± 14*	45***	12 ± 3**	67	19 ± 3
IGF-I + TGF-β1	0	—	64*	19 ± 8	10	1 ± 0*	83	12 ± 2
IGF-I + bFGF + TGF-β1	29	18 ± 7*	68**	45 ± 10**	95***	53 ± 10***	100**	110 ± 16***

[a] Somites were cultured in BSM alone or in BSM plus defined factors at the following concentrations: IGF-I (40 ng/ml), bFGF (30 ng/ml), and TGF-β1 (10 ng/ml).
[b] Somites I–IV from stage 10–13 and somites I–III from stage 14 embryos contain no cells which are committed to myogenesis.
[c] Somites V–VIII from stage 10–11 and 13–14 embryos contain increasing numbers of cells already committed to myogenesis.
[d] Percentage of cultures with at least one MyHC-positive cell when stage 10–14 explants were cultured in BSM and the indicated factor(s). At least 24 different cultures were examined for each set of conditions. Statistical differences between control and treated cultures: $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.
[e] Number of MyHC-positive cells in MyHC-positive culture(s); mean ± SEM is reported for all treatment conditions with more than one positive culture. Statistical difference indicated as above.

levels 10- to 100-fold higher. Factor concentrations for subsequent studies were selected so as to be in the midrange of doses giving an optimal myogenic response (e.g., IGF-I was typically used at 40 ng/ml). The only conditions under which all three factors exhibited additive effects were concentrations below their optimal levels, e.g., in the 1 ng/ml range (data not shown).

IGF-I, bFGF, and TGF-β1 Act Synergistically to Enhance the Myogenic Response in Somites and Segmental Plate

Stern *et al.* (1997) showed that the combination of bFGF and TGF-β1 has strong myogenic promoting activity on uncommitted somites and segmental plate, while either factor alone has little to no myogenic promoting activity. To determine whether IGF-I signals can interact with those from bFGF and TGF-β1 to promote myogenesis, paraxial mesoderm explants from stage 10–11 and 13–14 embryos were cultured with various combinations of these growth factors (Figs. 1E and 1F, Table 1). Uncommitted somites (somites I–III/IV) exhibited significant developmental differences in their myogenic response to IGF-I with and without the simultaneous presence of bFGF and TGF-β1 (Table 1). Most somites from stage 10–11 embryos exhibited no myogenic response to IGF-I, bFGF, or TGF-β1 when only one or two of the factors were present, and only about 30% responded when all three factors were present. In contrast, 50% of the uncommitted somites from stage 13–14 embryos exhibited MyHC-positive cells in the presence of IGF-I alone, and virtually all of the somites exhibited high numbers of differentiated muscle cells in the presence of all three factors. At both developmental stages a greater percentage of older somites (V–VIII) also exhibited MyHC-positive cells in the presence of IGF-I alone, and the mean number of these cells was significantly enhanced by the presence of all three factors. Interestingly, with older somites from stage 13–14 embryos the simultaneous presence of bFGF and TGF-β1 consistently increased the myogenic response over that to IGF-I alone, while the addition of either single factor to medium containing IGF-I was often inhibitory to myogenic differentiation relative to that with only IGF-I. Analogous studies with IGF-II and insulin plus or minus bFGF and TGF-β1 gave qualitatively similar results.

The myogenic response of stage 10–11 and 13–14 segmental plate explants to IGF-I was qualitatively similar to that of uncommitted somites. For example, 4 and 32% of the rostral segmental plate explants from stages 10–11 and 13–14, respectively, exhibited MyHC-positive cells in the presence of IGF-I, and 42 and 71% of caudal segmental plate explants from these stages exhibited a myogenic response to IGF-I (data not shown). The greater myogenic response of caudal than rostral segmental plate was observed previously when these regions were cultured with neural tube or bFGF + TGF-β1 (Stern *et al.,* 1997). Caudal segmental plate

Copyright © 2000 by Academic Press. All rights of reproduction in any form reserved.

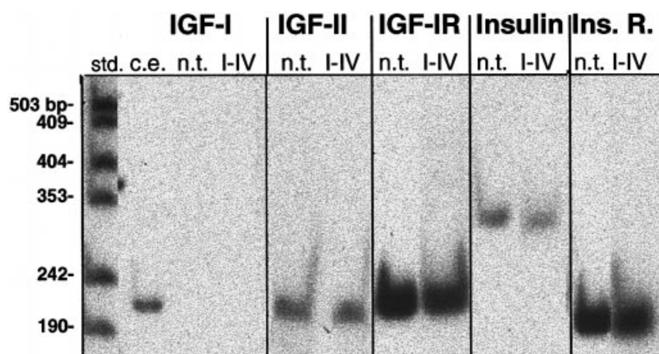

FIG. 2. RT-PCR detection of IGF-I, IGF-II, insulin, IGF-I receptor, and insulin receptor mRNAs in neural tube (n.t.) and somites I–IV (I–IV) from stage 10–14 embryos and detection of IGF-I mRNA in whole stage 13 embryos (c.e.). IGF-II, insulin, IGF-I receptor, and insulin receptor transcripts were detected in both the neural tube and somites I–IV, whereas IGF-I mRNA was not detected in somites or neural tube, but was detected in whole embryos. All samples contained amplifiable S6 ribosomal protein transcripts (data not shown). Abbreviations: IGF-IR, IGF-I receptor; Ins. R., insulin receptor.

explants also exhibited a weak myogenic response to IGF-II and insulin. The combination of IGF-I with bFGF + TGF-β1 increased the percentage of MyHC-positive caudal and rostral explants from stage 10–11 to about 30% and from stage 13–14 to nearly 80%. Thus some paraxial mesoderm cells that are not destined to form somites for an additional 12 h are responsive to myogenic induction by insulin and the IGFs alone; but these factors can also act synergistically with bFGF + TGF-β1 to increase the percentage of differentiated muscle cells.

Considering the complete set of stage 10–11 and 13–14 data, it is clear that myogenesis can be induced in some somite and segmental plate cells by insulin, IGF-I, or IGF-II alone, but that IGF-I is the most potent inducer. It is also evident that myogenic induction by these factors is enhanced by the simultaneous presence of bFGF + TGF-β1. These data suggest that the optimal induction of somite myogenesis involves an interplay of multiple signals.

Are Insulin and IGFs and Their Receptors Expressed in Early Chicken Embryos, Neural Tubes, and Somites?

To address the possibility that insulin, IGF-I, and IGF-II may affect myogenic development *in vivo* as components of neural tube and/or somite-derived signals, we investigated whether their mRNAs are present in stage 10–14 neural tubes and uncommitted somites. RT-PCR analysis showed that mRNA encoding insulin and IGF-II is present in both the neural tube and the somites (Fig. 2), and while IGF-I mRNA was not detected in these tissues, it was found in whole stage 13 embryos. This concurs with previous studies which reported IGF-I mRNA in the head portion of 2-day chick embryos (Perez-Villamil *et al.,* 1994). To determine whether somites could potentially respond to these ligands, we also looked for expression of mRNAs encoding the insulin and IGF-I receptors in the somites of stage 10–14 embryos. (Both of these receptors bind insulin, IGF-I, and IGF-II, albeit with varying affinity.) Insulin receptor and IGF-I receptor transcripts were found in somites as well as in the neural tube (Fig. 2). Therefore mRNAs encoding insulin, IGF-II, and their receptors are present in the appropriate embryonic tissue locations to permit their encoded proteins to participate in the myogenic signaling pathway. Moreover, IGF-I has been demonstrated to signal in a paracrine fashion (Florini *et al.,* 1996). Thus, while its synthetic site is not localized adjacent to paraxial mesoderm, the possibility of IGF-I participating in early myogenesis is supported by detection of its mRNA in whole embryos at stages in which somites express IGF-I receptor transcripts and in which *in vitro* studies demonstrate somite myogenic responsiveness to IGF-I.

Are Insulin/IGFs and Their Receptors Involved in Myogenic Induction of Somites by the Neural Tube?

When somites I–IV from stage 10–11 embryos are cocultured with neural tube, they survive and exhibit myogenesis in a CEE-free BSM. As shown in Table 2, 94% of cultures responded to neural tube induction with an average of 60 ± 7 MyHC-positive cells. To determine whether insulin or IGF-mediated signals are involved in neural tube-induced somite myogenesis, neural tube–somite cocultures were exposed to antisera against these factors. Neutralization experiments were performed in CEE-free BSM so that the presence of unknown factors would not complicate the analysis. Anti-IGF-I, anti-IGF-II, and anti-insulin antibodies were used at concentrations capable of blocking myogenic induction effects of IGF-I, IGF-II, and insulin on somites when present at 20 ng/ml. Addition of goat anti-IGF-II or anti-insulin antibodies to the BSM decreased the MyHC-positive cell number from 60 ± 7 to 39 ± 6 ($P < 0.05$) and 32 ± 5 ($P < 0.01$), respectively, whereas addition of equivalent amounts of nonimmune goat IgG had no effect on myogenesis (Table 2). A combination of anti-insulin and anti-IGF-II antibodies decreased MyHC-positive cultures to 42% ($P < 0.001$) and the average number of MyHC-positive cells to 15 ± 4 ($P < 0.001$). As anticipated, because neural tubes do not express IGF-I, anti-IGF-I antibody alone did not block neural tube-induced myogenesis, and it did not increase blocking effects of anti-insulin and anti-IGF-II antibodies on neural tube–somite cocultures (Table 2). These data suggest that insulin and IGF-II could be involved in neural tube-mediated signals for somite induction, whereas any involvement of IGF-I would have to be via systemic sources. This is consistent with our finding that insulin and IGF-II transcripts are present in the neural tube while those of IGF-I

Copyright © 2000 by Academic Press. All rights of reproduction in any form reserved.

TABLE 2
Effects of Anti-insulin, Anti-IGF-I, Anti-IGF-II, and Anti-IGF Receptor and Anti-insulin Receptor Blocking Antibodies on Myogenic Induction by the Neural Tube

Tissue	Antibody	n	MyHC+ explants (%)[a]	MyHC+ cells (mean + sem)[b]
Somite I–IV + neural tube[c]	None	32	94	60 ± 7
	Anti-IGF-I	16	100	70 ± 7
	Anti-IGF-II	16	100	39 ± 6*
	Anti-insulin	24	88	32 ± 5**
	Anti-IGF-II and anti-insulin	20	42***	15 ± 4***
	Anti-IGF-I + anti-IGF-II + anti-insulin	16	44***	20 ± 7***
	Anti-IGF receptor[d]	10	79	20 ± 4**
	Anti-insulin receptor	10	75	11 ± 3**
	Goat IgG[e]	16	94	67 ± 12
	Rabbit IgG[f]	37	80	53 ± 10

[a] Percentage of cultures with at least one MyHC-positive cell. Statistical differences between control and antibody-treated cultures: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.
[b] Number of MyHC-positive cells in MyHC-positive culture(s). Results represent means ± SEM. Statistical difference indicated as above.
[c] Neural tube pieces were cocultured with somites I–IV from stage 10–11 embryos in BSM *without CEE.*
[d] Previous control studies with irrelevant mouse monoclonal antibodies showed no effects on neural tube-induced somite myogenesis (Stern *et al.,* 1997; Table 4, line C).
[e] Control for anti-IGF-I and anti IGF-II antisera.
[f] Control for anti-insulin receptor antiserum.

are absent from the neural tube but present in whole embryos.

IGF-I receptor and insulin receptor are both signal transducers for IGF-I, IGF-II, and insulin (Florini *et al.,* 1996). When neural tube–somite cocultures were treated with anti-IGF-I receptor or anti-insulin receptor antibodies that block receptor function, fewer MyHC-positive cells were observed (20 ± 4, $P < 0.001$, and 11 ± 3, $P < 0.001$, respectively, versus 60 ± 7 without antibodies), whereas control cultures treated with equivalent amounts of nonimmune rabbit IgG exhibited no significant decrease in MyHC-positive cells (Table 2). These data imply that both the insulin and the IGF receptors transduce neural tube signals which promote somite myogenesis.

Induction of Somite Myogenesis by Shh

Comparison of the maximal myogenic induction of uncommitted somites from stage 10–11 embryos by IGF-I + bFGF + TGF-β1 (Table 1) to that of somites cocultured with neural tube (Table 2) indicates that the neural tube stimulates about threefold more cells to become MyHC-positive. This implies that the neural tube may supply components other than insulin/IGF-II and bFGF. (Previous antisera inhibition studies showed that TGF-β1 was not a component of neural tube-mediated somite myogenesis; Stern *et al.,* 1997.) Earlier studies by our group and others suggested involvement of both the Wnts and Sonic hedgehog (Stern *et al.,* 1995; Münsterberg *et al.,* 1995; Hirsinger *et al.,* 1997; Borycki *et al.,* 1998; Cann *et al.,* 1999). Although Shh had not been tested directly in our single-somite assay system, previous data (Stern *et al.,* 1997) and results from the present studies (e.g., uncommitted somites exhibit myogenesis in CEE-free BSM in response to IGF-I plus bFGF +TGF-β1) implied that *in vitro* myogenesis can occur *without* Shh. Furthermore, Münsterberg *et al.* (1995b) demonstrated that Shh alone was insufficient to induce myogenesis in presegmental plate mesoderm from stage 10 embryos. However, given the unanticipated myogenic inductive effects of insulin and the IGFs, the synergism of these with bFGF + TGF-β1, the higher inductive levels achieved in neural tube cocultures than with any combination of defined factors, and the likelihood that somites would have been exposed to Shh prior to their dissection, it was of interest to assess Shh as an additional defined factor that might be involved in the array of signals affecting somite myogenesis *in vitro.*

The role of Shh was examined by adding Shh to uncommitted somites from stage 13–14 embryos cultured in BSM. Shh promotes substantial myogenesis by itself (Fig. 3A; see below for BSM minus CEE). Further studies showed that the effects of Shh were dose-dependent. Addition of 1 μl Shh-conditioned medium to 100 μl culture BSM (~1 nM final Shh concentration) induced myogenesis in 20% of the explants, while about 60% of the cultures became MyHC-positive in the presence of 5–10 μl (~5–10 nM) Shh-conditioned medium, and about 80% of the cultures became MyHC-positive in the presence of 20–30 μl (~20–30 nM) Shh-conditioned medium. Higher levels of Shh did not cause further increases in somite myogenesis. As shown previously by Münsterburg *et al.* (1995), and in contrast to our data for insulin, the IGFs, and bFGF + TGF-β1, Shh alone was not able to promote myogenesis in caudal and rostral segmental plate cultures (data not shown).

Do IGF, bFGF, TGF-β1, and Shh Signals Interact to Promote Myogenesis?

Synergistic effects were studied by adding different factor combinations to uncommitted somites cultured in BSM as well as CEE-free BSM. Somites from stage 13–14 embryos cultured in BSM plus either IGF-I or Shh alone, or with both factors, differentiated well in the presence of either single factor, but exhibited the greatest myogenesis when both factors were present (Figs. 1C, 3A, and 3B). When cultured in CEE-free BSM, so as to preclude effects of unknown

Copyright © 2000 by Academic Press. All rights of reproduction in any form reserved.

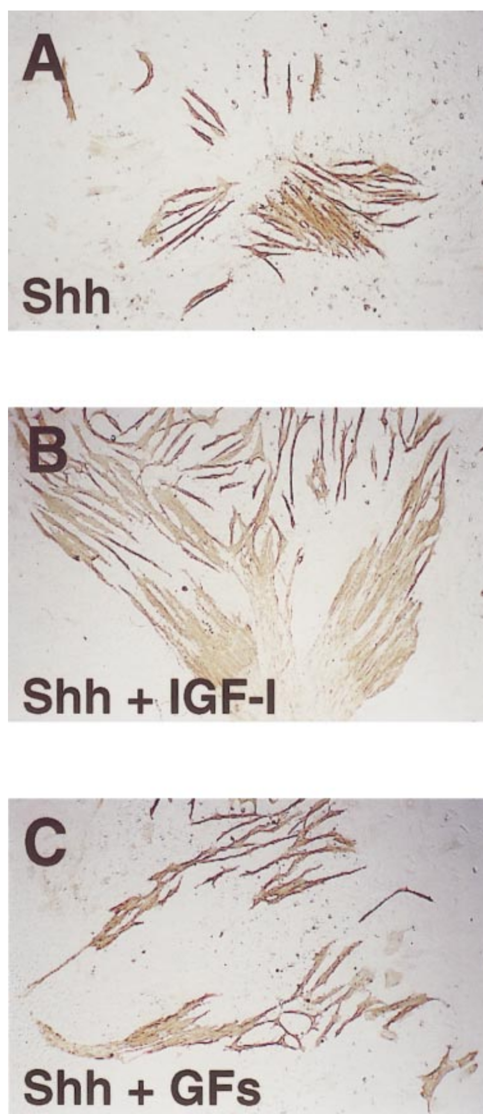

FIG. 3. *In vitro* somite myogenesis in response to Sonic hedgehog plus other defined factors. Somite III explants from stage 13 embryo were cultured for 3 days in BSM plus Shh (A), Shh + IGF-I (B), or Shh + IGF-I +bFGF + TGF-β1 (C) and were then fixed and stained for MyHC. Factor concentrations were 40 ng/ml IGF-I, 10 μl Shh-conditioned medium (~10 nM), and, for the combination of growth factors (GFs), 40 ng/ml IGF-I, 30 ng/ml bFGF, and 10 ng/ml TGF-β1. See Fig. 1A for comparison with no added factors.

factors, somites appeared unhealthy and exhibited no myogenesis unless bFGF, insulin/IGFs, or Shh was present (Fig. 4). In CEE-free BSM, TGF-β1 (10 ng/ml) had no myogenic inducing effect, insulin or IGF-II alone at 40 ng/ml caused only about 5% of the explants to become myogenic (data not shown), and bFGF alone induced low levels of myogenesis in only about 20% of the somites (Fig. 4). In contrast, either IGF-I or Shh induced substantial myogenesis, and the combination of IGF-I and Shh was highly synergistic; 100% of somite cultures were myogenic with an average of 113 ± 21 MyHC-positive cells ($P < 0.001$). The combination of bFGF, TGF-β1, and IGF-I ("GFs") also induced myogenesis in 100% of the somites but only about half as many cells became MyHC-positive, and interestingly, while nearly 90% of the somites exhibited myogenesis when cultured with all four factors (IGF-I, bFGF, TGF-β1, Shh) the number of MyHC-positive cells per explant decreased by more than fivefold in CEE-free BSM (Fig. 4) and by severalfold in BSM (Fig. 3C). Taken together these data suggest that IGF-I and Shh are strongly synergistic in promoting myogenesis, but that their inductive effects may also be repressed at the level of terminal differentiation by the presence of multiple proliferation-stimulating growth factors (see below).

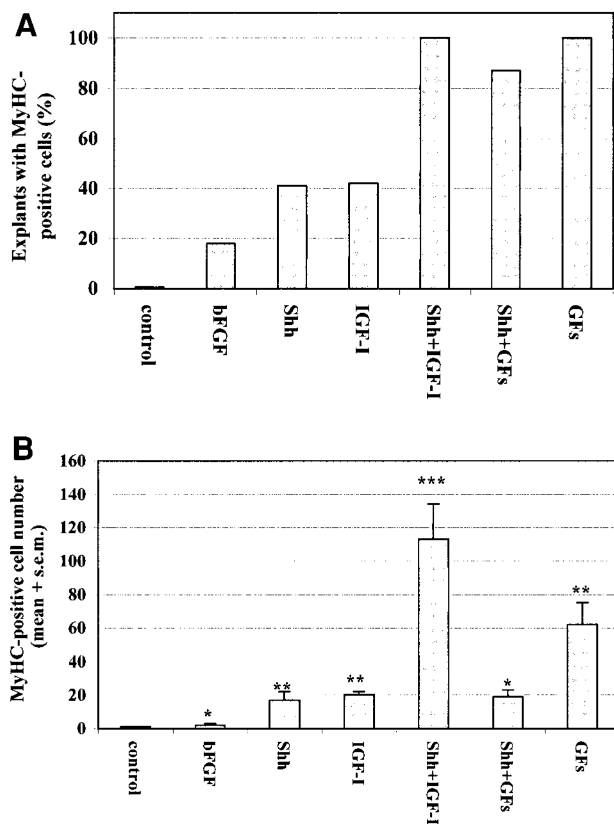

FIG. 4. *In vitro* somite myogenesis in response to the addition of defined factors to *CEE-free* BSM. Somites I–III/IV from stage 13–14 chicken embryos were cultured in BSM without CEE. Concentrations for defined factors were 40 ng/ml IGF-I, 30 ng/ml bFGF, 10 μl Shh-conditioned medium (~10 nM), and, for the combination of the growth factors (GFs), 40 ng/ml IGF-I, 30 ng/ml bFGF, and 10 ng/ml TGF-β1. Results are reported as the means ± SEM from at least 16 explants. Statistical differences between control and treated cultures are indicated as $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

Copyright © 2000 by Academic Press. All rights of reproduction in any form reserved.

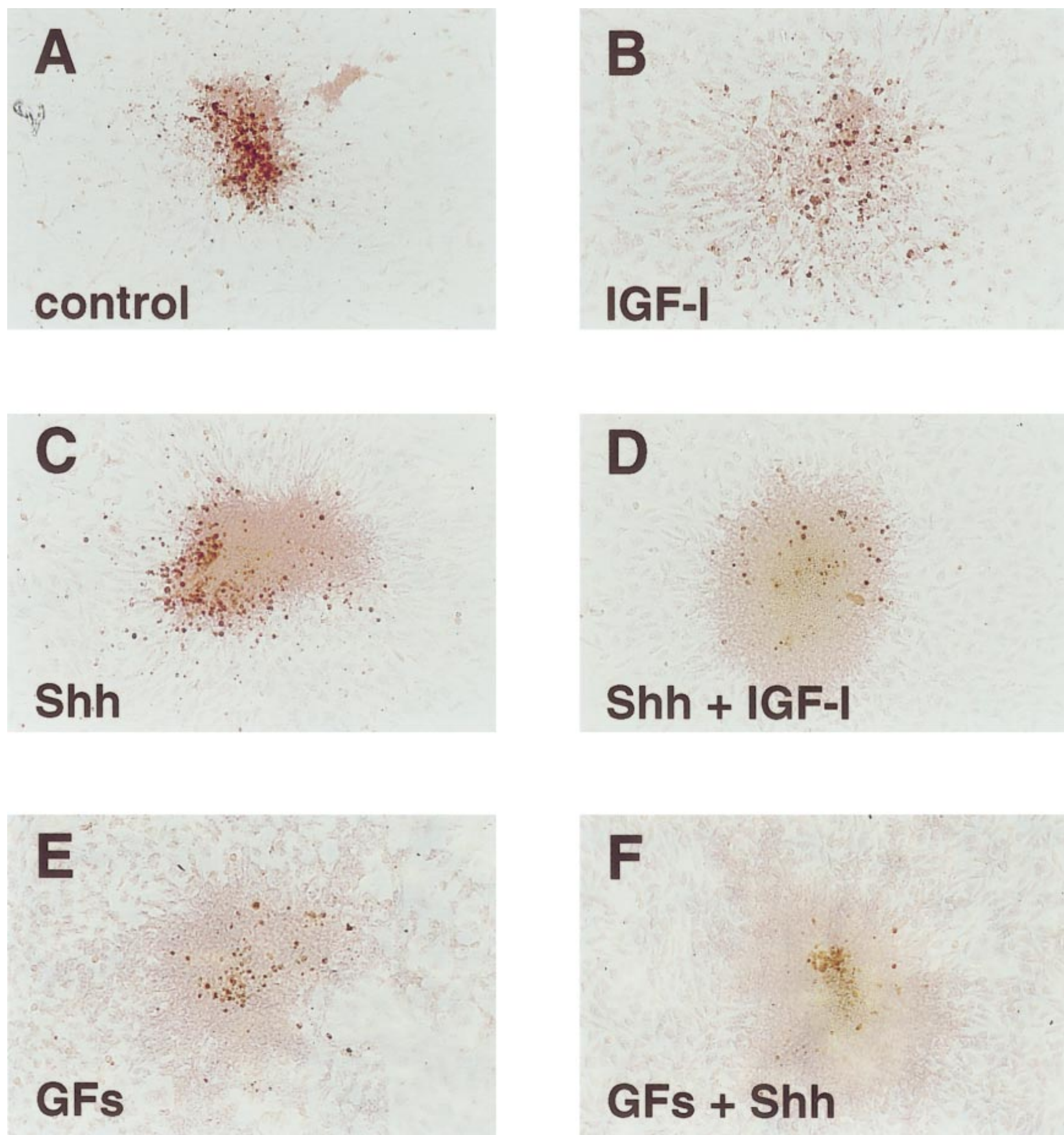

FIG. 5. Effects of defined factors on apoptotic cell death in somite cultures. Somites I–IV from stage 10–11 embryos were cultured in BSM. After 24 h cultures were fixed and apoptotic cells (brown nuclei) were determined as described under Materials and Methods. Cultures were counterstained with eosin to show nonapoptotic cells. Concentrations of defined factors were 40 ng/ml IGF-I, 10 μl Shh-conditioned medium (~10 nM), and, for the combination of growth factors (GFs), 40 ng/ml IGF-I, 30 ng/ml bFGF, and 10 ng/ml TGF-β1.

IGFs, bFGF, and Shh Affect Cell Survival and Proliferation in Somite Cultures

A consistent observation in BSM assays with and without single factor additions is that cell numbers do not increase at the rate anticipated for growing somites, and in many cases the original cell numbers decrease. Since cell survival and proliferation could have major effects on myogenic induction, these parameters were examined. Somites I–IV from stage 10–11 embryos have 1663 $\pm$ 88 cells; yet after 3 days culture in BSM the mean cell number per somite culture decreased to 481 $\pm$ 25, indicating extensive cell death. When added alone IGF-I, IGF-II, insulin, bFGF, and Shh significantly reduced cell loss (Table 3), but they did not increase cell numbers (TGF-β1 had no effect). Furthermore, IGF-I and bFGF together or with the further addition of TGF-β1 supported only small increases in cell numbers. However, when Shh was combined with either IGF-I alone, or with all three factors, the explant cell number increased 7- and 10-fold, respectively.

Copyright © 2000 by Academic Press. All rights of reproduction in any form reserved.

TABLE 3
Somite S-Phase Cells at 24 h *in Vitro* and Total Cell Numbers after 3-Day Exposure to Defined Factors

Condition	% cells in S phase at 24 h[a]	Mean cell number at 3 days[b]
Noncultured control	N.D.	1660 ± 90
BSM, no added factors	3.7 ± 0.4	480 ± 20
IGF-I	11.3 ± 1.2[c]	1010 ± 90[c]
IGF-II	11.0 ± 0.8[c]	990 ± 70[c]
Insulin	9.1 ± 1.1[c]	600 ± 20[c]
bFGF	10.3 ± 0.9[c]	1240 ± 60[c]
TGF-β1	3.8 ± 0.2	460 ± 20
bFGF + IGF-I	18.8 ± 0.8[c,d]	1960 ± 120[c,d]
TGF-β1 + IGF-I	10.0 ± 0.9[c]	970 ± 50[c]
bFGF + TGF-β1 + IGF-I	19.4 ± 0.7[c,d]	2864 ± 397[c,d]
Sonic hedgehog (Shh)	12.5 ± 0.9[c]	1090 ± 100[c]
Shh + IGF-I	27.5 ± 1.5[c,d]	11950 ± 1100[c,d]
Shh + bFGF + TGF-β1 + IGF-I	35.0 ± 2.8[c,e]	16940 ± 1490[c,e]

[a] Somites I–IV from stage 11 embryos were cultured in BSM alone or BSM plus defined factors for 23 h. Factor concentrations were the following: 40 ng/ml IGF-I, 40 ng/ml IGF-II, 40 ng/ml insulin, 30 ng/ml bFGF, 10 ng/ml TGF-β1, and 10 μl Sonic hedgehog-conditioned medium (~10 nM). BrdU was then added to the culture medium at a final concentration of 100 μM for the final 1 h of culture. The cultures were fixed and stained for incorporated BrdU as described under Materials and Methods. Percentages are means ± SEM from at least 12 different explants.

[b] Somites I–IV from stage 10–11 embryos were utilized for cell count experiments. After 3 days of culture explants were trypsinized and the cells counted by a hemacytometer. Cell numbers are means ± SEM from at least 12 different somites subjected to each culture environment.

[c] Statistical difference between the BSM control and factor-treated cultures $P < 0.001$.

[d] Statistical difference between the cultures with IGF-I plus a single additional factor and IGF-I alone $P < 0.001$.

[e] Statistical difference between mean cell number in cultures with Shh + bFGF + TGF-β1 + IGF-I and Shh + IGF-I $P < 0.01$.

To determine whether proliferation was occurring under culture conditions in which cell numbers declined, explants were treated with 100 μM BrdU for 1 h starting at 23 h and were then fixed and stained for BrdU as an indication of cells in S phase (Table 3). Only about 4% of nuclei in control cultures stained for BrdU (Table 3), whereas the addition of 40 ng/ml IGF-I, IGF-II, or insulin; Shh (10 μl, ~10 nM); or bFGF (30 ng/ml) all increased the S-phase-positive nuclei to about 10%. As anticipated from the cultured somite cell counts (Table 3), the combination of IGF-I and Shh increased cell proliferation further so that 27.5% of nuclei were BrdU-positive, while 35% of the cells were in S phase in media containing Shh, IGF-I, bFGF, and TGF-β1. When cultures containing all of these factors were incubated continuously with 10 μM BrdU between 24 and 48 h and then fixed, greater than 95% of the cells were BrdU-positive, indicating that nearly all cells replicated at least once during this period. Explants exposed to BrdU for 24 h without added factors exhibited only 5% BrdU-positive cells.

To study whether these factors also affect cell apoptosis, somites I–IV from stage 10–11 embryos were cultured for 24 h either with or without different factors and then subjected to TUNEL assays. Apoptosis was observed under all conditions studied, and most apoptotic cells were seen in the center of the spreading explants. Compared to control cultures, apoptotic cells were decreased at least twofold by the presence of any single factor, except TGF-β1 (Figs. 5A, 5B, and 5C). Media with either Shh + IGF-I or bFGF + TGF-β1 + IGF-I or with all four factors caused at least another twofold decrease in apoptotic cells (Figs. 5D, 5E, and 5F). (Precise numerical analysis of apoptosis could not be performed since apoptotic cells were often located in the centers of explants where cell density is too great for accurate counting.) Taken together the BrdU-labeling and TUNEL assays indicate that insulin, the IGFs, and Shh stimulate somite cell proliferation and decrease somite apoptotic cell death. In both cases the insulin/IGF factors act synergistically with Shh as well as with bFGF + TGF-β1.

DISCUSSION

Skeletal muscle development is regulated by multiple polypeptide factors. Wnts, Shh, bFGF, and possibly a TGF-β1 family member play roles in the initial induction of somite myogenesis (Münsterberg *et al.,* 1995a,b; Du *et al.,* 1997; Fan *et al.,* 1997; Stern *et al.,* 1995, 1997; Borycki *et al.,* 1998, 1999), while FGFs, TGF-β1, IGF-I, and many other mitogenic factors control muscle differentiation by stimulating proliferation and repressing the onset of terminal differentiation (Linkhart *et al.,* 1981; Lathrop *et al.,* 1985; Clegg *et al.,* 1987; Seed and Hauschka, 1988; Florini and Magri, 1989; Campbell *et al.,* 1995; Weyman and Wolfman, 1998), and insulin, IGF-I, and IGF-II, and possibly TGF-β1 increase the level of differentiation (Florini *et al.,* 1996; Zentella and Massaque, 1992). Given that insulin and the IGFs can affect the onset of muscle differentiation as well as the extent of postdifferentiation muscle development, we sought to determine whether they were also involved in the initial induction of somite myogenesis and, if so, whether they acted synergistically with other known myogenic inducers.

Insulin and the IGFs were tested for myogenic inducing effects using single somite/segmental plate explants (Stern and Hauschka, 1995). Addition of insulin, IGF-I, or IGF-II to BSM promoted somite/segmental plate myogenesis in a dose-dependent manner while control contralateral explants exhibited no myogenic differentiation during a 3-day culture period in BSM; IGF-I also promoted myogenesis in BSM minus CEE. Insulin and IGFs were also able to synergistically promote muscle development with bFGF +TGF-β1 (Fig. 1, Table 1) or with Shh (Figs. 3 and 4).

Copyright © 2000 by Academic Press. All rights of reproduction in any form reserved.

Interestingly, bFGF or TGF-β1 did not exhibit synergism with Shh alone (data not shown), and when these two factors were added to BSM containing the otherwise highly potent IGF-I + Shh combination, the number of MyHC-positive cells was diminished (Fig. 4). While this could be due to an inhibition of myogenic induction per se, it could also be due to a secondary repression of terminal differentiation among cells that had been induced to enter the myogenic lineage. An analogous "induction/repression" phenomenon was described previously for FGF-dependent muscle colony-forming cells in developing chick limb buds (Seed and Hauschka, 1988). In contrast to the inducing factor synergisms described above, when IGF-I and Wnt-1 were combined by adding IGF-I to somites cultured on Rat-2 cells expressing Wnt-1 (Stern *et al.,* 1995), no synergism was observed (data not shown). This result does not, however, preclude the possibility of interactions between the IGF-I and the Wnt signal transduction pathways *in vivo,* because analysis of Wnt effects in our assay system is complicated by inhibitory effects of the Rat-1 cell monolayer (see Fig. 6 in Stern *et al.,* 1995).

Our study also examined the neural tube and somites for mRNAs encoding IGF-I, IGF-II, insulin, and their receptors to determine whether these components were present in the appropriate interacting tissues *in vivo.* mRNAs for insulin and IGF-II and for the insulin and IGF receptors were present in the neural tube and somites of stage 13–14 embryos (Fig. 2) and, although IGF-I transcripts were not found in either tissue, IGF-I mRNA was detected in whole stage 13–14 embryos. Thus insulin and IGF-II could be secreted by the neural tube or produced endogenously by somites, and IGF-I could be available systemically. Other investigators have also detected mRNAs for insulin, IGF-I and their receptors in early chick embryos (Perez-Villamil *et al.,* 1994; Holzenberger *et al.,* 1996; Morales *et al.,* 1997), and *in situ* hybridization analyses indicate widespread distribution of preproinsulin mRNA in a pattern similar to that of insulin receptor mRNA beginning from the blastoderm stage (Perez-Villamil *et al.,* 1994; Morales *et al.,* 1997). *In situ* hybridization studies of stage 13–14 embryos have also revealed high levels of IGF receptor mRNA in the neuroepithelia, notochord, and somites (Holzenberger *et al.,* 1996); biologically active insulin has been detected in 2-day-old chick embryos as well as in the yolk of unfertilized eggs (De Pablo *et al.,* 1982), and yolk has also been shown to contain IGF activity (Scavo *et al.,* 1989).

Combining our RT-PCR results with data from the above studies suggests that the IGFs, insulin, and their receptors almost certainly exhibit a temporal and spatial occurrence which would be appropriate for their participation as components of the *in vivo* somite myogenic induction process. However, a complexity raised by our studies concerns why somites which are expressing insulin and IGF-II mRNAs nevertheless require addition of these ligands to the BSM in order to undergo myogenesis *in vitro.* An analogous paradoxical situation occurs in certain myoblasts which synthesize bFGF but nevertheless require exogenous FGF in order to replicate (Hannon *et al.,* 1996).

Further evidence for normal developmental roles of insulin and the IGFs in these early phases of muscle development was obtained by treating neural tube–somite cocultures with antibodies to either the ligands or their receptors. Neural tube-mediated somite myogenesis was diminished upon the addition of anti-insulin, anti-IGF-II, anti-insulin receptor, or anti-type I-IGF receptor antibodies (Table 2). Together, these results are consistent with the hypothesis that insulin and IGF-II produced by the neural tube or somites are involved in early somite myogenesis. Although anti-IGF-I antibody did not diminish neural tube-induced myogenesis *in vitro,* this is not surprising since IGF-I transcripts were not detected in the neural tube; the antisera inhibition data thus do not exclude the *in vivo* involvement of systemic IGF-I.

While our *in vitro* studies as well as the *in vivo* IGF/insulin/receptor distribution studies summarized above are consistent with the IGFs and insulin playing myogenic inductive roles *in vivo,* data from *in vivo* somite/neural tube manipulation studies raise additional interpretive complexities. Namely, somites that remain in place following neural tube removal (Rong *et al.,* 1992), and segmental plates that have been spatially displaced from their normal close proximity to the neural tube/notochord (Borycki *et al.,* 1998), do not exhibit myogenesis, despite the fact that such somites should still be exposed to any systemically available and endogenously synthesized IGFs and insulin (Fig. 2). Systemic/endogenous IGFs and insulin are thus *not sufficient* to induce somite myogenesis *in vivo* under these experimental conditions. The absence of myogenesis could be due to insufficient levels of these ligands, as well as to an imbalance of positive and negative factors (e.g., Hirsinger *et al.,* 1997) at the abnormal position in which the somites are placed.

In vitro analysis of somite myogenesis is also complicated by effects of the culture environment on cell survival and replication (Table 3 and Fig. 5). Evidence that some somite cells die in BSM was obtained by direct cell counts after 3 days *in vitro* and by TUNEL assays. These studies indicated that significant cell death occurred under all conditions in which only a single putative myogenic inducing factor was added to the BSM and even when some myogenic differentiation was promoted, as in the cases of IGF-I or Shh. However, when IGF-I and Shh were combined with each other and/or with bFGF + TGF-β1, the total cell number in somite explants increased and fewer apoptotic cells were detected. To determine whether proliferation occurred under the conditions in which extensive cell death was observed, cultures were pulse-labeled with BrdU to assess the percentage of S-phase cells. In agreement with previous data (Florini *et al.,* 1996; Massagué, 1998; Tajbakhsh and Spörle, 1998), we found that insulin, IGF-I, IGF-II, bFGF, and Shh stimulated somite cell proliferation, while TGF-β1, alone, had no effect. IGF-I, Shh, and bFGF were roughly equivalent inducers of somite cell prolifera-

Copyright © 2000 by Academic Press. All rights of reproduction in any form reserved.

tion (Table 3); however, IGF-I and Shh were each considerably more potent than bFGF in increasing the number of MyHC-positive cells (Table 1, Fig. 4). This implies that the myogenic inducing effects of IGF-I and Shh are not solely attributable to an increase in cell proliferation.

The mechanistic similarities between insulin/IGF effects during somite myogenesis and during terminal differentiation and muscle hypertrophy are unknown. Many *in vitro* studies have demonstrated positive myogenic effects of insulin and the IGFs (Florini *et al.,* 1986, 1996; Damon *et al.,* 1998). For example, insulin, IGF-I, or IGF-II alone potentiate differentiation of L6 rat myogenic cells (Minotti *et al.,* 1998), and inhibition of IGF-II expression in C2 mouse myoblasts abolishes their ability to undergo autonomous differentiation (Montarras *et al.,* 1996). Furthermore, chicken myoblasts from 11- to 12-day embryos undergo both cell hyperplasia and myofiber hypertrophy in response to insulin and IGF-I, while IGF-II stimulates only cell hyperplasia (Vandenburgh *et al.,* 1991). In addition, mouse gene knockout studies of IGF-I, IGF-II, and IGF receptor indicate severe decreases in muscle mass and significant effects on muscle development (Baker *et al.,* 1993; Powell-Braxton *et al.,* 1993). In contrast, IGF-I overproduction in transgenic mice after the onset of terminal muscle differentiation causes muscle hypertrophy (Barton-Davis *et al.,* 1998; Musaro *et al.,* 1999).

The effects of insulin and IGFs on cell proliferation and differentiation of muscle cells from later developmental stages are mediated by both the mitogen-activated protein (MAP) kinase and phosphatidylinositol 3 (PI3) kinase cascades (Florini *et al.,* 1996; Kaliman *et al.,* 1996, 1998; Gredinger *et al.,* 1998; Weyman and Wolfman, 1998). Interestingly, one downstream target of the MAP kinase/PI3 kinase-mediated insulin/IGF signals is p300/CBP (Klemm *et al.,* 1998), which in transactivation studies is known to function as a coactivator of both MyoD and MEF2C (Sartorelli *et al.,* 1997) and which may affect myogenesis via its association with PCAF histone acetyltransferase (Puri *et al.,* 1997; Sartorelli *et al.,* 1999). Perhaps these pathways are also used for controlling early events in myogenic determination.

The differentiation of older myoblasts in response to IGF can occur in the absence of proliferation (Turo and Florini, 1982; Florini *et al.,* 1986). Similarly, the increase in MyHC-positive cells after treating somite cultures with growth factors was not directly correlated with increased cell numbers (compare values in Tables 1 and 3, e.g., IGF-I vs bFGF for somites I–III/IV). However, we cannot rule out the possibility that these factors stimulate myogenesis by selectively increasing the survival of cells that had *already* become committed to myogenesis. The same interpretive complexity applies to the mechanism of Shh, a widely accepted "inducer" of somite myogenesis.

Our studies indicate that Shh has a potent effect upon somite cell proliferation and survival when combined with IGF-I, bFGF, and TGF-β1 (Table 3 and Fig. 5). These data are consistent with the hypothesis that a major component of Shh's positive effects on somite myogenesis involves stimulating cell proliferation and/or inhibiting cell death; but we have no direct evidence proving that the affected cells are myogenic precursors. Working with more developed somites, Cann *et al.* (1999) have also shown that Shh stimulates cell proliferation and prevents apoptosis; but in addition they discovered that Shh potentiates the development and/or survival of cells that become primary slow-type muscle fibers. *In vivo* studies also suggest multiple Shh effects on myogenesis. Du *et al.* (1997) reported that ectopic Shh expression induced slow muscle precursors throughout the zebrafish somite; Teillet *et al.* (1998) found that the survival of somite myogenic and chondrogenic cell lineages is mediated by Shh; Borycki *et al.* (1999) reported that Shh is required for sclerotomal cell survival but is *not* essential for the survival or proliferation of epaxial myogenic precursor cells; Marcelle *et al.* (1999) proposed that mitogenic effects of Shh mediate the selective expansion of subpopulations within developing somites. Similarly, Shh and Wnt signals compete in the specification of presomitic mesoderm to become sclerotome or dermamyotome (Lee *et al.,* 2000). Taken together these studies indicate that Shh mediates a broad spectrum of processes in somite development which impinge upon proliferation, cell survival, and differentiation. How these processes affect myogenic induction remains to be determined.

The relationship of MyoD and Myf5 gene expression to myogenic induction is not fully understood. While these genes have been described as "activated" in response to myogenic inducing tissues and factors (Münsterberg *et al.,* 1995b; Borycki *et al.,* 1999), they are, in fact, expressed in both the epiblast and the paraxial mesoderm prior to the time at which *intact* somites exhibit cells committed to myogenesis. For example, MyoD and Myf5 mRNAs are detected by RT-PCR assays prior to somite segmentation in both mouse and chick (Kopan *et al.,* 1994; Münsterberg *et al.,* 1995b; Lin Jones and Hauschka, 1996; George-Weinstein *et al.,* 1996; J. Kiefer and S. D. Hauschka, unpublished). However, MyoD and Myf5 gene expression is reduced to undetectable levels in somites cultured without inducing factors (Münsterberg *et al.,* 1995b; J. Kiefer and S. D. Hauschka, unpublished) or when the neural tube and/or notochord are removed from chick embryos (Teillet *et al.,* 1998) or when somites/segmental plates are physically separated from proximity to the neural tube/notochord *in vivo* (Pownall *et al.,* 1996; Spence *et al.,* 1996; Borycki *et al.,* 1998). These observations indicate that the initial expression of Myf5/MyoD is *not sufficient* to maintain somite myogenesis either *in vivo* or *in vitro* if the inducing components are withdrawn. Defined factors such as Wnts, Shh, bFGF+TGF-β1, and insulin/IGFs might thus function as "permissive" as well as *de novo* "inductive" signals.

In conclusion, insulin and the IGFs have been shown to promote/induce somite myogenesis *in vitro,* to increase somite cell proliferation, to decrease apoptosis, and to be synthesized at the correct times and places to affect all of

Copyright © 2000 by Academic Press. All rights of reproduction in any form reserved.

these activities. Since insulin and IGFs are able to synergize *in vitro* with other factors, bFGF + TGF-β1 and Shh, to promote even higher levels of somite muscle formation, cell proliferation, and inhibition of apoptosis, the entire set of factors may play similar roles *in vivo,* even if some are not the primary muscle-inducing signals. Identifying primary and secondary gene targets of the multiple signal transduction pathways activated by these factors will be necessary for obtaining a full understanding of somite myogenesis.

ACKNOWLEDGMENTS

The authors thank John Angello for helping with the photomicroscopy, John Angello and Jean Buskin for discussions and comments regarding the manuscript, and Frank Stockdale and Gordon Cann for discussions and for providing unpublished data concerning Shh's effects on skeletal muscle differentiation. The authors are very grateful to their colleagues Henk Roelink for providing recombinant Shh and Steve Kaufman for providing anti-BrdU antibody. This work was supported by grants from the National Institutes of Health (NIH, AR18860). A. Pirskanen was supported by a training grant from the Academy of Finland, and J. Kiefer was supported by a training grant from the National Institute of General Medical Sciences (T32GM07270).

REFERENCES

Bader, D., Mawaki, T., and Fischman, D. (1982). Immunochemical analysis of myosin heavy chain during avian myogenesis *in vivo* and *in vitro. J. Cell Biol.* **95,** 763–770.

Baker, J., Liu, J. P., Robertson, E. J., and Efstratiadis, A. (1993). Role of insulin-like growth factors in embryonic and postnatal growth. *Cell* **75,** 73–82.

Barton-Davis, E. R., Shoturma, D. I., Musaro, A., Rosenthal, N., and Sweeney, H. L. (1998). Viral mediated expression of insulin-like growth factor I blocks the aging-related loss of skeletal muscle function. *Proc. Natl. Acad. Sci. USA* **95,** 15603–15607.

Borycki, A. G., Brunk, B., Tajbakhsh, S. Buckingham, M., Chiang, C., and Emerson, C. P., Jr. (1999). Sonic hedgehog controls epaxial muscle determination through Myf5 activation. *Development* **126,** 4053–4063.

Borycki, A. G., Mendham, L., and Emerson, C. P. (1998). Control of somite patterning by Sonic hedgehog and its downstream signal response genes. *Development* **125,** 777–790.

Buffinger, N., and Stockdale, F. E. (1994). Myogenic specification in somites: Induction by axial structures. *Development* **120,** 1443–1452.

Campbell, J. S., Wenderoth, M. P., Hauschka, S. D., and Krebs, E. G. (1995). Differential activation of mitogen-activated protein kinase in response to basic fibroblast growth factor in skeletal muscle cells. *Proc. Natl. Acad. Sci. USA* **92,** 870–874.

Cann, G. M., Lee, J. W., and Stockdale, F. E. (1999). Sonic hedgehog enhances somite cell viability and formation of primary slow muscle fibers in avian segmented mesoderm. *Anat. Embryol. (Berlin)* **200,** 239–252.

Capdevila, J., Tabin, C., and Johnson, R. L. (1998). Control of dorsoventral somite patterning by Wnt-1 and β-catenin. *Dev. Biol.* **193,** 182–194.

Christ, B., Brand-Saberi, B., Grim, M., and Wilting, J. (1992). Local signaling in dermomyotomal cell type specification. *Anat. Embryol.* **186,** 505–510.

Clegg, C. H., Linkhart, T. A., Olwin, B. B., and Hauschka, S. D. (1987). Growth factor control of skeletal muscle differentiation: Commitment to terminal differentiation occurs in G1 phase and is repressed by fibroblast growth factor. *J. Cell Biol.* **105,** 949–956.

Cohick, W. S., and Clemmons, D. R. (1993). The insulin-like growth factors. *Annu. Rev. Physiol.* **55,** 131–153.

Cossu, G., Kelly, R., Tajbakhsh, S., Di Donna, S., Vivarelli, E., and Buckingham, M. (1996). Activation of different myogenic pathways: myf-5 is induced by the neural tube and myoD by the dorsal ectoderm in mouse paraxial mesoderm. *Development* **122,** 429–437.

Damon, S. E., Haugk, K. L., Birnbaum, R. S., and Quinn, L. S. (1998). Retrovirally mediated overexpression of insulin-like growth factor binding protein 4: Evidence that insulin-like growth factor is required for skeletal muscle differentiation. *J. Cell. Physiol.* **175,** 109–120.

de la Rosa, E. J., Bondy, C. A., Hernandez-Sanchez, C., Wu, X., Zhou, J., Lopez-Carranza, A., Scavo, L., and De Pablo, F. (1994). Insulin and insulin-like growth factor system components gene expression in the chicken retina from early neurogenesis until late development and their effect on neuroepithelial cells. *Eur. J. Neurosci.* **6,** 1801–1810.

De Pablo, F., and de la Rosa, E. J. (1995). The developing CNS: A scenario for the action of proinsulin, insulin, and insulin-like growth factors. *Trends Neurosci.* **18,** 143–150.

De Pablo, F., Roth, J., Hernandez, E., and Pruss, R. M. (1982). Insulin is present in chicken eggs and early chick embryos. *Endocrinology* **111,** 1909–1916.

Du, J. S., Devoto, S. H., Westerfield, M., and Moon, R. T.(1997). Positive and negative regulation of muscle cell identity by members of the *hedgehog* and *TGF-β* gene families. *J. Cell Biol.* **139,** 146–156.

Fan, C. M., Lee, C. S., and Tessier-Lavigne, M. (1997). A role for WNT proteins in induction of dermomyotome. *Dev. Biol.* **191,** 160–165.

Florini, J. R., Ewton, D. Z., Falen, S. L., and Van Wyk, J. J. (1986). Biphasic concentration dependency of stimulation of myoblast differentiation by somatomedins. *Am. J. Physiol.* **250,** C78–82.

Florini, J. R., and Magri, K. A. (1989). Effects of growth factors on myogenic differentiation. *Am. J. Physiol.* **256,** C701–C711.

Florini, J. R., Ewton, D. Z., and Coolican, S. A. (1996). Growth hormone and the insulin-like growth factor system in myogenesis. *Endocr. Rev.* **17,** 481–517.

Foster, R. F., Thompson, J. M., and Kaufman, S. J. (1987). A laminin substrate promotes myogenesis in rat skeletal muscle cultures: Analysis of replication and development using antidesmin and anti-BrdUrd monoclonal antibodies. *Dev. Biol.* **122,** 11–20.

George-Weinstein, M., Foster, R. F., Gerhart, J. V., and Kaufman, S. J. (1993). *In vitro* and *in vivo* expression of α7 integrin and desmin define the primary and secondary myogenic lineages. *Dev. Biol.* **156,** 209–229.

George-Weinstein, M., Gerhart, J. V., Foti, G. J., and Lash, J. W. (1994). Maturation of myogenic and chondrogenic cells in the presomitic mesoderm of the chick embryo. *Exp. Cell Res.* **211,** 263–274.

George-Weinstein, M., Gerhart, J., Reed, R., Flynn, J., Callihan, B., Mattiacci, M., Miehle, C., Foti, G., Lash, J. W., and Weintraub, H.

Copyright © 2000 by Academic Press. All rights of reproduction in any form reserved.

(1996). Skeletal myogenesis: The preferred pathway of chick embryo epiblast cells *in vitro*. *Dev. Biol.* **173,** 279–291.
Gredinger, E., Gerber, A. N., Tamir, Y., Tapscott, S. J., and Bengal, E. (1998). Mitogen-activated protein kinase pathway is involved in the differentiation of muscle cells. *J. Biol. Chem.* **273,** 10436–10444.
Hamburger, V., and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol.* **88,** 49–92.
Hannon, K., Kudla, A. J., McAvoy, M. J., Clase, K. L., and Olwin, B. B. (1996). Differentially expressed fibroblast growth factors regulate skeletal muscle development through autocrine and paracrine mechanisms. *J. Cell Biol.* **132,** 1151–1159.
Hirsinger, E., Duprez, D., Jouve, C., Malapert, P., Cooke, J., and Pourquié, O. (1997). Noggin acts downstream of Wnt and Sonic hedgehog to antagonize BMP4 in avian somite patterning. *Development* **124,** 4605–4614.
Holzenberger, M., Lapointe, F., Leibovici, M., and Lievre, C. A. (1996). The avian IGF type 1 receptor: cDNA analysis and in situ hybridization reveal conserved sequence elements and expression patterns relevant for the development of the nervous system. *Brain Res. Dev. Brain Res.* **97,** 76–87.
Kaliman, P., Canicio, J., Shepherd, P. R., Beeton, C. A., Testar, X., Palacín, M., and Zorzano, A. (1998). Insulin-like growth factors require phosphatidylinositol 3-kinase to signal myogenesis: Dominant negative p85 expression blocks differentiation of L6E9 muscle cells. *Mol. Endocrinol.* **12,** 66–77.
Kaliman, P., Viñals, F., Testar, X., Palacin, M., and Zorzano, A. (1996). Phosphatidylinositol 3-kinase inhibitors block differentiation of skeletal muscle cells. *J. Biol. Chem.* **271,** 19146–19151.
Klemm, D. J., Roesler, W. J., Boras, T., Colton, L. A., Felder, K., and Reusch, J. E. (1998). Insulin stimulates cAMP-response element binding protein activity in HepG2 and 3T3-L1 cell lines. *J. Biol. Chem.* **273,** 917–923.
Konigsberg, I. R. (1968). *In* "Methods in Developmental Biology" (F. Wilt and N. K. Wessels, Eds.), pp. 520–521. Crowell–Collier, New York.
Kopan, R., Nye, J. S., and Weintraub, H. (1994). The intracellular domain of mouse Notch: A constitutively activated repressor of myogenesis directed at the basic helix-loop-helix region of MyoD. *Development* **120,** 2385–2396.
Lathrop, B., Olson, E., and Glaser, L. (1985). Control by fibroblast growth factor of differentiation in the BC3H1 muscle cell line. *J. Cell Biol.* **100,** 1540–1547.
Lee, C. S., Buttitta, L. A., May, N. R., Kispert, A., and Fan, C.-M. (2000). SHH-N upregulates Sfrp2 to mediate its competitive interaction with Wnt1 and Wnt4 in the somitic mesoderm. *Development* **127,** 109–118.
Lin-Jones, J., and Hauschka, S. D. (1996). Myogenic determination factor expression in the developing avian limb bud: An RT-PCR analysis. *Dev. Biol.* **174,** 407–422.
Linkhart, T. A., Clegg, C. H., and Hauschka, S. D. (1981). Myogenic differentiation in permanent clonal myoblast cell lines: Regulation by macromolecular growth factors in culture. *Dev. Biol.* **86,** 19–30.
Marcelle, C., Ahlgren, S., and Bronner-Fraser, M. (1999). *In vivo* regulation of somite differentiation and proliferation by sonic hedgehog. *Dev. Biol.* **214,** 277–287.
Marcelle, C., Stark, M. R., and Bronner-Fraser, M. (1997). Coordinate actions of BMPs, Wnts, Shh and noggin mediate patterning of the dorsal somite. *Development* **124,** 3955–3963.
Massagué, J. (1998). TGF-β signal transduction. *Annu. Rev. Biochem.* **67,** 753–791.
Minotti, S., Scicchitano, B. M., Nervi, C., Scarpa, S., Lucarelli, M., Molinari, M., and Adamo, S. (1998). Vasopressin and insulin-like growth factors synergistically induce myogenesis in serum-free. *Cell Growth Differ.* **9,** 155–163.
Montarras, D., Aurade, F., Johnson, T., Ilan, J., Gros, F., and Pinset, C. (1996). Autonomous differentiation in the mouse myogenic cell line, C2, involves a mutual positive control between insulin-like growth factor II and MyoD, operating as early as at the myoblast stage. *J. Cell Sci.* **109,** 551–560.
Morales, A. V., Serna, J., Alarcon, C., de la Rosa, E. J., and De Pablo, F. (1997). Role of prepancreatic (pro)insulin and the insulin receptor in prevention of embryonic apoptosis. *Endocrinology* **138,** 3967–3975.
Münsterberg, A. E., and Lassar, A. B. (1995a). Combinatorial signals from the neural tube, floor plate and notochord induce myogenic bHLH gene expression in the somite. *Development* **121,** 651–660.
Münsterberg, A. E., Kitajewski, J., Bumcrot, D. A., McMahon, A. P., and Lassar, A. B. (1995b). Combinatorial signaling by Sonic hedgehog and Wnt family members induces myogenic bHLH gene expression in the somite. *Genes Dev.* **9,** 2911–2922.
Musaro, A., McCullagh, K. J., Naya, F. J., Olson, E. N., and Rosenthal, N. (1999). IGF-1 induces skeletal myocyte hypertrophy through calcineurin in association with GATA-2 and NF-ATc1. *Nature* **400,** 5581–585.
Perez-Villamil, B., de la Rosa, E. J., Morales, A. V., and De Pablo, F. (1994). Developmentally regulated expression of the preproinsulin gene in the chicken embryo during gastrulation and neurulation. *Endocrinology* **135,** 2342–2350.
Pourquié, O., Coltey, M., Breant, C., and Le Douarin, N. M. (1995). Control of somite patterning by signals from the lateral plate. *Proc. Natl. Acad. Sci. USA* **92,** 3219–3223.
Pourquié, O., Fan, C. M., Coltey, M., Hirsinger, E., Watanabe, Y., Bréant, C., Francis-West, P., Brickell, P., Tessier-Lavigne, M., and Le Douarin, N. M. (1996). Lateral and axial signals involved in avian somite patterning: A role for BMP-4. *Cell* **84,** 461–471.
Powell-Braxton, L., Hollingshead, P., Warburton, C., Dowd, M., Pitts-Meek, S., Dalton, D., Gillett, N., and Stewart, T. A. (1993). IGF-I is required for normal embryonic growth in mice. *Genes Dev.* **7,** 2609–2617.
Pownall, M. E., Strunk, K. E., and Emerson, C. P., Jr. (1996). Notochord signals control the transcriptional cascade of myogenic bHLH genes in somites of quail embryos. *Development* **122,** 1475–1488.
Puri, P. L., Sartorelli, V., Yang, X. J., Hamamori, Y., Ogryzko, V. V., Howard, B. H., Kedes, L., Wang, J. Y., Graessmann, A., Nakatani, Y., and Levreo, M. (1997). Differential roles of p300 and PCAF acetyltransferases in muscle. *Mol. Cell* **1,** 35–45.
Reshef, R., Maroto, M., and Lassar, A. B. (1998). Regulation of dorsal somitic cell fates: BMPs and Noggin control the timing and pattern of myogenic regulator expression. *Genes Dev.* **12,** 290–303.
Roelink, H., Porter, J. A., Chiang, C., Tanabe, Y., Chang, D. T., Beachy, P. A., and Jessell, T. M. (1995). Floor plate and motor neuron induction by different concentrations of the amino-terminal cleavage product of sonic hedgehog autoproteolysis. *Cell* **81,** 445–455.
Rong, P. M., Teillet, M.-A., Ziller, C., and Le Douarin, N. M. (1992). The neural tube/notochord complex is necessary for vertebral

Copyright © 2000 by Academic Press. All rights of reproduction in any form reserved.

but not limb and body wall striated muscle differentiation. *Development* **115,** 657–672.

Sartorelli, V., Huang, J., Hamamori, Y., and Kedes, L. (1997). Molecular mechanisms of myogenic coactivation by p300: Direct interaction with the activation domain of MyoD and with the MADS box of MEF2C. *Mol. Cell. Biol.* **17,** 1010–1026.

Sartorelli, V., Puri, P. L., Hamamori, Y., Ogryzko, V., Chung, G., Nakatani, Y., Wang, J. Y., and Kedes, L. (1999). Acetylation of MyoD directed by PCAF is necessary for the execution of the muscle program. *Mol. Cell* **4,** 725–734.

Scavo, L., Alemany, J., Roth, J., and De Pablo, F. (1989). Insulin-like growth factor I activity is stored in the yolk of the avian egg. *Biochem. Biophys. Res. Commun.* **162,** 1167–1173.

Seed, J., and Hauschka, S. D. (1988). Clonal analysis of vertebrate myogenesis. VIII. Fibroblast growth factor (FGF)-dependent and FGF-independent muscle colony types during chick wing development. *Dev. Biol.* **128,** 40–49.

Seed, J., Olwin, B. B., and Hauschka, S. D. (1988). Fibroblast growth factor levels in the whole embryo and limb bud during chick development. *Dev. Biol.* **128,** 50–57.

Serna, J., Gonzáles-Guerrero, P. R., Scanes, C. G., Prati, M., Morreale, G., and De Pablo, F. (1996). Differential and tissue-specific regulation of (pro)insulin and insulin-like growth factor-I mRNAs and levels of thyroid hormones in growth-retarded embryos. *Growth Regul.* **6,** 73–82.

Spence, M. S., Yip, J., and Erickson, C. A. (1996). The dorsal neural tube organizes the dermamyotome and induces axial myocytes in the avian embryo. *Development* **122,** 231–241.

Stern, H. M., Brown, A. M. C., and Hauschka, S. D. (1995). Myogenesis in paraxial mesoderm: Preferential induction by dorsal neural tube and by cells expressing *Wnt-1. Development* **121,** 3675–3686.

Stern, H. M., and Hauschka, S. D. (1995). Neural tube and notochord promote *in vitro* myogenesis in single somite explants. *Dev. Biol.* **167,** 87–103.

Stern, H. M., Lin-Jones, J., and Hauschka, S. D. (1997). Synergistic interactions between bFGF and a TGF-β family member may mediate myogenic signals from the neural tube. *Development* **124,** 3511–3523.

Tajbakhsh, S., and Spörle, R. (1998). Somite development: Constructing the vertebrate body. *Cell* **92,** 9–16.

Teillet, M. A., Watanabe, Y., Jeffs, P., Duprez, D., Lapointe, F., and Le Douarin, N. M. (1998). Sonic hedgehog is required for survival of both myogenic and chondrogenic somitic lineages. *Development* **125,** 2019–2030.

Turo, K. A., and Florini, J. R. (1982). Hormonal stimulation of myoblast differentiation in the absence of DNA synthesis. *Am. J. Physiol.* **243,** C278–C284.

Vandenburgh, H. H., Karlisch, P., Shansky, J., and Feldstein, R. (1991). Insulin and IGF-I induce pronounced hypertrophy of skeletal muscle fibers in tissue culture. *Am. J. Physiol.* **243,** C475–C484.

Weyman, C. M., and Wolfman, A. (1998). Mitogen-activated protein kinase kinase (MEK) activity is required for inhibition of skeletal muscle differentiation by insulin-like growth factor 1 or fibroblast growth factor 2. *Endocrinology* **139,** 1794–1800.

Williams, B. A., and Ordahl, C. P. (1997). Emergence of determined myogenic precursor cells in the somite. *Development* **124,** 4983–4997.

Zentella, A., and Massaqué, J. (1992). Transforming growth factor beta induces myoblast differentiation in the presence of mitogens. *Proc. Natl. Acad. Sci. USA* **89,** 5176–5180.

Zhou, M., Ma, Z., and Sly, W. S. (1995). Cloning and expression of the cDNA of chicken cation-independent mannose-6-phosphate receptor. *Proc. Natl. Acad. Sci. USA* **92,** 9762–9766

Received for publication February 24, 2000
Accepted May 16, 2000

Copyright © 2000 by Academic Press. All rights of reproduction in any form reserved.