Mechanisms Underlying the Differentiation of the Avian Iris and Ciliary Body

by Brian Alexander Link

A Dissertation

Presented to the Department of Cell and Developmental Biology and the
Oregon Health Sciences University School of Medicine
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy.

December 1997

School of Medicine

Oregon Health Sciences University

CERTIFICATE OF APPROVAL

This is to certify that the Ph.D. thesis of

Brian A. Link

has been approved

[redacted]

Professor in charge of thesis

[redacted]

Member

[redacted]

Member

[redacted]

Member

[redacted]

Member

[redacted]

Associate Dean for Graduate Studies

TABLE OF CONTENTS

List of Figures and Tables

Chapter I

Chapter II

Chapter III.

Chapter IV.

ACKNOWLEDGEMENTS

Many accolades -much more than can be written here- are due to all who have guided and supported me through the development and completion of this project. However:

Very special thanks to my advisor Dr. Rae Nishi for all the patience, guidance, scientific inspiration, and for creating a truly remarkable environment to do research. Enough can't be said. . .

Thanks too, to my advisory and thesis committee members Dr. Phil Copenhaver, Dr. Mike Danilchik, Dr. Caroline Enns, Dr. Steve Matsumoto, and Dr. Matt Thayer.

I would also like to extend my gratitude to Dr. Gary Reiness, who always had time to listen and help out.

More thanks to all my labmates, expecially those who I shared the grad school adventure with: Vivian Lee, Gillian Bunker, Tom Finn, and Diane Darland - wonderful collaborators, but more importantly wonderful friends.

Not to be forgotten are three other close friends: Tristan Darland, Gwen Schafer, and Bill Chang.

Finally, I am indebted to my family, especially my parents Bill and Kit Link, my sister Karie Link, and my grandparents Bill and Virgina Link, for providing endless love, support, and humor.

ABSTRACT

I have used the developing avian iris and ciliary body to investigate mechanisms that regulate cellular differentiation and maturation. During development, the muscles of the iris undergo a conversion from smooth-type to striated-type. This transition is coincident with establishment of neuropeptide phenotype and developmental cell death in the ciliary ganglion, which innervates structures in the eye including the iris and ciliary body. The focus of my thesis is to understand the cellular mechanisms and molecules that regulate the smooth to striated muscle transition in the iris and ciliary body. In addition I have investigated the role of these target tissues in regulating neuronal maturation of ciliary neurons.

Previous work established that activin regulates neuropeptide phenotype in cultures of ciliary ganglion neurons. In vivo, the neuropeptide somatostatin is expressed only in choroid neurons; however, in culture, activin induces this peptide in both neuron types of the ciliary ganglion - choroid and ciliary. Activin is a TGFβ superfamily member that signals through cell surface receptors. Activin signaling is antagonized by follistatin, an activin-binding protein. Both activin and follistatin are secreted molecules that have diverse developmental modulatory effects. I investigated the distribution of activin and follistatin proteins in the ocular target regions and found that activin was present in targets for both neuron types. During the time of somatostatin induction within choroid neurons, follistatin was detectable only in the iris-ciliary muscles. To show that functional antagonism was secreted by iris cells, I used iris cell conditioned medium to prevent activin-mediated somatostatin induction in cultured ciliary ganglion neurons. Immunodepletion demonstrated follistatin as a component of the conditioned medium. During these studies, activin was found to repress the development of striated muscles while potentiating smooth muscle in cultures of iris-ciliary body. This observation was the basis for asking whether activin-follistatin antagonism, in addition to regulating neuropeptide phenotype, could organize target muscle differentiation.

I first addressed the role of activin and follistatin in striated muscle differentiation using homogeneous cultures of pectoral myoblasts. Markers

for successive stages of muscle differentiation were analyzed after activin or follistatin treatment. Activin inhibited while follistatin potentiated each marker for muscle differentiation, without altering proliferation or survival of the myoblasts. These data suggest that the two cytokines modulate differentiation of committed myoblasts to postmitotic, fusion-competent myocytes. The physiological significance of these findings are supported by the in vivo expression of activin, activin receptors, and follistatin at sites of muscle differentiation, including the chick pectoral muscles.

To address muscle differentiation within the iris and ciliary body, the smooth to striated muscle conversion was described at the cellular and molecular levels. The expression of multiple smooth and striated muscle markers were analyzed, including those for contractile protein isoforms, actylcholine receptor sub-types, and striated specific transcription factors. To test if smooth and striated muscle cells are derived from a common progenitor, single cell clonal assays were developed and performed. All clones differentiated as single cell types, regardless of enviromental influences. Transient co-expression of multiple smooth and striated markers were observed in single iris cells isolated during the transition. These results suggest that transdifferentiation of smooth muscle, in addition to determined myoblasts, contribute to the formation of myotubes in the iris.

Finally, to investigate the regulation of this transition, heterochronic chick-quail iris cell co-culture experiments were performed to understand the role of intrinsic, cell-autonomous versus extrinsic influences. Undifferentiated iris cells placed into a more mature environment showed a greater propensity to form striated muscle demonstrating the role of locally-derived, extrinsic influences in striated iris muscle differentiation. This activity was found to be secreted from the mature iris cells and could be removed from conditioned medium with an activin-affinity column. Furthermore, activin inhibited striated muscle development in undifferentiated iris explants, while smooth muscle differentiation was potentiated. The converse was true for these two cell types following follistatin treatment. The results from my thesis research support a model where locally produced signaling molecules, activin and follistatin, coordinate the differentiation of neurons - neuropeptide phenotype, and their targets - smooth and striated muscle development.

INTRODUCTION

Overview of developmental themes: coordinated differentiation.

During embryogenesis, several salient features of metazoan development exist (reviewed in Gurdon, 1992). These include fertilization, the combining of two individual genomes with the union of egg and sperm; cleavage, the rapid mitotic divisions of the zygote to form the blastula; gastrulation, the cellular movements that establish the three germ layers - the ectoderm, mesoderm, and endoderm; and organogenesis/morphogenesis, the formation of the various specialized, functional units that interact to enable complex behaviors. At the cellular level, specification - the establishment of positional information and structural identity, and determination - the commitment of individual cells and their subsequent progeny to specific cell-types or lineages, result from progressive restrictions in developmental potentials. Differentiation is the cellular equivalent to morphogenesis: the expression of cell-type specific characteristics. Two general modes of signaling regulate progressive restrictions in development. They can be classified as intrinsic (cell-autonomous) regulation, the ability of a cell to control its own development independent of its environment, and extrinsic (non-cell-autonomous) regulation, where signals derived from outside the cell influence specification, determination, or differentiation. Examples of intrinsic regulation include the segregation of cytoplasmic determinants during cleavage and the initiation of genetic programs that limit the competence to respond to subsequent signals. Systemic or hormonal signals, locally secreted signals, extracellular matrix cues, cell surface signaling molecules, and electrical signaling are all examples of extrinsic regulation in development. During organogenesis and morphogenesis, the various cells that compose the tissues that will eventually give rise to specialized organs, must integrate multiple developmental signals to coordinate differentiation. The governing questions of my thesis address these issues of coordinated differentiation.

Specification and differentiation of muscle

Muscle classifications

The development of muscle cells is one example of cellular specification and differentiation. Muscle can be classified into three general catagories based on embryonic origin, contractile properties, and morphology as cardiac, smooth, and skeletal. Cardiac muscle, is similar to skeletal in that the intracellular contractile aparatus appears cross-banded, but it is composed of mono-nucleated cells that are tightly associated and linked by gap junctions. Skeletal muscle (also refered to as striated muscle) is multi-nucleated and also displays a well organized contractile apparatus. Smooth muscle cells are more diverse in function and morphology, are mono-nucleated, and do not show banded striations.

Embryonic origins and specification

The three major muscle cell-types all arise from mesodermal precursors following gastrulation. Cardiac muscle develops from cells in the anterior lateral plate mesoderm; skeletal muscle arises from specific regions of somitic and head mesoderm; and most smooth muscle is derived from splanchnic lateral plate mesoderm. Cardiac muscle cells are specified early in gastrulation through interactions with the underlying endoderm (Nascone and Mercola, 1995; Schultheiss et al., 1995). The endoderm exerts an instructive signal that is mediated in part by the secreted molecule bone morphogenetic protein 2 (BMP2) (Schultheiss et al., 1997). Specification of smooth muscle is not as well understood, owing to a lack of molecular markers for undifferentiated but specified smooth muscle myoblasts. Differentiation of smooth muscle, however, is better understood, and experiments in cell culture suggest that several secreted molecules, primarily Transforming Growth Factor βs (TGFβs) and Fibroblast Growth Factors (FGFs), and multiple matrix components regulate expression of smooth muscle traits (reviewed in Owens, 1995). Skeletal muscle specification is among the best understood for any cell-type. The patterning or specification of precursor cells within the somites depends on interactions with proximal

tissues including lateral mesoderm, the neural tube, overlying ectoderm, and the more distant notochord. Studies using embryonic tissue and genetic manipulations, as well as cell culture experiments, indicate that both cell surface molecules, for example Notch and Delta, and secreted signaling molecules, such as Sonic Hedgehog, Wnt proteins, and BMPs, act to specify the myogenic lineage from other cell lineages in the developing somites (reviewed in Cossu et al., 1996).

Transcription factors in muscle determination and differentiation.

The regulation of gene transcription is what ultimately dictates determination and subsequent differentiation of any cell type. The restriction of unspecified mesodermal cells to either cardiac, smooth, or skeletal muscle lineages has recently been suggested to be regulated in common by a family of transcription factors termed myocyte enhancer factors (reviewed in Olson et al., 1995). The restriction and divergence of the three types of muscle is thought to be controled by different sets of transcription factors specific for each muscle lineage. Indeed, transcription factors specific for each muscle cell-type have now been identified. Again, those for the skeletal muscle lineage are the best understood. Basic helix-loop-helix (bHLH) muscle regulatory factors (MRFs) - for example myoD, control the differentiation and maintenance of this muscle type (reviewed in Weintraub, 1993; Ludolph and Konieczny, 1995). These nuclear phosphoproteins dimerize with a second bHLH protien of the E-box family to activate transcription of skeletal muscle specific genes. Several post-translational mechanisms have also been described that negatively regulate MRF function. For example, MRF associations with Id protein (Inhibitor of differentiation), an HLH that lacks a basic domain and prevents the heterodimer from binding DNA. Id can also repress by occupying available E protein partners (Benezra et al., 1990). Another mechanism is the sequestration of MRFs from the nucleus. This mode of inhibition was first described in Xenopus (Rupp et al., 1994) and recently a mammalian protein, I-mf (Inhibitor of MyoD family), has been shown to exhibit such activities (Chen et al., 1996). In addition, MRF function can be repressed through protein kinases C-dependent phosphorylations of a conserved threonine located within the basic domains of MRFs. The inhibitory activities of FGFs and TGFβs on differentiation have been shown

to induce such phosphorylations and prevent MRF mediated transactivation (Li et al., 1992; Martin et al., 1992).

Genetic alteration experiments in Drosophila and mice suggest that analogous regulatory transcription factors exist for cardiac and smooth muscle. In Drosophila, cardiac muscle specification is facilitated by the gene *tinman*, which encodes a homeodomain-containing protein (Bodmer, 1993). The vertebrate homolog, Nkx2.5, is expressed in early cardiac progenitors and is considered the earliest marker of cardiac specification. In mice deficient for Nkx2.5, however, some cardiac myocytes are specified and do differentiate, suggesting that this factor is not a myoD equivalent in the strictest sense. However, specification of the ventricular myocyte lineage in Nkx2.5 knockout mice is deficient, as is later morphogenic events (Lyons et al., 1995). Similar results with another homeodomain factor, *Bagpipe*/BAPX1 have been obtained in Drosophila and mice with respect to one smooth muscle lineage - visceral smooth muscle (Aziazu and Frasch, 1993; Tribioli et al., 1997). Drosophila larva with a null mutation in *bagpipe* fail to specify visceral mesoderm, which normally would give rise to gut muscle. The mouse homolog BAPX1 is first detectable in splanchnic mesoderm, the presursors of abdominal mesenteric smooth muscle. Not only do cells of a specific lineage become progressively restricted in their developmental potential, they must do so in a temporally and spacially regulated fashion with respect to surrounding cells which they will eventually function with in concert.

Eye development: migration, morphogenic movements, and local inductions.

While muscle development serves as an example for regulation of cellular commitment and maturation, the vertebrate eye is an excellent example of the developmental regulation between cells of both equivalent and diverse embryonic origins that must interact to form a complex organ. Development of the vertebrate eye progresses through a series of steps including:

1. Specification of the anterior neural plate to ocular structures.
2. Outpocketing of the optic vesicles from the ventral forebrain and the concurrent migration of neural crest and head mesodermal cells to form the periocular mesenchyme.
3. Invaginations of both the lens placode and the optic vesicle to form the lens and the optic cup, respectively.
4. Determination and differentiation of cells that form the multiple layers and structures of the eye.

Early events in eye development

Regional fate maps in Xenopus and zebrafish have demonstrated that the cells of the anterior-medial portion of the neural plate become specified as eye progenitors (Eagleson et al., 1995; Woo and Fraser, 1995). This single eye field, through influences from the underlying axial mesoderm and the ventral midline of the prospective CNS, is subdivided into two bilateral eye regions. These progenitors for ectodermally derived eye components can be identified through the expression of *Rx* (Retinal homeobox) gene family members which encode nuclear Paired-like homeodomain factors that mediate eye specification (Mathers et al., 1997). Expression of *Rx* genes is then sculpted to neuroectodermal cells that comprise the primary optic vesicle. As the optic vesicle evaginates from the wall of the forebrain, it becomes closely associated with the surface epithelium and induces lens formation (Coulombre, 1965; Henry and Grainger, 1990). The optic vesicle then undergoes dramatic morphogenesis to become a bilayed cup.

Posterior segment development

At the time when the optic vesicle contacts the surface ectoderm, all vesicle cells are committed to ocular phenotypes. Despite their similar embryonic origin, the pigmented epithelium (from the outer layer) and neural retina (from the inner layer) differentiate into distinct tissues. If the vesicle is stripped of the surrounding mesenchyme and epidermis, differentiation is arrested, and the pigmented epithelium and neural retina fail to diverge (reviewed in Reh, 1992). Recent data indicate that members of the FGF family are expressed in the surface ectoderm and specify neural retina from the pigmented epithelium (Pittack et al., 1997). Classic embryological experiments suggest that the specification of the pigmented epithelium is induced by the surrounding periocular mesenchyme. If the optic vesicle is flattened and surrounded entirely with periocular mesenchyme, only pigmented epithelium will develop (Lopashov and Strovea, 1972). Subsequent differentiation of the pigmented epithelium and neural retina are reviewed in Cepko et al., 1996.

Anterior segment development

Coincident with specification of the posterior segment, progressive restrictions occur in the anterior region of the optic cup. Following optic vesicle invagination, the lens organizes the anterior chamber by instructing periocular neural crest cells to form the corneal endothelium. In addition, lens derived signals specify the iris and ciliary body epithelium at the margin of the optic cup (Strovea, 1967; Dhawan et al., submitted; Beebe et al., submitted). The remaining periocular mesenchymal cells then form the corneal, iris, and ciliary body stromas as well as extraocular structures through lens-independent regulation. While the cellular inductive interactions responsible for specification of anterior chamber structures have been shown through lens extripation and transplantation studies, the signals mediating these events and the subsequent lens-independent differentiation and maturation of the iris and ciliary body have not been identified.

The avian iris and ciliary body as an experimental model to study differentiation and maturation during organogenesis

The iris and ciliary body provide an excellent opportunity to study cell-cell interactions and local signaling events during organogenic development. The avian iris and ciliary body are derived from cells with diversity in embryonic origins: surface ectodermal, neuroectodermal, cranial neural crest, and mesodermal cells all contribute to these structures of the ocular anterior chamber. The avian iris and ciliary body are particularly attractive for study, as the morphogenesis of these structures has been described in detail, and the embryo is readily accessible to in ovo manipulation as well as cell culture. Furthermore, the avian ciliary ganglion, composed of neurons that innervate the iris and cilary body and other ocular targets, has served as an informative model for developmental neuron-target interactions. The iris functions as the aperture for the eye by the action of two muscle groups, the constrictor and dilator, which control pupillary responses to the external environment. The ciliary body is bipartite in function: the epithelium produces aqueous humor - the lymph-like fluid that circulates through the anterior chamber, while the muscles located in the stroma control the shape of the lens and facilitate visual accomodation. An understanding of the development of these structures may give insight into pathologic conditions such as glaucoma - the imbalance of aqueous humor production and drainage that results in raised intraocular pressure, or anterior uveitis - an inflammation of the iris and ciliary body that can compromise vision and eventually result in tissue neovascularization or degeneration.

Muscle ontogeny in the avian iris: embryonic transition from smooth to straited muscle.

The avian iris and ciliary body undergoes a smooth-to-striated muscle transition during development. This gradual switch from an initial smooth muscle phenotype to the final multinucleated, striated muscle phenotype has been described morphologically by light and electron microscopy, immunohistochemically, and electrophysiologically. While not all vertebrates undergo a smooth-to-striated muscle transition in the iris, all

avian species investigated, as well as some reptiles and mammals, have been shown to contain striated muscle in the iris and the ciliary body. The chick iris becomes a distinguishable structure at stage 30 (embryonic day (E) 6.5) (Hamburger and Hamilton, 1951) as a small extension of the optic cup (Ferrari and Koch, 1984). The two layers of the anterior optic cup differentiate into the pigmented (outer, proximal to the cornea) and non-pigmented (inner, proximal to the lens) epithelia of the iris and ciliary body. At this stage, undifferentiated cranial-derived mesenchyme overlie the epithelial layers and will form the stroma of the iris, ciliary body, and cornea. Starting at E8, the two epithelial layers invaginate as buds into the overlying stroma, differentiate into smooth muscle cells, and migrate peripherally to form a transient structure, the iridial lamella (Gabella and Clarke, 1983). Overall growth of the iris is driven by mitosis primarily at the periphery in both the ectodermal and mesodermal layers (Stroeva, 1967). The smooth-to-striated muscle transition is first apparent at E10, with the emergence of striated muscle midway along the length of the iris and within close proximity to the smooth muscle cells. Differentiation of striated muscle continues through hatching, while the smooth muscle cells stop growing at E15, and are eventually confined to the pupillary margin. Both the initial epithelial budding of smooth muscle precursors and the emergence of striated muscle begin in a temporal and ventral region and differentiation progresses in a centripetal fashion into dorsal and nasal regions. (Lewis, 1903; Gabella and Clarke, 1983; Ferrari and Koch, 1984).

The embryonic origin of the striated muscle is ambiguous. Cell lineage experiments utilizing the chick-quail transplant technique suggest that some of the striated muscle precursors are derived from cranial mesenchymal cells (Nakano and Nakamura, 1985; Yamashita and Sohal, 1987). By performing various isotopic transplants (cranial mesoderm, neural crest, or forebrain) and heterotopic transplants (replacing cranial neural crest with trunk neural crest), Yamashita and Sohal (1987) concluded that striated muscle in the dorsal iris is of cranial neural crest origin, while striated muscle in the ventral iris is not derived from the neural crest and may arise from the cranial mesoderm. Other researchers have found that markers specific for either smooth or striated muscle are co-expressed in single cells within the iris during the muscle transition. These data were interpreted as supporting a

neuroectodermal origin of the striated muscle through transdifferentiation of smooth muscle cells (Pilar et al., 1987; Volpe et al., 1993). The cellular basis and regulation of the smooth-to-striated muscle transition has been further investigated in my thesis research.

Neuronal innervation

The muscles of the iris and ciliary body are innervated by parasympathetic ciliary neurons of the ciliary ganglion. Ciliary neurons extend axons from E4.5 when the ciliary ganglion is still condensing posterior to the eye, subjacent to the optic nerve. The first axons reach the prospective iris and cilairy body by E6 (Landmesser and Pilar, 1974; Weaver et al., 1995). Using electrophysiology, Pilar et al. (1987), demonstrated that neuromuscular transmission begins at E8 as the first smooth muscle cells differentiate. Initial contractile responses in the iris to tetanic stimulation of the nerves are long and protracted, characterized by a slow rise and decay time. By E11, fast responses were evoked with single-shock stimulation, as were the long-protracted responses to tetanic stimulation. At the time of hatching, only fast responding and rapid decay responses were observed. These results suggest that both the smooth and striated muscles are functionally innervated during the transition. Fast kinetic responses, but not the slow responses, could be blocked with the nicotinic acetylcholine receptor antagonist, alpha-bungarotoxin. Ultrastucturally, the initial neuromuscular contacts are typical of "en passant" synapses of other smooth muscle. They lack synaptic specializations such as vesicle clusters, pre- or post-synaptic membrane thickenings, and a defined basal lamina. From E11 to E16, synaptic morphology is heterogenous with some displaying well organized motor-end plates, while others remained unspecialized (Pilar et al., 1981). Myelination is evident by E14 and continues through hatching (Narayanan and Narayanan, 1981).

Neuronal maturation: target dependent influences

Following innervation, the ciliary neurons undergo maturation, of which several aspects, including neurotransmitter phenotype and developmental cell death, are target dependent. Although both ciliary and choroid neurons,

which together comprise the ciliary ganglion, utilize acetylcholine as their primary neurotransmitter, only choroid neurons express the modulatory neuropeptide somatostatin (Epstein et al., 1988; De Stephano et al., 1993). Expression of somatostatin immunoreactivity within the ganglion increases during the period of target differentiation (Smet and Rush, 1993). Previous studies have shown that choroid muscle cells induce the synthesis of the neuropeptide in both neuron types in vitro (Coulombe and Nishi, 1991). This activity was found to be secreted and indistinguishable from the signaling molecule activin A (Coulombe et al., 1993; reviewed in Nishi, 1994). Whether this molecule regulates neuropeptide induction in choroid neurons in vivo or, conversely, whether iris- and ciliary body-derived signals repress such induction is investigated in this thesis.

In addition to neuropeptide phenotype, developmental cell death in the ciliary ganglion has been shown to be target-dependent. This period of naturally occuring cell death is coincident with muscle maturation in both the iris and ciliary body, as well as the choroid. Removal of the these targets dramatically increases neuronal cell death, while transplanting an extra optic vesicle enhances survival (reviewed in Nishi, 1994). Efforts succeeding these observations led to the identification of a secreted molecule, chicken ciliary neurotrophic factor (chCNTF), which is expressed in the target cells and can promote the survival of ciliary ganglion neurons in vitro and in vivo (Manthorpe et al., 1980; Nishi and Berg, 1981; Eckenstien et al., 1990; Leung et al., 1992; Finn and Nishi, 1996; Finn et al., in press). Whether other target-derived signals work in conjunction with chCNTF to regulate cell death in the ciliary ganglion is unknown.

Cytokine signaling in development

A recurrent theme in embryogenesis is that cellular communication is often mediated by cytokines. Cytokines are secreted proteins that bind transmembrane receptors and induce intracellular signaling cascades that regulate diverse cellular processes such as specification, determination, differentiation, proliferation, migration, and survival. The specific response of a given cell depends on both the milieu of other signals currently impinging on the cell and the signaling history or competence state of that cell. Most cytokines are members of a limited number of families grouped by structural similarity. Examples of cytokine families important in development include the Fibroblast Growth Factors (FGFs), Neurotrophins (NTs), *wingless* proteins (Wnts), Hedgehogs (HHs), and Transforming Growth Factor βs (TGFβs). TGFβs are catagorized as a superfamily that can be subgrouped. TGFβ subfamilies include Bone Morphogenic Proteins (BMPs), *decapentaplegic-vg1* related (DVR), activins, and the original TGFβs.

Activins: regulation by multifunctional cytokines in development

The activins serve as an example of the precision and complexity in cytokine regulation. Activin isoforms, like other TGFβ superfamily members, are secreted as homodimers consisting of β subunits that contain 7 conserved cysteines. Six of these form three intramolecular disulfide bonds, which facilitate a "cysteine knot" tertiary structure (McDonald and Hendrickson, 1992). The seventh cysteine forms an intermolecular disulfide bond to link the dimer. Upon secretion, proteases cleave a pro-region to activate the homodimer. Three activin β subunits have been identified: A, B, and C (reviewed in Moses and Serra, 1996). In addition, one α subunit exits and can form heterodimers with the β subunits to function as dominant negative ligands termed inhibins (Xu et al., 1995). Both activins and inhibins were originally identified by their function in anterior pituitary regulation (Ling et al., 1986). In addition, activin signaling has been shown to have roles in early developmental patterning events such as mesoderm induction (Thomsen et al., 1990; Hemmati-Brevanlou and Melton, 1992) and specification of left-right asymmetry (Levin et al., 1995), as well as regulation of growth and

differentiation of multiple cell types during organogenesis (Murata et al., 1988; Vassalli et al., 1994; Matzuk et al., 1995c). Consistent with their multifunctional roles in development, the activins are widely expressed throughout embryogenesis (Albano et al., 1994; Feijen et al., 1994; Verschueren et al., 1995).

Follistatin: a secreted antagonist

In addition to dominant negative effects of inhibins, the activins and other TGFβ superfamily members are negatively regulated by secreted binding proteins. Follistatin is one such antagonist. Follistatin shows highest affinity for the activins (K_d ~ 400 pM, Kogawa et al., 1991), but will also bind and antagonize BMP 2, 4, and 7 (Yamashita et al., 1995; Fainsod et al., 1997). Follistatin transcipts are alternatively spliced to give two distinct protein products that are each differentially glycosylated (reviewed in Michel et al., 1993). The smaller protein species binds heparin proteoglycans, but the functional significance of this interaction is not understood. Like the activins, follistatin is widely expressed, and the local concentration of activin:follistatin is thought to control activin bioactivity.

Activin receptors and intracellular signaling

Biochemical and genetic evidence has shown that the activins bind and signal through heteromeric receptor complexes, composed of type I and type II transmembrane serine/threonine receptors (reviewed in Wrana et al., 1994). Type II receptors alone can recognize activin in solution, whereas type I receptors do not recognize ligands without out the presence of type II receptors. In addition, type II kinases are constitutively active, while type I kinase activity is phosphorylation-dependent. These observations, as well as mutational analyses in both receptor types, suggest that activin-type II binding promotes receptor heterodimerization, phosphorylation, and activation of type I receptors. Type I receptors then induce phosphorylation of members of the Smad family of TGFβ superfamily signal transducers (Macias-Silva et al., 1996). Smad proteins are vertebrate homologs to the *sma* and *mad* genes of C. elegans and Drosophila, respectively (reviewed in Massagué, 1996). Upon phosphorylation, these proteins are translocated to the nucleus where they

regulate gene transcription. To date, greater than 7 Smad family members have been identified, and mRNA localization studies suggest both overlapping and distinct patterns of expression. Thus Smad proteins represent another way to regulate precision in TGFβ superfamily signaling.

Questions addressed in this thesis

How does the avian iris and ciliary body develop? This is the overall question that has governed my thesis research. Specifically, what are the cellular mechanisms that facilitate, as well as the underlying molecules that regulate, the differentiation of iris and ciliary body muscle cells and the neurons that innervate these structures? I have focused on two coincident differentiation events during the development of the iris and ciliary body: the smooth-to-striated muscle transition and the regulation of neuropeptide phenotype. Based on expression studies and functional analyses of activin and follistatin in other developmental systems, I have investigated the role of these proteins in coordinating cellular differentiation and maturation in the iris and ciliary body.

MATERIALS and METHODS

Cell culture

Dissociated iris and ciliary body

Embryonic iris-ciliary body were isolated at various ages (described in figure legends). The dissection was performed by removing the overlying cornea by grasping the surface epithelium with one forceps while holding the back of the head with another forceps. The scleral ossicle ring and periocular mesenchyme were then dissected away to expose the iris and ciliary body. The iris and ciliary body were removed by cutting inside the ora serrata boundary with curved iridectomy scissors and lifting the tissues away from the vitreous. The iris and ciliary body were then carefully stripped of their attachments to the lens and remaining retinal tissue. Iris-ciliary body tissue, with epithelium intact, was then placed in modified Pucks glucose (MPG) solution (123 mM NaCl, 5.3 mM KCl, 10 mM Na_2HPO_4, 10mM NaH_2PO_4, 0.1% phenol red, 0.01% glucose, pH 7.2) and cut into 1 mm^3 pieces before mechanical dissociation in MPG by trituration through a reduced-bore pipette. Following dissociation, cells were filtered through 15 μm^2 polyamide nylon mesh (Tetko, Inc.), centrifuged and resuspended in modified L15 CO_2 (Mains and Patterson, 1973) supplemented with 10% horse serum (plating medium). Cells were counted with a hemocytometer and seeded into rat tail collagen coated wells at various densities (described in figure legends). After 1 to 2 days in vitro, cultures were switched to serum free L-15 to promote smooth muscle differentiation and myotube formation. Serum free L-15 was supplemented with 2.5 mg/ml bovine serum albumin, 25 µg/ml ovotransferrin, 30 nM selenium, and 2.5 µg/ml insulin (Sigma Chemical Co.). Both serum and serum free L-15 included 6 µg/ml glucose, 20 U/ml penicillin, and 2 mg/ml streptomycin (Sigma Chemical Co.).

Iris and ciliary body explants

Iris-ciliary body were isolated as above and explanted on collagen-agarose cushions in a 35 mm^2 dish. Collagen-agarose cushions were prepared by mixing the following at a 1:2:1 ratio: collagen stock (1.5 mg/ml)/plating medium/1.0% melted agarose. 400 µl of this mixture was placed onto a 35 mm^2 dish. Once the collagen-agarose cushion was set, the dish was flooded with plating medium to equilibrate the cushion. The explant was then placed on top of the cushion, and excess medium was removed to leave only a thin layer over the explant thus promoting oxygen exchange. After 1 day in vitro, the explants were changed to supplemented serum free medium and changed daily, thereafter.

Pectoral muscle cultures

Embryonic day 11 (E11) chick pectoral muscles were isolated in Earle's balanced salt solution (GIBCO-BRL) modified by replacing $NaHCO_3$ with 15 mM HEPES (Sigma) (Nishi, 1996). Muscle stroma was dissected away from condensing cartilage and placed into a dish of MPG. Muscle tissue was cut into 2.0 mm^3 pieces and mechanically dissociated in MPG (1 pectoralis per ml MPG) by trituration through a reduced bore Pasteur pipette. Following dissociation, cells were filtered through 15 µm polyamide nylon mesh, centrifuged, and resuspended in modified L15 CO_2 supplemented with 10% horse serum (plating medium). The cells were then incubated on 100 mm^2 tissue culture dishes (Falcon #1029, Becton Dickinson Labware) for 20 minutes at 37°C, during which many of the fibroblasts adhered to the plastic. Non-adherent, myoblast enriched cells were then plated onto rat tail collagen-coated 48-well tissue culture plates (Falcon #3078) at 1 x 10^4 cells/ml in 200 µl plating medium. A limited exposure to 10% (v/v) horse serum was found necessary to obtain optimal plating efficiency. After 16 hours in 10% horse serum/L15, this medium was replaced with supplemented serum-free L15 medium.

Embryonic fibroblast cultures

Head skin from E8 embryos was removed and cut into 1 mm^3 peices before enzymatic digestion in 0.5% trypsin in MPG for 20 minutes at 37°C. Cells were then dissociated by trituration as with iris cultures and seeded into rat tail collagen coated wells at a density of $1x10^4$ cells/ml of plating medium. After 1 day in vitro, cultures were switched to supplemented serum-free L15 medium.

Choroid cultures

Choroid cells were prepared as previously described (Coulombe and Nishi, 1991). Cells were plated on tissue culture dishes coated with rat tail collagen and grown in modified L-15 medium supplemented with 10% chick serum and penicillin/streptomycin/glucose, as described above, for 2 days. After 2 days in vitro, cells were transferred to supplemented serum free conditions.

Iris Cell Clonal Analyses

Clonal Experiment type 1

E7 quail iris, the unfolded band located central to the ciliary body, was isolated and cells were dissociated as described above. Cells were seeded at approximately one cell per well in 72 well, conical collagen-coated Terasaki plates (Nunc) containg 25 µl of 5% E10 chick embryo extract (CEE)/10% HS/modified L15. Wells with a single cell were identified under phase-contrast optics with a 10X objective. Once clones reached approximately 100 cells (5-6 days), they were removed using 0.1% trypsin in MPG (25 µl total volume) and 1/2 of the cells were placed into culture alone, while the other 1/2 were added to E11 iris-ciliary body cultures. For both conditions, serum-free medium in collagen-coated 35 mm^2 dishes was used. A 1 cm hole had been drilled in the center and covered with Petriperm biomembrane (Heraeus Instruments) to form a 250 µl volume well within the center of each 35 mm^2 dish. Quail clones alone or in co-culture were fixed with Zamboni's fixative (4% paraformaldehyde, 15% picric acid, 0.05 M phosphate buffer, pH 7.2) after

4 days in the new environment. The clonal derivatives were then analyzed for smooth and striated muscle markers.

Clonal Experiment type 2

Dissociated E7 quail iris cells were incubated with 0.8 μM calcein-acetoxymethyl ester (calcein-AM, Molecular Probes, 4mM stock diluted in MPG) for 15 minutes prior to filtration. Cells were seeded at low density (approximately one cell per well) on 3 day cultured E8 chick head skin fibroblasts or chick E11 iris-ciliary body. Although the membrane permeability of calcein-AM is dramatically reduced when cleaved by endogenous esterases of intact and healthy cells, the fluorescent molecule does diffuse from the cytoplasm with a half-life of three hours. Therefore, wells were assessed for the presence of single, fluorescent cells three hours after seeding. These wells were marked and clones were co-cultured for 4 days at which time the cultures were fixed. Clones were identified by the quail nuclear marker QCNP and analyzed for smooth markers and the ability to fuse with myotubes.

Cytokine Additions

Activin A, follistatin, FGF 2, and TGFβ1 were added independently or in combinations in plating medium as described in the figure legends. Growth factors were added daily in fresh serum free medium. Recombinant human activin A (lot # 15365-23 III) and recombinant human TGFβ1 (lot # GO98AD) were generous gifts from Genentech, San Francisco, CA. Follistatin (lot # B3904), was obtained from the National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases, Rockville, MD. Recombinant bovine FGF 2 was a generous gift of Dr. Felix Eckenstein, OHSU, Portland OR., and was produced as a GST-fusion protein which was isolated on a glutathione column. The GST was subsequently cleaved with thrombin, and FGF 2 was then purified on a heparin affinity column. Recombinant and native FGF 2 were indistinguishable in several bioassays (Eckenstein, 1991). Cytokine stock solutions were stored at -80°C in PBS with 1 mg/ml BSA as a carrier protein at the following concentrations: activin A (0.6mg/ml), follistatin (1.0 mg/ml), FGF 2 (0.4mg/ml), and TGFβ1 (0.1mg/ml).

Immunolocalization

Antibodies

Rabbit anti-follistatin antiserum (Sugawara et al., 1990) was a generous gift from Dr. Shunichi Shimasaki, The Whittier Institute, La Jolla, CA. Rabbit anti-Inhibin βA antiserum and activin A peptide$^{81\text{-}113}$ (Vaughan et al., 1989) were generous gifts from Dr. Wylie Vale, The Salk Institute, La Jolla CA. MF20 [anti-striated myosin heavy chain (MyHC)], and QCPN [quail specific nuclear marker] mouse monoclonal supernatants were obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Biological Sciences, University of Iowa, Iowa City, IA 52242. Rabbit anti-chicken myoD antiserum was a generous gift from Dr. Bruce Paterson (Natl. Cancer Inst., NIH, Bethesda, MD.). Rabbit antiserum for chicken muscarinic acetylcholine receptor sub-types 2, 3, and 4 were generous gifts from Dr. Neil Nathanson (Dept. of Pharmacology, University of Washington, Seattle, WA.). The following antibodies were purchased commercially: anti-myoD (M-318sc#760, Santa Cruz Biotechnology), anti-mrf4 (242sc#-784, Santa Cruz Biotechnology) anti-smooth muscle specific alpha-actin (αSMA, Sigma), anti-calponin (αCLPN, Sigma), anti-smooth muscle specific myosin light chain kinase (αMLCK, Sigma).

Immunocytochemistry

Cultures were fixed in 500 µl Zamboni's Fixative (4% paraformaldehyde, 15% picric acid, 0.05M phosphate buffer, pH 7.2) for 20 minutes at 25°C followed by washing in PBS. For activin βA and follistiatin detection, fixed cultures were incubated at 4°C overnight in blocking solution. Polyclonal rabbit anti-activin βA or anti-follistatin antiserum diluted 1:500 in blocking solution were then incubated for 2 hours at 25°C. Endogenous peroxidases were then inactivated in 30% EtOH/1% hydrogen peroxide/PBS. Antibody localization was detected using single peroxidase-antiperoxidase staining. Specificity was controlled for by comparing staining in the absence of a primary antibody or incubating with normal rabbit serum instead of primary. Anti-activin βA specificity was further tested by preabsorbing 15 ml of the antiserum with 100

mg of the activin peptide$^{81\text{-}113}$ used to generate the antiserum. For muscle marker detection, fixed cultures were blocked overnight with 10% horse serum/5% chick serum/2% lamb serum/0.5% Triton X-100/0.1% sodium azide/PBS. Primary antibodies were incubated 4 hrs at 25°C or overnight at 4°C at the following dilutions in blocking solution: MF20 (1:6), QCNP (1:4), anti-SMA (1:1000), anti-CLPN (1:1000), anti-MLCK (1:1800); anti-cMyoD (1:250), anti-myoD[SC#] (1:500), and anti-cCM2 (1:200), anti-cCM3 (1:400), anti-cCM4 (1:1000). Cultures were subsequently processed for single (Anti-activin βA and anti-follistatin) or double (anti-cMuscarinic Receptor sub-types) peroxidase-antiperoxidase immunoreactivity as described previously (Darland et al., 1995). For immunofluorescence, goat-anti-mouse-fluorescein isothiocyanate (1:800, Cappel) or goat anti-rabbit-tetramethylrhodamine (1:800, Cappel) were incubated 1 hr. at 25°C prior to Hoechst staining (1μg/ml, Sigma) and equilibration in Anti-Fade (Molecular Probes). Specimens were viewed using a Sony digital camera attached to a Zeiss conventional microscope. Images were captured using Sony DKC-5000 software.

Immunohistochemistry

Embryonic eyes were removed and fixed in 10 mls fresh Zamboni's Fixative for 40 minutes at 25°C and washed in PBS prior to equilibration in graded dilutions of sucrose (3%, 6%, 15%, 22.5%, 30%) and then embedded in OCT freezing medium (TissueTek, Elkhart, IN). Serial sections (8 μm) were cut on a Leica Jung Frigocut 2800N cryostat. Sections were mounted on gelatin-coated glass slides, dried 2-3 hours at 25°C, and processed for immunolocalization as described above.

Alpha–Bungarotoxin labeling

Dissociated iris-ciliary body cells were cultured for 4 days as described. On day 4, medium was replaced with fresh serum free medium with 10 μg/ml α–Bungarotoxin-FITC (Molecular Probes) and incubated for 20 minutes to label the cell surface pool of nicotinic acetylcholine receptors. Cultures were then washed in PBS and fixed with fresh Zamboni's prior to processing for MyHC immunoreactivity using a rhodaminated secondary antibody.

RNase Protection Assay for Activin and Follistatin

Riboprobe Constructs

A fragment of chick ribosomal protein S17 (CHRPS) of 361 base pairs was obtained by RT/PCR (Beverly, 1991) using the primer pair based on the published sequence (Trüeb, et al, 1988): forward, 49-67 and reverse, 389-409, with cycling conditions of 93°C (melting), 54°C (annealing) and 72°C (extension), one minute each (30 cycles) with one unit/reaction of Taq DNA polymerase (Promega, Madison, WI). The fragment was subcloned into pBluescript SK+/- (Stratagene). A fragment of chick follistatin was obtained by RT/PCR from embryonic day 17 (E17) ovary using Xenopus primers from a region conserved between Xenopus and human follistatin sequences (Hemmati-Brivanlou and Melton, 1994b). The primers recognized an equivalent region in the rat follistatin gene that crossed the exon 4/5 boundary (Shimasaki et al., 1989). In chick, there does not appear to be an exon boundary in the region covered by the primers, as no shift in size of the 223 base pair product was observed when the primers were used to amplify genomic DNA. The 223 base pair fragment was generated with the primer pair from the published sequence: forward, 639-658 and reverse, 842-862, with the same amplification conditions used for CHRPS. The chick follistatin was cloned into the pCR^{TM}II vector (Invitrogen). The sequence of the inserts was confirmed by Sequenase sequencing analysis. A full length chick inhibin bA cDNA in pBluescript was a generous gift from Dr. Patricia Johnson, Cornell University, Ithaca, NY.

Template and Probe Production

Templates for riboprobe production were generated by linearizing constructs in the multiple cloning site or within the insert to generate blunt ends or 5' overhangs. Linearized templates were treated with 50 µg/ml proteinase K and 1% SDS, 30 minutes at 37°C. The solution was extracted with an equal volume of phenol:chloroform:isoamyl alcohol in a ratio of 25:24:1. The aqueous phase was precipitated with 10% volume of 3M sodium acetate, pH 7.0 and 2.5 volumes of 100% ethanol and kept at -20°C for at least one hour. Pellets were spun and washed one time with 70% ethanol. The DNA was resuspended in H_20 for storage at -20°C. Riboprobes were generated in the following reaction mix: 12.5 µM cold rCTP (250 µM cold rCTP for CHRPS); 1X transcription buffer, 10 mM DTT, 10 units RNasin, 500 µM each rUTP, rATP, rGTP; 750 ng template; 25 µCi ^{32}P rCTP, 3000 Ci/mMole and 10 units bacterial RNA polymerase. The free nucleotides and the RNasin were purchased from Promega and the remaining reagents were from Gibco/BRL. The mix was incubated one hour at 37°C and the remaining template was removed by digestion with 10 units of RNase-free DNase I. Proteinase K treatment and extraction were as described for template production. The probes were precipitated with 1 µl of 10 mg/ml glycogen plus sodium acetate and ethanol, as before. Probes were routinely generated with activity greater than 10^7 dpm prior to purification. Full length probes were purified essentially as described (Gilman, 1991) with the following modification: the gel band was crushed with a tube pestle and extracted in 500 µl of 0.3M NaCl, 0.5% SDS and 10 mM Tris, pH 7.5 for 45 minutes on a rocker at 37°C. Purified probes were suspended in 40 µl of hybridization buffer, 40 mM PIPES, pH 6.7, 0.4 M NaCl, 1mM EDTA, and combined in the hybridization mix at 100,000 dpm each for activin A and follistatin and 10,000 dpm for CHRPS.

Hybridization and Protection

Total RNA was obtained from embryonic tissue or cells in culture using acid phenol extraction (Chomczynski and Sacchi, 1987). Purified pellets were resuspended in hybridization buffer. Total RNA and probes were mixed and hybridized 15-18 hours at 42°C. Protection and processing were done as described (Gilman, 1991). Protected fragments for activin A, follistatin and

CHRPS were 410 bp, 223 bp and 122 bp, respectively. The unprotected probes ran 20-70 bases higher than the protected fragments. Fragments were run on 6% acrylamide/8M urea/0.5X TBE gels. Dried gels were exposed to autoradiographic film (X-omat; Imaging Products International) and phosphorimager screens (Molecular Dynamics).

Analysis of mRNA levels

The phosphorscreen image was scanned using Scanner Control SI (Molecular Dynamics) at 200μ resolution. Relative signal intensity from the scanned phosphorimage was determined using the IPLab Gel software (Signal Analytics, Vienna,VA) to generate the values for protected fragments. Relative intensity for activin A and follistatin mRNA were expressed as a ratio to the relative intensity of CHRPS mRNA to obtain the RNA value. Normalization to the CHRPS loading control allowed for comparison between samples and eliminated variation in sample handling.

Competitive Reverse Transcription - Polymerase Chain Reaction (RT-PCR) Assay for MyoD and Myf5

cDNA Synthesis

RNA was isolated from pectoral muscle cultures grown in 48 well plates as described above. Two μg total RNA resuspended in 20 μl H_2O was added to 0.5 μg oligo dT (Promega Corp., Madison, WI) and incubated at 70°C for 10 minutes. RNA+oligo dT was then placed on ice and added to 20 μl of reaction mix containing 1X First Strand Buffer (GIBCO-BRL), 0.1 M DTT, 10 mM 2'-deoxynucleoside 5'-triphosphates, and 200 U (1 μl) Superscript reverse transcriptase (GIBCO-BRL). cDNA synthesis was carried out for 50 minutes at 37°C.

Competitive PCR

Competitive PCR was used to take advantage of linear internal standardization between sample comparisons (Gilliland et al., 1990). All PCR reactions were carried out in 25 μl volumes consisting of: 1μl cDNA, 1μl of

purified diluted competitive template (optimal dilution was established empirically using a dilution of competitive template with control cDNA as shown in results), 0.5 mM forward and reverse primers, 1X ThermoPol reaction buffer (2 mM final $MgSO_4$ concentration) (New England Biolabs), 2.5 mM 2'-deoxynucleoside 5'-triphosphates, and 0.4 Units Vent (exo-) DNA polymerase (New England Biolabs). 20 µl of vitamin E stabilized mineral oil was added prior to cycling to prevent evaporation. Reactions were cycled with the following parameters: [94°C denaturing for 1 minute, 52-54°C annealing for 1 minute, 73°C extension for 1 minute] x30 cycles. 10 µl of each reaction was separated on a 1.4 % agarose gel containing 5 µg ethidium bromide. Reaction products for each primer pair showed single bands which were sub-cloned and sequenced in initial experiments to confirm specificity. A minimum of three independent amplifications were performed for each comparison.

Construction of Competitive Templates

Competitive templates were generated by first amplifying phage X174 DNA at low stringency, [91°C denaturing for 1 minute, 45°C for 3 minutes for low stringency annealing, 73°C extension for 1 minute] x8 cycles, with myoD or myf5 primer pairs. This was followed by amplification at higher stringency, [91°C for 1 minute, 54°C for 1 minute, 73°C for 1 minute] x20 cycles. The band of desired size was then isolated by gel purification and subcloned into the pCRII vector (Invitrogen). The competitive template generated in this way thus contained the primer sequences of the myoD and myf5 cDNAs, but coded for different sequences between the primer sites.

Primers and Annealing Temperatures

mRNA	Forward/Reverse Primers	Annealing Temp.
CHRPS	F: CGTCTGGGCAACGACTTCC	54°C
	R: AAGCAACATAACGAGCGGCTC	
myf5	F: GAGGGAATCGCTGCATTGTG	52°C
	R: GCACCTCCTCAAGGTCTCGAATG	
myoD	F: CACGGAATCACCAAATGACCC	52°C
	R: TGGACCTGCCTTTATAGCACTTG	

In Situ Hybridization

Riboprobes specific for the chicken muscarinic acetylcholine receptor subtypes (CM2, 3, 4), corresponding to the heterologous third cytoplasmic loop, were generated from plasmids kindly provided for by Neil Nathanson (Dept. of Pharmacology, University of Washington, Seattle, WA.). Constructs were linearized and purified as described before (Darland et al., 1995). T7 or SP6 RNA polymerases (Gibco-BRL) with digoxigenin-11-UTP (Boehringer Mannheim) were used to label anti-sense probes. In addition chicken myoD anti-sense riboprobes, corresponding to region 776-1063 bp subcloned into pCRII vector (Stratagene), were synthesized to use as positive controls with muscle cultures. For each reaction, 1 µl was analyzed on a 1.5% agarose gel to check synthesis fidelity and estimate probe concentration. Probe purification, hybridization, and immunodetection were performed as described in Neito et al., 1996. In brief, cultures were fixed in 4% paraformaldehyde/4% sucrose/PBS and transferred to 70% EtOH and stored at -20°C until processed for in situ hybridization. Following re-hydration, cells were pre-hybridized 1-3 hours at 50°C. Pre-hybridization buffer was replaced with warmed, fresh hybridization buffer with 100 ng riboprobe. Cultures were hybridized 16 hrs. at 50°C in a humidified chamber. Cells were washed 3 times for 15 minutes in each of the following: 2XSSC/0.1% CHAPS (50°C); 0.2XSSC/0.1% CHAPS (37°C); KTBT buffer (25°C). Cultures were then processed for immunodetection with anti-digoxigenin Fab fragment antibodies (1:1000 in 20% lamb serum/PBS, Boehringer Mannheim) and a secondary antibody coupled to alkaline phosphatase. BM purple was used as the reaction

substrate (Boehringer Mannheim). Immunohistochemical reactions were allowed to develop 6-8 hrs prior to quenching with KTBT washes.

Preparation of conditioned medium

Confluent fibroblast and E8 or E11 iris-ciliary body cultures were grown in serum-free medium for 3 days. Cultures were then feed with fresh serum-free medium and conditioned medium was collected 48 hrs later and centrifuged at 3000 x g at 4°C in a 10 kDa-cutoff Centriprep filter unit (Amicon) to concentrate 10-fold. Unconditioned serum-free medium was also concentrated 10-fold and used as control medium. Concentrated conditioned medium was sterile filtered through a 0.22 μm filter and stored at -80°C prior to use in muscle induction assays and depletion of activin-binding proteins.

Bioassay for Somatostatin Induction

Neuronal culture

Somatostatin expression was assayed by immunoreactivity in cultured E8 ciliary ganglion neurons as previously described (Coulombe and Nishi, 1991). Briefly, ciliary ganglia were dissociated and plated at a density of 1 ganglion per well into drilled 35 mm^2 tissue culture dishes. 1.5 cm^2 cut Petriperm™ biomembrane (Heraeus Instruments, Inc.) had been glued under the 1 cm hole and the resulting well was coated with poly-D-lysine (100 μg/ml) and then laminin (2 μg/ml) prior to plating. Neurons were grown in the presence of 1 ng/ml recombinant chCNTF (GPA; Finn and Nishi, 1996) to promote survival (Eckenstein et al., 1990). chCNTF has no somatostatin induction capacity in this assay (Coulombe et al., 1993). Dose responses (0-50 ng/ml) of recombinant human activin A (Genentech, San Francisco, CA) were tested in the presence of 2X concentrated serum free L-15 or 2X concentrated iris conditioned medium for 5 days in a volume of 200 ml. Medium was changed every 36 hours.

Immunocytochemistry

After the 5 day culture in the described medium, neurons were processed for double peroxidase-antiperoxidase immunoreactivity (Vacca et al., 1980) using a rabbit anti-somatostatin antiserum (INC Star Corp.) as described (Coulombe and Nishi, 1991) with the following modifications. Cultures were incubated with goat anti-rabbit antiserum (Sternberger Immunochemicals) at a dilution of 1:125 in blocking solution and Activity Select™ peroxidase-rabbit antiperoxidase complex (Sternberger Immunochemicals) was used at 1:200 in blocking solution. Blocking solution consisted of 10% horse serum, 5% chick serum, 2% lamb serum, 1% Triton X-100 in PBS. Normal rabbit serum staining was performed as a control for non-specific immunoreactivity.

Immunodepletion

Immunodepletion was accomplished by repeatedly passing 10 ml of ICM through a 1 ml Hi-trap™ protein G column (Biorad) to which 50 µl of follistatin antiserum (Sugawara et al., 1990) had been bound. Antibody binding to the column was monitored by the absence of immunoreactivity on cultured iris myotubes with the flow through.

Immunoreactivity scoring

The number of neurons containing somatostatin immunoreactivity (SOM IR) was viewed under a Zeiss microscope with a 40X objective using bright field optics. Neurons were defined as an ovoid cell with an axonal process at least two cell diameters long. SOM IR was scored positive if the cytoplasm contained punctate dark brown diaminobenzidine reaction product. Ten fields of view were scored for SOM IR positive neurons and total neurons for each well. Three wells for each condition tested were included in each experiment. Percent SOM IR was determined by summing the SOM IR positive neurons per well and dividing by the total neurons per well. Mean percent SOM IR and the standard deviation (SD) were determined using percent SOM IR from each well from one representative experiment. A one-

way ANOVA followed by Scheffe's multiple range test was used to assess the statistical significance of the results.

Determination of neuronal survival and choline acetyltransferase activity

Neuronal survival per culture was determined by adding the number of neurons in ten fields of view at 400X. Reported values were the average of 3 cultures from one representative experiment. A one-way ANOVA followed by Scheffe's multiple-range test was used to assess the statistical significance of the results. Choline acetyltransferase activity was determined as described (Coulombe and Nishi, 1991).

Bioassay for activin A and follistatin

Erythroid cell differentiation

Activin A-dependent erythroid differention assay was adapted from Schwall and Lai, 1991. The K562 human erythroleukemia cell line was maintained in T75 flasks at ~5X10^6 cells per ml. in 10%FCS/RPMI. K562 cells were plated at 2.5 x 10^4 cells per well in 24 well plates with final volume of 400 µl 10%FCS/RPMI. Activin A with recombinant follistatin or 10X conditioned medium was pre-incubated for 30 minutes at 25°C prior to addition to K562 cells. Cells were grown for 4 days without medium changes. On day 4, cells were removed and pelleted by centrifugation at 4°C. Medium was removed and cells were washed two times with cold PBS. Cells were then lysed in 60 µl cold H_2O, vortexed briefly, and lysates were stored at -80°C prior to hemoglobin synthesis and lactate dehydrogenase (LDH) assays.

Hemoglobin synthesis assay

Lysates were thawed, vortexed again, and cellular debris was removed by centrifugation. For each sample, 40 µl of lysate was removed and added to one well of a 96 sample plate. In addition, 5-5000 ng dilutions of purified hemoglobin (Sigma) was added to establish the linear range for the standard curve. Once all lysates and standards had been transfered to the 96 sample plate, a reaction mix of 10 mg/ml tetramethylbenzidine (Sigma), 1% H_2O_2,

and 45% acetic acid was prepared. The reaction was initiated with addition 160 µl of this mix. The assay plate was incubated 20 minutes in the dark and then OD_{595nm} was measured with a UV Max microplate reader (Molecular Devices, Sunnyvale, CA). Data were analysed with Softmax software (Molecular Devices).

LDH assay

Total cell numbers for each sample were estimated by quantitation of LDH, a constitutively synthesized cytoplasmic enzyme. LDH activity was spectrophotometrically assayed as described previously (Eckenstein et al., 1990). Briefly, for each sample 10 µl of the lysate was added to a well of a 96 sample plate. In addition, lysates with known cell numbers (500-250,000 K562 cells) were added to the assay plate to establish a standard curve and determine the linear range. 90 µl of reaction buffer was added to each well. The reaction was initiated by addition of 100 µl of reaction substrate mix. After reaching maximum reaction velocity (mOD/min.), the rate of OD_{490nm} change over 2 minutes was measured.

Activin-A affinity column

An activin A affininty column was prepared as described in Inouye et al., 1991 with the following scaling modifications. 100 µg of recominant activin A was coupled to 400 µl of activiated N-hydroxisuccinimide agarose (Affigel 10, Bio-Rad, Richmond, CA) in 100mM HEPES, pH. 7.4. Control-agarose column matrix was prepared by linking 400 µl of 1M ethanolamine with 400 µl of Affigel 10. All procedures were carried out at 4°C. The coupling reactions proceeded for 16 hours with gentle rocking. Remaining reactive sites for the activin-agarose matrix were blocked with 400 µl of 1 M ethanolamine. The matricies were then washed ten times with 1 ml of 100 mM HEPES, pH. 7.4. A coupling efficiency of 75% for the activin A matrix was estimated by determining the total amount of unbound protein. Columns were prepared using sterile cotton plugged 1 cc syringes. Control and activin affinity columns were then equilibrated with serum free medium. Conditioned medium was divided into three pools: two for repeated passage through the

activin or control column and one to test directly in the muscle induction and erythroid differentiation assays.

Creatine Phosphokinase Activity Assay

Cultures were washed with PBS and homogenized on ice with 80 µl of 1% nonidet P-40, 100 mM sodium phosphate buffer (pH 7.0). Homogenates were stored at -80°C prior to assaying. CPK activity was determined by reacting 10 µl lysate with 200 µl of the following reaction mixture: 5 mM a-D-Glucose, 1.5 mM ADP, 20 mM DTT, 0.5 U/ml G-6-PDH, 1.3 U/ml hexokinase, 0.7 mM NADP, 9mM phosphocreatine (Sigma CPK assay no.45UV). After reaching constant reaction velocity (mOD/min), the rate of change of absorbance at 340 nm over 2.5 minutes was measured spectrophotometrically (UV Max kinetic microplate reader run with Softmax software, Molecular Devices). V_{max} remained linear throughout the assay. All samples were assayed at least in triplicate. Each experiment was repeated three times.

Quantitative Muscle Cell Differentiation Assay

After processing cultures for MyHC or αSMA immunoreactivity, cells were incubated for 10 minutes with the nuclear stain acridine orange (10 µg/ml in PBS, Sigma). Nuclei per MyHC or αSMA positive cell and total nuclei were scored by counting and summing 10 non-overlapping fields of view per well. Cultures were plated in triplicate for each condition. Each experiment described was repeated three times.

Bromodeoxyuridine labeling

Dissociated E8 iris-ciliary body cells were cultured for 4 days as described in the figure legend. On day 4, medium was replaced with fresh serum free medium containing 10 µM bromodeoxyuridine (BrdU, Sigma) and pulsed for 1 hr. Cultures were then fixed with ice cold 100% methanol for 10 minutes and then allowed to air dry. Cells were then rehydrated with PBS. Cellular DNA was denatured by incubating in 2N HCl for 1 hr at 37°C. Acid neutralization was accomplished by two washes with 100 mM borate buffer, pH 8.5 and cultures were processed for single peroxidase-antiperoxidase

immunoreactivity using an anti-BrdU monoclonal antibody (Boehringer Mannheim, 1170-376; 1:500 in blocking solution).

Mitogenic Assay for FGF1- and FGF2-like Bioactivity

Preparation of extracts

Extracts were prepared as described in Eckenstein et al., 1991. Briefly, iris-ciliary bodies or ciliary ganglion were dissected in EBSS and frozen at -80°C. Tissues were later thawed on ice and homogenized with a dounce pestle in ice cold protease inhibitor buffer. Homogenates were centifuged at 1500 x g for 20 minutes. The supernatant was removed and the protein concentration was estimated by OD_{280}. The supernatant was then adjusted to 100 mM Tris and applied to a heparin column. The flow-through and 300 mM NaCl/50 mM Tris washes were pooled as non-heparin binding fractions. Heparin-binding fractions were eluted with 600 mM NaCl/50 mM Tris. Each fraction was then dialyzed against 50 mM Tris and protein concentration was estimated by OD_{280}. Equivalent amounts of each fraction were then assayed for mitogenic activiy in the presence or absence of exogenous heparin.

^{3}[H]-Thymidine Incorporation Assay

The mitogenic effects of iris and ciliary ganglion extracts were tested in a ^{3}[H]-thymidine incorporation assay according to Shipley, 1986. Briefly, AKR-2B mouse fibroblast cells were grown in 24 well plates with McCoy's 5A medium supplemented with 5% (v/v) fetal calf serum until confluent and contact growth-inhibited. The medium was then replaced with serum free medium (MCDM 402) and cells were incubated an additional 2 days. Dilutions of extracts were added with fresh serum free medium with or without added heparin proteoglycan (Sigma). Twenty-two hours after extract additions the cells were pulsed for 1 hour with 1.0 µCi ^{3}[H]-thymidine (New England Nuclear). Labeled DNA was assayed by acid precipitation followed by liquid scintillation spectroscopy. Dilutions of extracts used for comparison between ages were all within the linear range of this assay.

Electrophysiology

Intracellular recordings were obtained using current-clamped methods of Furshpan et al., 1986, from pectoral and iris-ciliary body myotubes 2 days after serum withdrawal. In brief, prior to recording the muscle cultures were equilibrated in recording medium consisting of the following (per 100 mls): 10 ml L15 CO_2 basal medium; 1 ml 10X Hanks balanced salt solution without Ca^{2+} or Mg^{2+}; choline cloride, 1 mg (72 μM); glucose, 600 mg (33 mM); glutamine, 29.2 mg (2 mM); penicillin, 20,000 units; streptomycin, 20 mg; calcium chloride, 311 mg (2.8 mM). Glass microelectrodes were pulled to 75-95 MΩ. and filled with 3M KCl. Recording medium and antagonist solutions were perfused with a flow rate of approximately 400 μl/min. Cells were impaled and showed membrane potentials between -45 to -70 mV. Two large diameter (5-10 μm) pipettes were used to pressure apply acetylcholine (10μM) or muscarine (10μM) (Choi and Fischbach, 1981). Recording medium was replaced with either curare (1μM, Sigma) or atropine (1μM, Aldrich) in recording medium. Antagonist reversal was assessed following reprofusion with recording medium.

Statistics

Two tailed t-test and Chi-square analyses were performed using Statistica statistics software (Statsoft, Inc.). One-way ANOVA followed by Scheffe's multiple range tests were performed using Statview statistics software (Abacus Concepts, Inc.).

CHAPTER I

Activin A and Follistatin Expression in Developing Targets of Ciliary Ganglion Neurons Suggests a Role in Regulating Neurotransmitter Phenotype

Diane C. Darland†, Brian A. Link† and Rae Nishi*

Department of Cell and Developmental Biology, L215
Oregon Health Sciences University
3181 S.W. Sam Jackson Park Road
Portland, Oregon 97201

*Author for correspondence
†Co-first authors

The manuscript that comprises this chapter was co-written with Diane Darland.
The experiments for Figures 1, 2, 3, and 5 were performed by Diane Darland.
The experiments for Figures 4, 6, and 7 were performed by Brian Link.

Summary

The avian ciliary ganglion contains choroid neurons that innervate choroid vasculature and express somatostatin and ciliary neurons that innervate iris-ciliary body, but do not express somatostatin. We have previously shown in culture that activin A induces somatostatin immunoreactivity in both neuron populations. We now show in vivo that both targets contain activin A; however, choroid expressed higher levels of activin A mRNA. In contrast, follistatin, an activin A inhibitor, was higher in iris-ciliary body. Iris cell conditioned medium also contained an activity that inhibited activin A and could be depleted with follistatin antibodies. These results suggest that development of somatostatin is limited to choroid neurons by differential expression of activin A and follistatin in ciliary ganglion targets.

Introduction

The influence of target tissues on the neurotransmitter phenotype of developing neurons has been well established (reviewed in Landis, 1990; Dryer, 1994; Nishi, 1994). For example, in the developing sympathetic nervous system, noradrenergic, sympathetic neurons switch to a cholinergic phenotype upon contact with the sweat gland target cells in the rat footpad (Leblanc and Landis, 1986; Landis, 1994). This transition is regulated by a diffusible target-derived factor (Schotzinger and Landis, 1988; Stevens and Landis, 1990; Habecker et al., 1995). Neural plasticity of a similar type regulated by exogenous molecules has been demonstrated in vitro. Sympathetic neurons have been shown to undergo an adrenergic-to-cholinergic switch when co-cultured with cardiac myocyte-conditioned medium (Furshpan et al., 1976; Weber, 1981; Potter et al., 1986). This effect has been shown to be due to the presence of leukemia inhibitory factor (LIF; Fukada, 1985; Yamamori et al., 1989) in the conditioned medium. Addition of ciliary neurotrophic factor (CNTF) to cultures of sympathetic neurons also induces an adrenergic to cholinergic switch (Saadat et al., 1988; Ernsberger et al., 1989). However, transgenic mice deficient for LIF were found to have normal sweat gland innervation and function (Rao et al., 1993). In addition vasoactive intestinal peptide immunoreactivity and choline acetyltransferase activity in the footpad sweat glands of transgenic mice deficient for CNTF were unchanged compared to wildtype (Masu et al., 1993). These results suggest that these factors are not essential to the regulation of neurotransmitter phenotype in vivo. Although evidence demonstrates that target-derived factors are important to the development of neurotransmitter phenotype, there is limited understanding of the specific molecules that regulate the processes in the developing embryo (reviewed in Patterson and Nawa, 1993).

We have used the avian ciliary ganglion to investigate the effect of target-derived molecules on the regulation of neurotransmitter phenotype. In the ciliary ganglion two distinct neuronal populations innervate separate targets in the eye (Marwitt et al., 1971). The ciliary neurons innervate the striated muscle of the iris and ciliary body to regulate pupilary dilation and lens

accommodation. The choroid neurons innervate the smooth muscle surrounding the arterial vasculature of the choroid layer and aid in oxygenation of the eye. These neuronal populations have the same neural crest derivation (Narayanan and Narayanan, 1978); receive preganglionic input from the accessory oculomotor nucleus (Narayanan and Narayanan, 1976); and synthesize acetylcholine as the primary neurotransmitter (Burt and Narayanan, 1976; Chiapinelli et al., 1976; Coulombe and Bronner-Fraser, 1990). However, only the choroid neurons express the neuromodulatory peptide somatostatin (Epstein et al., 1988; De Stefano et al., 1993), which has been shown to regulate acetylcholine release (Guillemin, 1976; Gray et al., 1990).

Neurons isolated from the ciliary ganglion and maintained in culture have been used to characterize the molecules that regulate somatostatin expression. Previous studies showed that choroid muscle cells will induce somatostatin expression in ciliary ganglion neurons in vitro. The ability of ciliary neurons to express somatostatin in culture was shown by retrograde labeling of the neuron cell bodies before placing them in culture (Coulombe and Nishi, 1991). The choroid cell- derived, somatostatin stimulating activity was shown to be due to activin A (Coulombe et al., 1993).

Activin A has been implicated as a regulator in many different physiological and developmental processes (for review see Ying, 1989). Activin A is a homodimer of the inhibin βA chain (Mason et al., 1985) and was originally described as having a stimulatory effect on the release of follicle stimulating hormone (FSH) from the pituitary gland. The action of activin A in vivo is negatively regulated by follistatin (Ling et al., 1985; Nakamura et al., 1990), an activin A binding protein (Kogawa et al., 1991; Sumitomo et al., 1995). During early embryonic development in Xenopus, the action of the more widely distributed activin A is regulated by localized expression of follistatin (Hemmati-Brivanlou and Melton, 1992; Hemmati-Brivanlou and Melton, 1994a and b). Exogenous activin A has been shown in vitro to promote survival of some neurons (Schubert et al., 1990) and to increase mRNA levels for several neuropeptides in sympathetic neurons (Fann and Patterson, 1994).

We have examined whether the differential expression of activin A and follistatin in targets innervated by ciliary ganglion neurons results in the differential induction of somatostatin expression. Our results suggest that sufficient activin A is available at the choroid layer to induce somatostatin in choroid neurons, but the presence of excess follistatin in the iris prohibits an induction of somatostatin in the ciliary neurons.

Results

Chicken-specific probes for follistatin and activin A

To investigate the expression of activin A and follistatin mRNA, chicken-specific probes were obtained. Cloning of follistatin from several species has shown that there is one gene product (Shimasaki et al., 1988a and b; Albano et al., 1994) that encodes several protein isoforms arising from alternatively spliced mRNA and post-translational modifications (reviewed in Sugino et al., 1993). A cDNA fragment of follistatin was amplified from embryonic chick ovary RNA using reverse transcriptase/polymerase chain reaction (RT/PCR). The primers were designed based on a conserved region of cloned follistatin sequences from other species (Hemmati-Brivanlou and Melton, 1994b; Shimasaki et al., 1988a and b). The PCR product was ligated into the pCR^{TM}II vector and sequenced (Figure 1A). Riboprobes generated from this construct were designed to recognize all mRNA splice variants described. Comparison of the deduced amino acid sequence encoded by this fragment with follistatin proteins from other species revealed a high degree of conservation (Figure 1B). A full length cDNA clone of chick activin A (inhibin βA) was obtained from Dr. Patricia Johnson (Chen and Johnson, 1994, abstract in Biology of Reproduction, Vol. 50 Suppl. 1, 161). The region used for activin A probe synthesis was not homologous to the inhibin α transcript or the inhibin βB transcript.

A

```
  1  TCGTGTGTGG  TGGATCAGAC  TAATAACGCC  TACTGTGTGA  CATGTAATCG   50
 51  AATTTGCCCT  GAGCCTACCT  CCCCTGAGCA  GTATCTCTGT  GGGAATGATG  101
102  GCATAACTTA  CGCCAGTGCC  TGCCACCTGA  GAAAAGCGAC  CTGCCTGCTG  152
153  GGCGAATCCA  TTGGATTAGC  CTACGAGGGA  AAATGCATCA  AAGCGAAGTC  203
204  CTGTGAAGAT  ATTCAGTGCA  GCG
```

B

```
C    S C V V D Q T N N A Y C V T C N R I C P E P T S P E Q Y L C G N D
X    * * * * * * * * * * * * * * * * * * * * * * * * D * * * * * * *
H    T * * * * * * * * * * * * * * * * * * * * * * * A * S * * * * * * *
M    T * * * * * * * * * * * * * * * * * * * * * * * S * S * * * * * * *
P    T * * * * * * * * * * * * * * * * * * * * * * * S * * * * * * * *

C    G I T Y A S A C H L R K A T C L L G E S I G L A Y E G K C I K A K
X    * * * * G * * * N * * * * * * * * * R * * * * * * * * * * * * * *
H    * V * * S * * * * * * * * * * * C * * R * * * * * * * * * * * * *
M    * V * * S * * * * * * * * * * * * * R * * * * * * * * * * T - * *
P    * V * * S * * * * * * * * * * * * * R * * * * * * * * * * * * * *

C    S C E D I Q C S
X    * * * * * * * *
H    * * * * * * * T
M    C * * * * * * G
P    * * * * * * * T
```

Figure 1. Chick Follistatin Fragment Sequence and Amino Acid Comparison with other Species. (A) The 223 bp fragment of chick follistatin was cloned by RT/PCR and sequenced. Primer regions were underlined. (B) A comparison of the amino acid translation of the chick fragment (C) against human (H), pig (P), Xenopus (X) and mouse (M) follistatin showed relative conservation at the amino acid level.

Multiprobe RNase Protection Assay

In order to compare both activin A and follistatin mRNA transcript levels in the same sample, a multiprobe RNase protection assay was used (Qian et al., 1993). In order to determine the sensitivity of this assay, the linear range of detection was determined for each of the probes. Activin A and follistatin mRNA levels were measured in the same sample. Messenger RNA levels of a constitutively expressed gene, chick ribosomal binding protein S17 (CHRPS; Trüeb et al., 1988), were measured as an internal loading control. Total RNA from embryonic day 17 (E17) chick ovary was used to establish multiprobe assay conditions, as both of these proteins are known to be present within this tissue (Mason et al., 1985; Vale et al., 1986). Probe sizes were chosen to prevent overlap in the protected fragments. To demonstrate the sensitivity of this assay, protected bands for activin A, follistatin and CHRPS were detected in RNA from embryonic ovary (Figure 2, left). Unprotected probe (Figure 2, right) showed the predicted size prior to processing. No protected fragments were detected when yeast tRNA was used in place of tissue RNA. Chick genomic DNA was also negative (data not shown). Relative intensity measurements of the signal from the protected fragments were obtained from a scanned phosphorimage. The signal increased with increasing amounts of total RNA and the signals were linear for all three probes tested (Figure 2B). In subsequent experiments we chose conditions from tissues that would yield signals within the linear range of sensitivity of this assay.

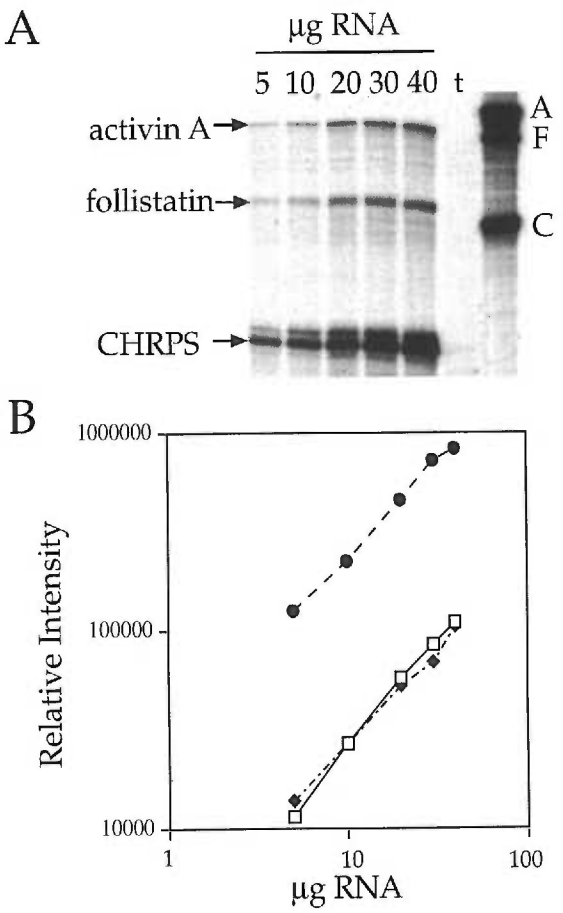


Figure 2. Multiprobe RNase Protection Assay (RPA) Standard Curve. (A) An autoradiograph of 5-40 μg of E17 chick ovary total RNA hybridized with activin A, follistatin and CHRPS probes. All three protected fragments (arrows) were visible in each sample. Unprotected probe (A, F and C) ran higher than the protected fragments. The tRNA negative control (t; 20 μg RNA) had no protected fragments. (B) A logarithmic plot of the scanned phosphorimage showed a linear increase in signal intensity relative to increases in RNA concentration ($r = 0.999$ for activin A; $r = 0.992$ for follistatin and $r = 0.994$ for CHRPS). Symbols represent CHRPS (circles), activin A (squares), follistatin (diamonds). This is a representative experiment from four repetitions which show similar results.

Activin A and Follistatin mRNA In The Developing Iris and Choroid

If activin A and follistatin regulate the expression of somatostatin in ciliary ganglion neurons in vivo, then mRNA for these factors should be detectable in the targets of the ciliary ganglion during development. Embryonic days 9-16 encompass a critical period of development during which both the iris/ciliary body (Pilar et al, 1980; Pilar et al., 1987) and choroid (Meriney and Pilar, 1987) tissues mature as they become innervated. In particular, somatostatin expression increases during this period until all the choroid neurons express the neuropeptide at E14 (Smet and Rush, 1993). RNase protection analyses of RNA from E9-E16 iris and E9-E16 choroid were done in parallel to compare RNA levels for activin A and follistatin (Figure 3A). Analysis of ciliary body mRNA was not performed because isolation of uncontaminated ciliary body separate from neural retina and lens epithelium is difficult to obtain. Follistatin and activin A transcripts in the iris increased from E9-E16 (Figure 3B). In contrast, follistatin mRNA levels in choroid remained low relative to the iris through all ages tested. Activin A mRNA was high from E9-E14, but dropped at E16 (Figure 3C). These results indicate a striking difference in mRNA expression for activin A and follistatin between the two target tissues of the ciliary ganglion neurons. The observations that follistatin mRNA levels in the choroid were lower than those in the iris, while the level of activin A was higher in the choroid than in the iris supports the hypothesis that differential expression of these factors in the two targets leads to a differential expression of somatostatin in the neurons that innervate them.

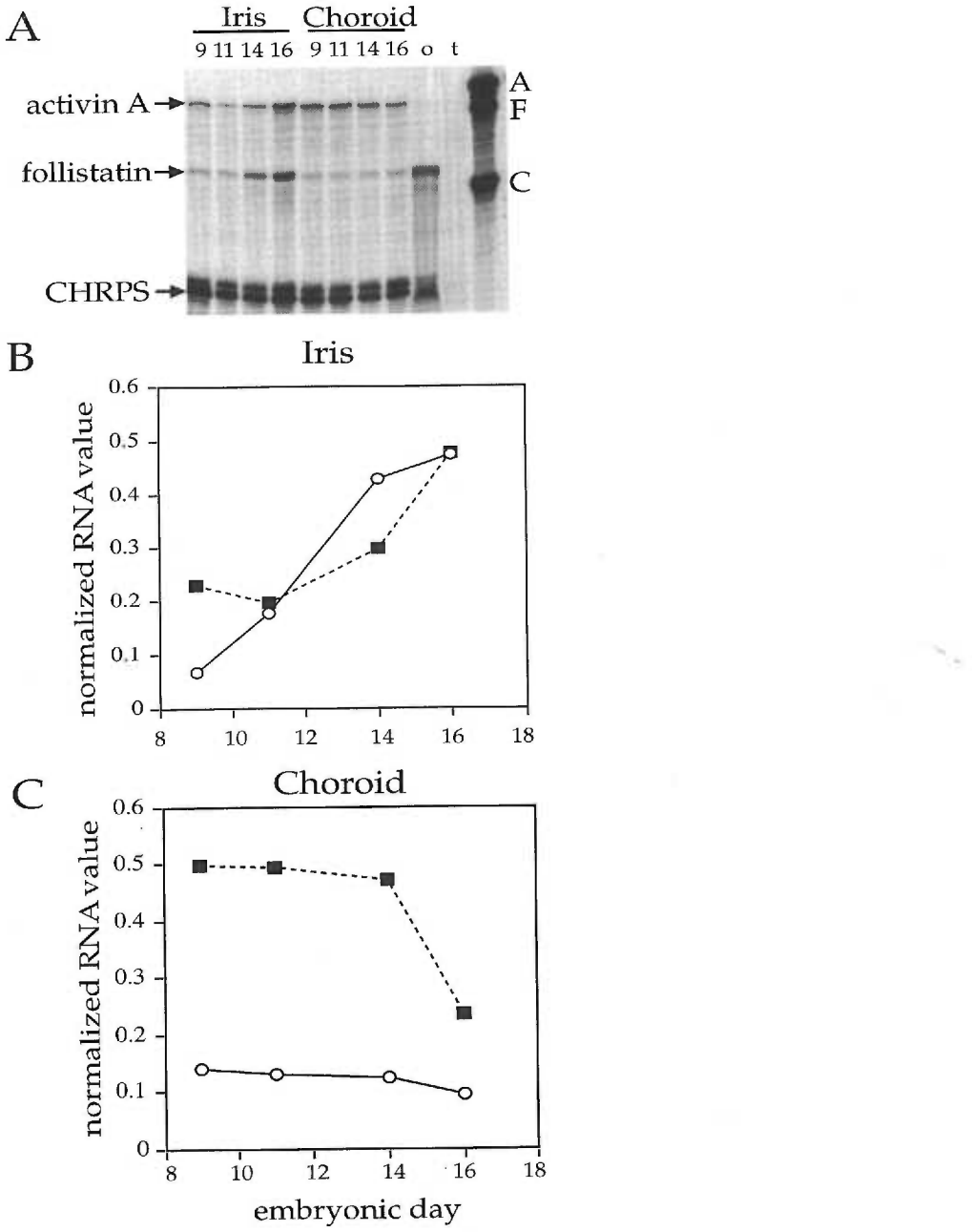


Figure 3. Follistatin and Activin A in Iris and Choroid Tissue during Development. (A) An autoradiograph of 20 μg each of E9-E16 iris and E9-E16 choroid total RNA after protection with activin A, follistatin and CHRPS probes showed all three protected fragments (arrows) were visible in each sample. Unprotected probe (A, F and C) ran slightly higher than the protected fragments. Adult ovary was run as a positive control (o; 20 μg of total RNA). The tRNA negative control (t; 20 μg) had no protected fragments. (B) Normalized RNA values for activin A and follistatin were determined as a ratio of relative signal intensity for each probe over the relative intensity for CHRPS, within the same sample. Follistatin and activin A mRNA in the iris increased from E9-E16. Follistatin in the choroid remained static, while activin A was high from E9-E14 and dropped at E16. Symbols respresent activin A (squares), follistatin (circles). This is a representative experiment from four repetitions showing similar results.

Immunolocalization of Activin A and Follistatin To Target Tissues of Ciliary Ganglion Neurons

To demonstrate that expression of activin A and follistatin protein correlates with their respective mRNA expression patterns, antisera produced against activin A (Vaughan et al., 1989; Kokan-Moore et al., 1991) and follistatin (Sugawara et al., 1990; Petraglia et al., 1994) were used to immunolocalize these proteins to targets of ciliary ganglion neurons. Activin A immunoreactivity was first detected at E11 in the developing ciliary body (Figure 4A) and iris (Figure 4D). In the choroid layer, activin A immunoreactivity could be seen at E12 (Figure 4G), the earliest age at which reliable histology could be obtained. Follistatin immunoreactivity was also observed at E11 in the musculature of the ciliary body (Figure 4B) and the iris stroma (Figure 4E); however, at E12 in the choroid layer follistatin immunoreactivity was not detectable (Figure 4H). Specificity of the activin A and follistatin immunostaining was assessed with normal rabbit serum (Figure 4C, F, and I). In addition preabsorption of the anti-activin A with the peptide used to generate the antiserum displayed no immunoreactivity above background on adjacent sections (data not shown).

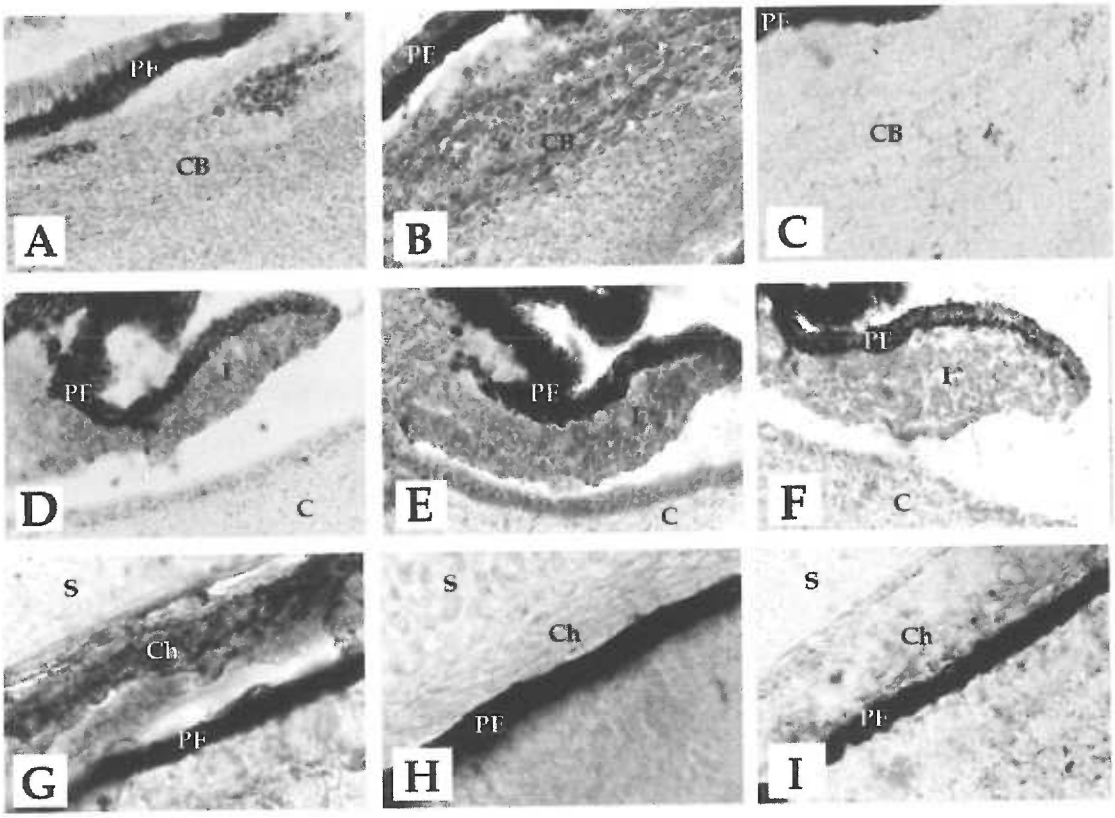


Figure 4. Activin and follistatin immunolocalization in ciliary ganglion neuron targets in vivo.

Cryostat sections of E11 ciliary body (A-C) and iris (D-F) show immunoreactivity for activin A (A and D) and follistatin (B and E). Sections of E12 choroid reveal strong staining for activin (G), but not for follistatin (H). Normal rabbit serum staining was included as a control. Arrows indicate comparable target areas. Pigmented epithelium staining could not be assessed owing to high pigment granule content in these cells. C, cornea; CB, ciliary body; Ch, choid layer; I, iris stroma; PE, pigmented epithelium; S, sclera. Bar, 700 μm (A-F), 450 μm (G-I).

Iris Cell Conditioned Medium Inhibits Somatostatin Induction in Ciliary Ganglion Neurons

The biological role of activin A and follistatin expression in the choroid and iris was investigated using cells derived from these target tissues in a bioassay that measured their effects on somatostatin induction in ciliary ganglion neurons. To confirm that cultured choroid and iris cells expressed activin A and follistatin, levels of mRNA were measured using RNase protection. Follistatin mRNA levels were similar in both iris and choroid cells in culture (Figure 5). Activin A was approximately four-fold higher in the choroid cells relative to the iris cells. These relative mRNA levels for activin A and follistatin were similar to the ratios seen at E11 in the targets in vivo (Figure 4). Synthesis of activin A and follistatin protein was assessed using immunocytochemistry (Figure 6). Activin A immunoreactivity was found as punctate perinuclear staining in multiple cell types of iris cultures (Figure 6A). Similar punctate staining was found in choroid smooth muscle cells (Figure 6D). Follistatin immunoreactivity was most dramatic in iris culture myotubes (Figure 6B), but was weak in choroid smooth muscle cells (Figure 6E). As a control, choroid and iris cultures were stained after replacing the primary antiserum with normal rabbit serum (Figure 6C and F).

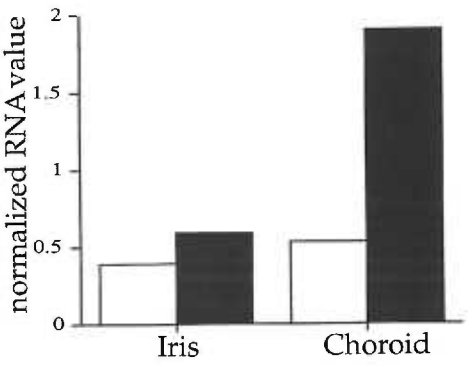


Figure 5. Follistatin and Activin A in Iris and Choroid Cells in 6 day old Cultures. 10 μg of total RNA from cells in culture were hybridized to activin A, follistatin and CHRPS probes. The histogram of the normalized RNA values from this protection are shown for activin A (filled columns) and follistatin (open columns). RNA values were obtained using the ratio of relative intensity of probe signal to the relative intensity of the loading control, CHRPS. The follistatin RNA value was similar in the iris and choroid cells in culture. In contrast the activin A value was approximately four fold higher in the choroid cells than in the iris cells. One representative experiment out of four repetitions with similar results is shown.

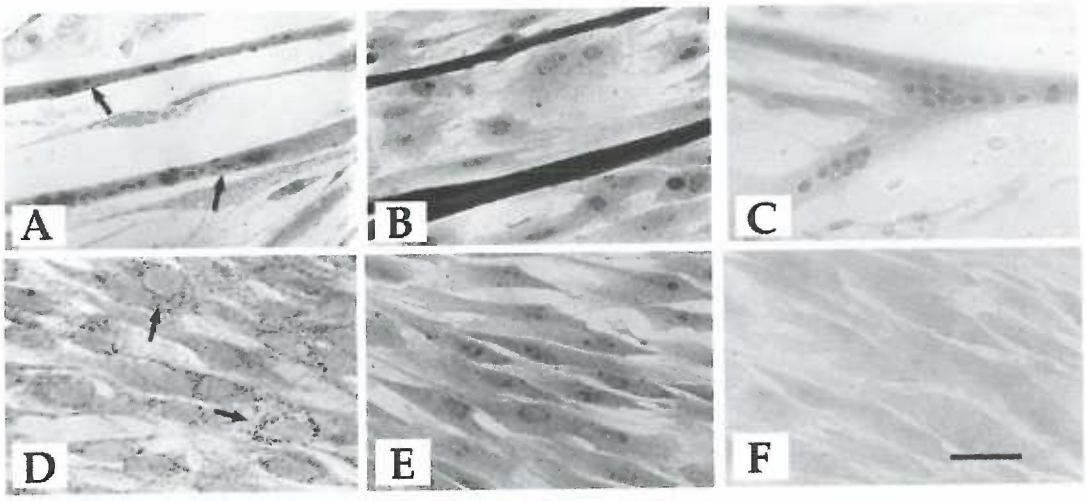


Figure 6. Activin and follistatin immunoreactivity in 6-day-old choroid and iris cultures.

Iris cultures (A-C) show punctate, perinuclear staining for activin A in multiple cell types, including multi-nucleated myotubes (A, arrows). Choroid culutres (D-F) reveal similar punctate patterns (D arrows). Follistatin immunoreactivity (B and E) is most dramatic in iris-derived myotubes (B, arrows). Normal rabbit serum was included as a control (C and F). Nuclei in myotubes showed non-specific staining as assessed with the normal rabbit serum (C, arrows), while choroid cells were negative (F, arrows). Bar, 75 μm.

To determine the ability of iris cell conditioned medium to inhibit the induction of somatostatin, we used the same bioassay that was previously used to identify activin A as an inducer of somatostatin expression in ciliary ganglion neurons (Coulombe et al., 1993). Serum-free conditioned medium from iris-derived cultures (iris conditioned medium, ICM) which contained myotubes was concentrated and added to E8 ciliary ganglion neurons cultured in the presence or absence of 10 ng/ml recombinant activin A. This dose of activin A was previously demonstrated to be within the linear range for somatostatin induction (Coulombe et al., 1993). ICM significantly reduced ($p<0.05$) the percentage of neurons that express somatostatin immunoreactivity when compared to neurons grown in control medium (Figure 7A). To investigate whether this inhibition of somatostatin immunoreactivity was mediated by the secretion of follistatin by iris cells, ICM was immunodepleted with a follistatin antiserum (Sugawara et al., 1990). Immunodepletion significantly restored somatostatin induction in the presence of activin A ($p<0.05$ as compared to ICM treatment). Immunodepleted ICM in the absence of activin A did not stimulate somatostatin induction, suggesting that activin A may be bound to follistatin and removed with follistatin immunodepletion. Quantitation of activin A and follistatin protein levels in ICM was not analyzed. To control for deleterious effects of ICM on ciliary ganglion neurons, neuronal survival and choline acetyltransferase (ChAT) activity were assayed. Neither survival nor ChAT activity were reduced in the presence of ICM (Figure 7B and C). The bioassay results, together with the different expression patterns of activin A and follistatin, support our hypothesis that activin A signaling in the iris is selectively inhibited by follistatin protein and regulates somatostatin phenotype in ciliary ganglion neurons.

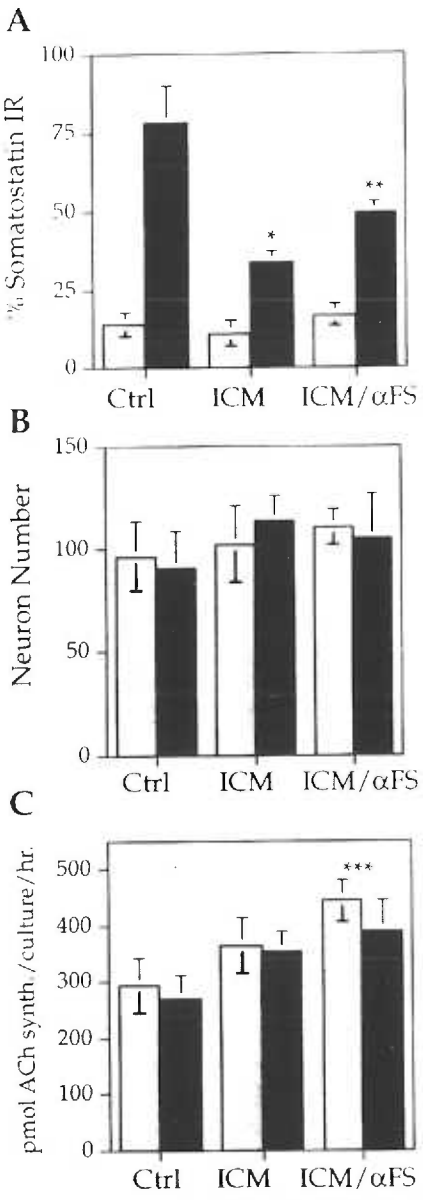


Figure 7. Somatostatin Induction, Neuron Survival, and ChAT Activity in Cultured Ciliary Ganglion Neurons

Percentage of somatostatin immunoreactivity (A), neuron survival (B), and ChAT activity (C) were measured in ciliary ganglion neurons cultured for 4 days in control medium (Ctrl), iris cell–conditioned medium (ICM), or ICM that had been immunodepleted with an antibody specific for follistatin (ICM/αFS). Ciliary ganglion neuron cultures from each experimental condition were maintained with 10 ng/ml activin A (closed bars) or without activin A (gray bars). The values indicated for each column are the mean of three different cultures from one representative experiment. Error bars represent SD. Single asterisk, significantly differs from control with activin A; double asterisk, significantly differs from ICM with activin A; triple asterisk, ICM/αFS with and without activin ChAT activity significantly differs from control with or without activin A. Significance at $p < .05$ by one-way ANOVA

Discussion

In the current study we have investigated the in vivo distribution of activin A and follistatin and the in vitro potential of iris conditioned medium to modulate somatostatin induction. Previous work from our laboratory has shown that activin A from choroid conditioned medium induces somatostatin expression in ciliary ganglion neurons (Coulombe et al., 1993). Using an RNase protection assay we have now demonstrated that activin A mRNA is present in both target tissues of the ciliary ganglion neurons, but is higher in the choroid than the iris. In contrast follistatin mRNA is higher in the iris. In agreement with these results, immunostaining for activin A and follistatin indicates that activin A is present in both choroid and iris, while follistatin is detectable only in the iris during the period of somatostatin induction. Moreover, the immunohistochemical results have localized both proteins to the iris/ciliary body and the choroid layer, regions which contain the target cells for ciliary and choroid neurons. As previously noted iris- and choroid-derived cells in culture produce factors that can influence transmitter phenotype. Choroid conditioned medium induces somatostatin and contains activin A, which alone can induce somatostatin (Coulombe et al, 1993). We have now shown that iris conditioned medium inhibits somatostatin induction and that this inhibition is significantly relieved by immunodepletion of the conditioned medium with follistatin antibodies. A model that accounts for these findings is that activin A produced in the choroid layer induces somatostatin in the choroid neurons, but activin A signaling in the iris and ciliary body is blocked by local production of follistatin.

Several additional observations led to other areas of inquiry. The increasing expression of activin A and follistatin in the iris correlates with increasing neuronal innervation in this target tissue. Although these studies do not address whether innervation may regulate activin A and follistatin expression, such regulation of target-derived factors has been demonstrated in sympathetic innervation of rodent sweat glands. Production of sweat gland factor activity which induces the noradrenergic-to-cholinergic switch is dependent on proper innervation (Habecker and Landis, 1994; Habecker et al.,

1995). While cultures of choroid and iris cells produce activin A and follistatin independently of innervation in vitro (Figures 5 and 6), the sources of these cultures are innervated at the time of removal. In addition there is a decrease in activin A mRNA levels seen consistently at E16 in the choroid (Figure 3). The dependence of ciliary ganglion neurons on activin A for maintenance of somatostatin expression has been shown up to E14 in vitro (Coulombe and Nishi, 1991). Potentially, this decrease at E16 reflects a loss of dependence on activin A once the adult neurotransmitter phenotype has been established. Finally, ChAT activity consistently increased in ciliary ganglion cultures treated with ICM or ICM which had been depleted with follistatin antibodies (Figure 7C), indicating that follistatin is not regulating ChAT activity. Although a target-derived ChAT induction in ciliary ganglion neurons has been demonstrated previously (Nishi and Berg, 1979; Tuttle et al., 1983), factors that affect ChAT activity remain to be purified.

The identification of follistatin transcripts and immunoreactivity in the developing iris is complemented by the appropriate biological activity in iris conditioned medium observed in the somatostatin induction assay. The significant but partial relief of inhibition of somatostatin induction by follistatin immunodepleted ICM (Figure 7A) demonstrates the presence of follistatin. The lack of complete relief of inhibition could be explained by several alternatives. One plausible explanation is that the antibodies used for the immunodepletion were generated against porcine follistatin, thus they may not be able to recognize all the possible isoforms of chicken follistatin. Another possibility is that other inhibitors of somatostatin induction may be present in the ICM. Interestingly, we do not see induction of somatostatin in immunodepleted ICM in the absence of exogenous activin A. This may indicate that secreted activin A is associated with follistatin and is removed upon immunodepletion of ICM with follistatin-specific antibodies. This possibility is consistent with our model of iris-derived follistatin as an inhibitor of somatostatin induction in ciliary neurons.

Activin A and follistatin may not be the only regulators of somatostatin expression. Activin A signals through a receptor serine/threonine kinase complex expressed in several isoforms (reveiwed in Vale et al., 1991; Mathews, 1994). Regulation may occur through expression of different levels

or isoforms of either the Type I or Type II receptors on the choroid and ciliary neurons. Post-translational processing has also been shown to regulate activity of several members of the TGFb superfamily (reviewed in Kingsley, 1994), and such processing may occur for activin A in this system by regulating its availability in an active form at the synapse. The inhibins, which share a common subunit with the activins, have been shown to block activin A function in some systems (Yu et al., 1987; Ying, 1989). However, inhibin βA was unable to affect somatostatin expression in our in vitro assay, suggesting that it does not function in the establishment of neurotransmitter phenotype in the ciliary ganglion (Coulombe et al., 1993). Our activin A reagents are specific for inhibin βA, but do not distinguish among family members that contain the common βA sub-unit. Thus our results do not rule out a role for other βA-containing proteins. Interestingly, the partial inhibition seen with follistatin-immunodepleted ICM (Figure 7A) suggests other factors, in addition to follistatin, may act to inhibit somatostatin expression. Finally, other molecules may be required to make cells competent to respond to activin signaling. Such a requirement has been demonstrated in the fibroblast growth factor-dependent, activin mediated induction of mesoderm in early Xenopus development (Cornell and Kimelman, 1994; LaBonne and Whitman, 1994).

Although these possibilities are intriguing, we do not think they detract from the model we propose based on the results of this study: that limited expression of somatostatin in the neurons of the ciliary ganglion results from a differential expression of activin A and its inhibitory binding protein, follistatin, in the distinct targets of these neurons. Our results show a unique role for the antagonistic functions of endogenous activin A and follistatin in the regulation of neurotransmitter phenotype during development. This study bridges the gap between an in vitro demonstration of neurotransmitter phenotype regulation and the appropriate temporal and spacial in vivo expression of the molecules.

Acknowledgments

The first two authors were equal contributors to this paper. The authors would like to thank Dr. Patricia Johnson for providing us with the full length chick inhibin βA cDNA prior to publication of the sequence. We would also like to thank Dr. Shunichi Shimasaki for the follistatin antiserum and Dr. Wylie Vale for generously providing us with activin A antiserum and peptide. We gratefully acknowledge technical advice from Dr. Miles Wilkinson and Tom Finn. We would also like to thank Dr. Jan Christian, Dr. Phil Copenhaver, Dr. John Fredieu, Dr. Gary Reiness and the members of the Nishi and Eckenstein labs for helpful discussions and for reading the manuscript. This work was supported by 5T32EY07123 (DD and BL) and by 5RO1NS25767 (RN).

CHAPTER II

Opposing Effects of Activin A and Follistatin on Developing Skeletal Muscle Cells

Brian A. Link* and Rae Nishi.

Department of Cell and Developmental Biology, L215
Oregon Health Sciences University
3181 SW Sam Jackson Park Rd.
Portland, Oregon 97201

*To whom correspondence should be addressed.

ABSTRACT

Activin and the activin-binding protein follistatin modulate a variety of biological processes and are abundant at sites of muscle development. Activin and follistatin were expressed in developing chick pectoral muscle in vivo and in primary cell culture. Addition of recombinant activin inhibited muscle development in a dose-dependent manner as measured by the number of nuclei in myosin heavy chain positive cells and creatine phosphokinase activity. Conversely, follistatin potentiated muscle development. The effects of activin were found to be distinct from those of the related protein transforming growth factor (TGF) β1. Muscle development was repressed by activin at all time points investigated and did not recover with the removal of activin following a limited exposure. In contrast, while myogenic differentiation in TGFβ1 was initially repressed, muscle marker expression recovered to control levels - even in the continued presence of TGFβ1. Fibroblast growth factor (FGF) had little effect on inhibiton of muscle development caused by activin A. However, inhibition of development produced by TGFβ increased with increasing concentration of FGF. Finally, early expression of myoD and myf5 mRNA by muscle cultures in the presence of activin and follistatin was analyzed. Activin treated cultures expressed reduced myoD and myf5 levels at 1.5 days after plating. Myf5 levels in follistatin treated cultures were elevated, but, surprisingly these cultures showed a reduction in myoD levels. These data suggest that endogenously expressed activin and follistatin are important modulators of muscle development.

INTRODUCTION

The development of skeletal muscle is characterized by a series of defined cellular events. First, mesodermal cells forming the somite become myoblasts, with axial and back musculature arising from the dorsal medial portion of the somite (myotome), while limb and ventral body musculature form from the lateral half of the somite (dermamyotome) (reviewed in Emerson et al., 1993; Cossu et al., 1996). Dividing myoblasts then undergo terminal differentiation to myocytes by withdrawing from the mitotic cycle and expressing contractile proteins characteristic of skeletal muscle (Holtzer et al., 1957; Cusella-DeAngelis et al., 1992). Finally, myocytes align and fuse, forming multinucleated myotubes. Functional muscle groups are sculpted by sequential myofiber formation. Primary fibers form from the first wave of myoblasts to arrive at the pre-muscle mass, while secondary fibers composed of late migrating myoblasts develop around the primary fibers. The precise mechanisms that coordinate muscle differentiation remain elusive.

A number of molecules that are involved in differentiation of skeletal muscle have been identified. The muscle regulatory factors myoD, myf5, myogenin, and MRF4 are a family of basic helix-loop-helix transcription factors that convert non-muscle cells in culture to the muscle lineage (Davis et al., 1987; Wright et al., 1989). Other transcription factors such as pax3 (Goulding et al., 1994; Williams and Ordahl, 1994; Epstein et al., 1995) and msx-1 (Hill et al., 1989; Robert et al., 1991; Song et al., 1992; Woloshin et al., 1995) are important in delaying terminal differentiation. A variety of cytokines, soluble molecules released by cells which function by activating extracellular receptors, have also been implicated in controlling muscle development. These include wnts, which regulate early determination of muscle (Takada et al., 1994; Stern et al., 1995a; Münsterberg et al., 1995); fibroblast growth factors (FGFs); insulin-like growth factors (IGFs), and transforming growth factor bs (TGFβs). A diversity of effects have been observed with FGF and TGFβ. For example, in muscle cell lines derived from adult rodents, FGF and TGFβ have been shown to inhibit muscle differentiation (Florini et al., 1986; Vaidya et al., 1989; Li et al., 1992; Olwin and Rapraeger, 1992). However, in the presence of mitogens, TGFβ rapidly

induces terminal differentiation of these muscle cells (Zentella and Massagué, 1992). FGF has been shown to delay, but not prevent the differentiation of myoblasts derived from embryonic chickens (Seed and Hauschka, 1988) and in vivo can stimulate proliferation while maintaining patterning activity in the developing limb bud (Niswander and Martin, 1993; Fallon et al., 1994). Finally, insulin-like growth factors (IGFs) potentiate muscle maturation by increasing the number and size of myotubes and enhancing muscle specific gene expression (Schmid et al., 1983; Vandenburgh et al., 1991; Coleman et al., 1995).

Activins and their receptors, as well as the activin-binding protein follistatin, are abundant at sites of muscle determination and differentiation. Activins form a subgroup of the TGFβ superfamily (Kingsley et al., 1994) and are secreted homodimer proteins comprised of disulfide linked βA or βB subunits (Mason et al., 1985). Follistatin is a secreted monomeric protein not homologous to the activins (Ueno et al., 1987; Michel et al., 1993). Although originally identified based on their ability to regulate the release of follicle-stimulating hormone from the pituitary, the activins and follistatin have been suggested to be important modulators of developmental decisions for many cell types (reviewed in Ying, 1989). Activins are thought to signal through high affinity binding to transmembrane activin type I and type II receptor complexes which induce serine/threonine kinase activity at the intracellular domain of the receptor complex (Mathews, 1994). Follistatin has been shown to antagonize activin signaling by binding to the activin β subunits (Shimonaka et al., 1991). Transcripts for activin βA and βB, the activin receptors type I, IB, IIA and IIB, and follistatin are all expressed in differentiating skeletal muscle groups (Ohuchi et al., 1992; Nohno et al., 1993; Tuuri et al., 1994; Verschueren et al., 1995). Earlier, in the developing somites, mRNA for follistatin and the activin type II receptors is expressed, but interestingly neither activin βA nor βB messages have been detected within these structures (Albano et al., 1994; Feijen et al., 1994; Stern et al., 1995b).

We hypothesized that endogenously expressed activin and follistatin are important regulators of normal skeletal muscle differentiation. In order to test this hypothesis, we have used primary embryonic chick pectoral muscle. We have characerized the temporal expression of activin A and follistatin protein in vivo and the effects of exogenous application of these factors on various stages of pectoral muscle development in culture. The effects of activin A and follistatin are compared to two other cytokines previously reported to modulate muscle developent, FGF-2 and TGFβ1.

RESULTS

Cells from the developing pectoral muscle express activin and follistatin.

We used immunohistochemistry to ask whether activin and follistatin are found within the developing chick pectoral muscle. Immunolocalization with antisera generated against an activin βA specific peptide (Vaughan et al., 1989) or purified follistatin (Sugawara et al., 1990) demonstrates that both of these secreted proteins are present in the developing pectoral muscle. At E6, some myoblasts within the pectoral premuscle mass begin to express sarcomeric myosin heavy chain (MyHC) (Figure 1A). Activin A immunoreactivity is diffuse and throughout the developing muscle (Figure 1G), while follistatin immunoreactivity co-localizes with MyHC posititve cells (arrows, Figure 1A and 1D). At E11, prior to myofiber formation, activin A immunoreactivity is lower (Figure 1H) and follistatin-positive cells strongly co-localize with MyHC-positive cells (arrows, Figure 1B and 1E). By E18, myofibers have formed bundles and both activin A and follistatin immunoreactivity are downregulated (Figure 1I and 1F).

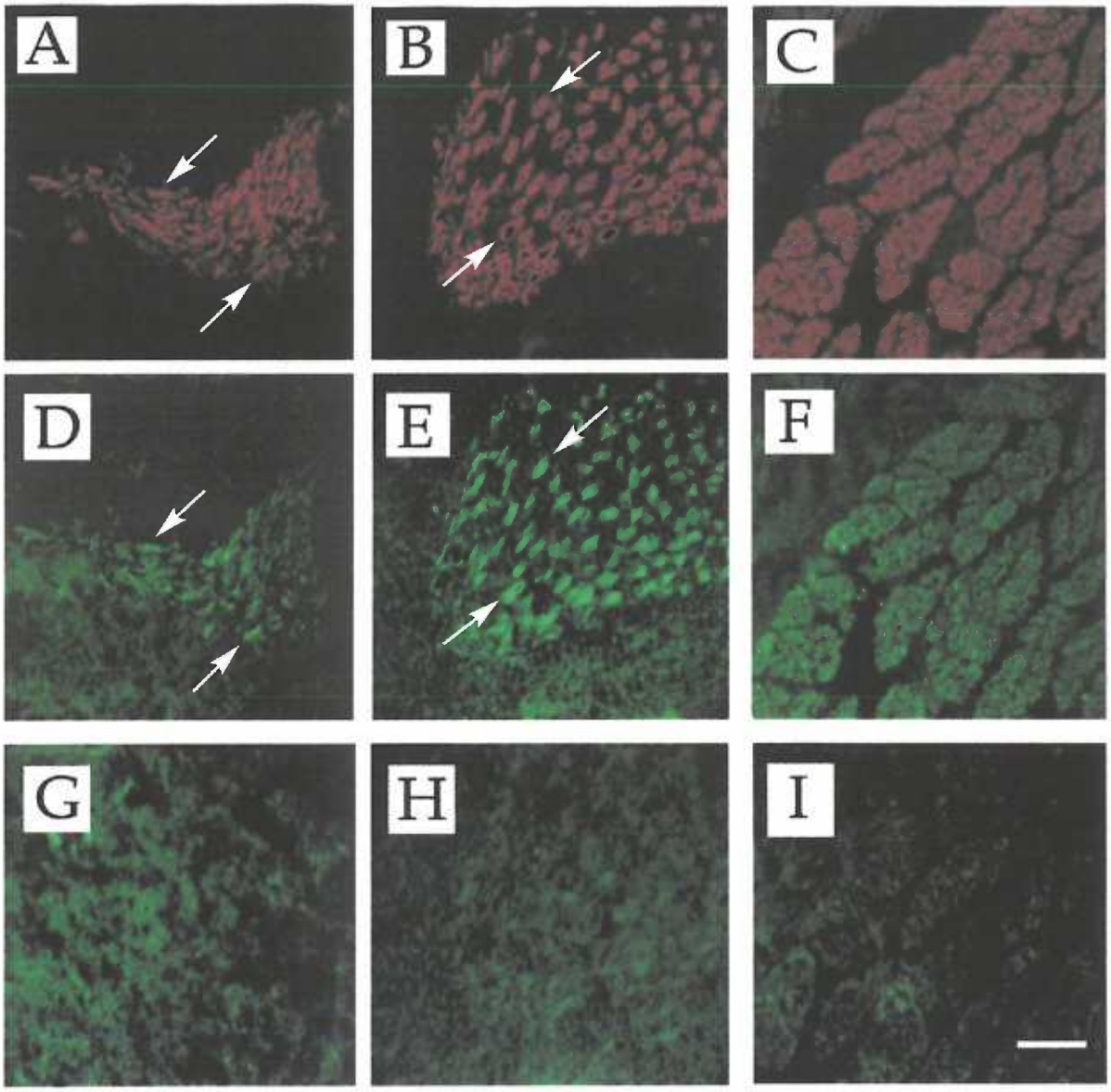


FIG.1. In vivo expression of activin and follistatin immunoreactivity in developing pectoral muscles. MyHC immunoreactivity (A, B, C), follistatin immunoreactivity (D, E, F), and activin A immunoreactivity (G, H, I) in embryonic day (E) 6 (A, D, G), E11 (B, E, H), and E18 (C, F, I) in cross-sectioned chick pectoral muscles. Adjacent sections were double-labeled for either MyHC and follistatin of MyHC and activin. Note the strong expression of follistatin by myocytes (MyHC+ cells) prior to muscle fiber formation (arrows A, B, D, E). Bar represents 150 μm.

RNAse protection assay and immunocytochemistry were employed to address whether activin and follistatin mRNA and protein are expressed in differentiating chick E11 pectoral muscle cells in culture. Transcripts for the activin βA subunit and follistatin were detected in cultures consisting primarily of unfused cells (1 day cultures, Figure 2, lane 4) as well as cultures composed primarily of myotubes (4 day cultures, Figure 2, lane 5). Immunolocalization demonstrates that both of these secreted proteins are translated and found in pectoral muscle cultures. The presence of both activin and follistatin, which are co-expressed in other tissues, suggests that a regulated balance of these proteins control activin signaling. Activin A immunoreactivity was strongest in unfused, spindle shaped cells from 1 day cultures. Punctate staining appeared throughout the cytoplasm, but was concentrated around the nucleus (arrow, Figure 3A). In 4 day cultures, activin A immunoreactivity was found in association with myotubes, but punctate intracellular staining was not observed, suggesting a decrease in the synthesis of activin with myocyte fusion (Figure 3B). Follistatin immunoreactiviy was intense in spindle shaped muscle cells from 1 day cultures (Figure 3C) as well as myotubes from 4 day cultures (Figure 3D). We were unable to discern whether follistatin immunoreactivity was intracellular or associated with the cell surface using conventional light microscopy. Normal rabbit serum showed low background staining (Figure 3E,F).

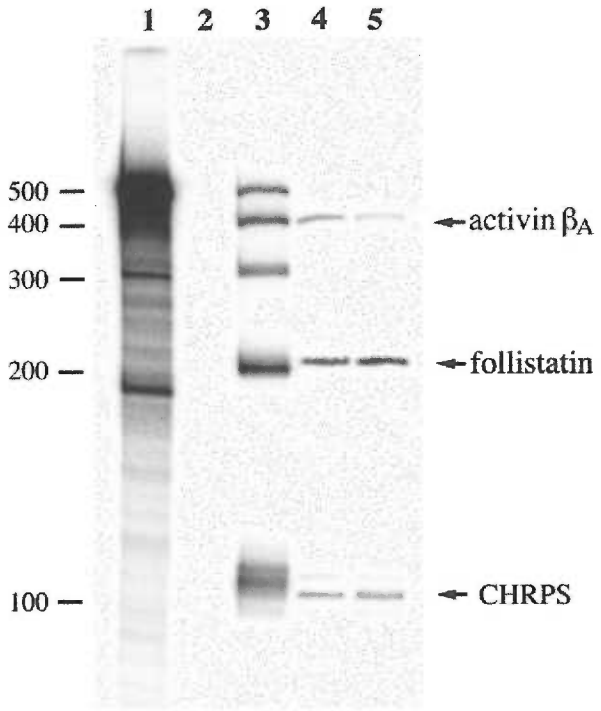


FIG. 2. Expression of mRNA for activin and follistatin in developing muscle cultures. RNase protection assays were performed using cRNA probes specific for chicken activin βA, follistatin, and ribosomal protein S17 (arrows) with 25 μg total RNA from E11 pectoral muscle cultures at 1 day (lane 4) and 4 days (lane 5). Undigested probes (lane 1) and molecular weight standards (lane 3) are included.

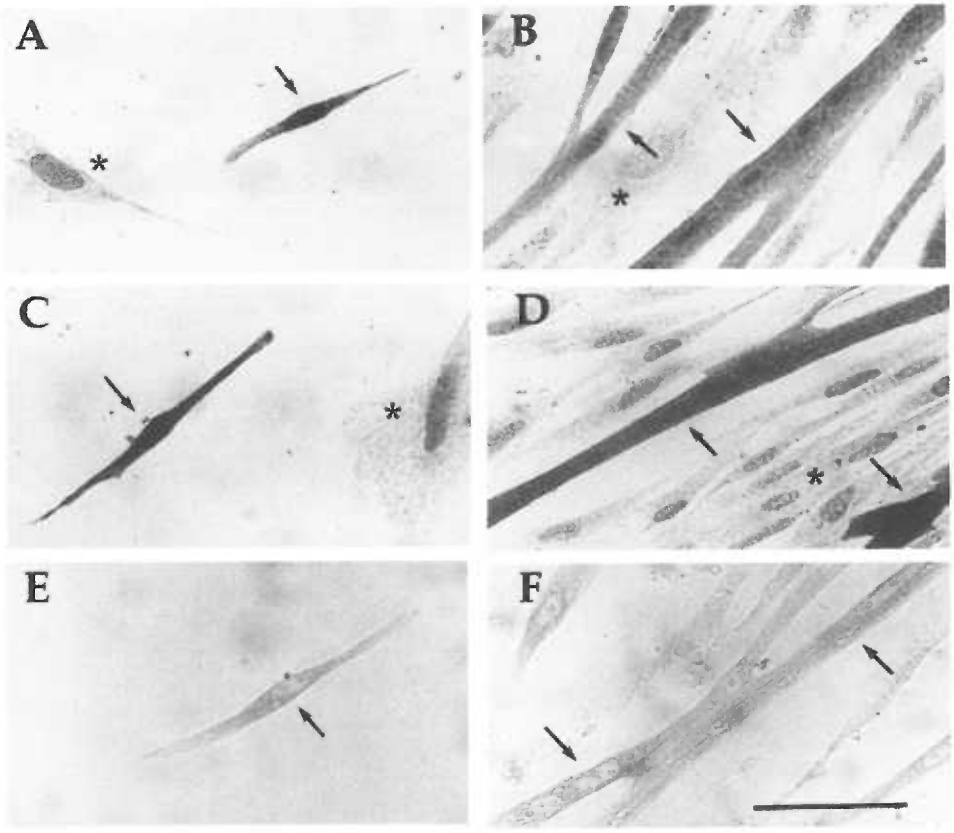


FIG. 3. Expression of activin and follistatin immunoreactivity in developing muscle cultures. Immunocytochemistry in 1-day (A, C, E) and 4-day (B, D, F) cultures using antisera against activin A (arrows, A and C), follistatin, (arrows, B and D), or normal rabbit serum (NRS; arrows, E and F) as control. Non-immunoreactive fibroblasts are labled with asterisks. Bar represents 60 μm.

Activin represses, while follistatin potentiates muscle development in culture.

The effects of activin and follistatin on muscle development were quantified by following two markers of differentiation: the number of nuclei found in MyHC immunoreactive cells and muscle creatine phosphokinase (CPK) activity. In chick pectoral muscle cultures MyHC immunoreactivity is found in both single and multinucleated muscle cells as early as 6 hours after cell cycle withdrawal, but not prior to terminal differentiation (Lin et al., 1994). Therefore, the number of nuclei found within MyHC immunoreactive cells represents the number of myoblasts that proceeded to terminal differentiation and became myocytes. CPK activity has been shown to increase markedly with myotube formation and with expression of other muscle markers such as contractile protiens and nicotinic acetyl choline receptors (Shainberg et al., 1971; Olson et al., 1983); thus, CPK activity is an index of myotube maturation.

Recombinant activin A was found to inhibit the increase in CPK activity in a dosage dependent fashion (Figure 4). The total number of nuclei in MyHC immunoreactive cells was also significantly decreased, along with the ratio of large myotubes found within activin treated cultures (Table 1). In contrast to activin, addition of increasing amounts of follistatin potentiated CPK activity and increased the total number of nuclei found in MyHC immunoreactive cells (Figure 4, Table 1). The distribution of muscle cells in follistatin treated cultures showed an increase in the number of myocytes as well as an increase in the number of nuclei found in large myotubes. The concentrations of activin and follistatin used in this study are similar to those used to experimentally manipulate differentiation in other systems (Hashimoto et al., 1992; Cornell and Kimelman, 1994; Pituello et al., 1995).

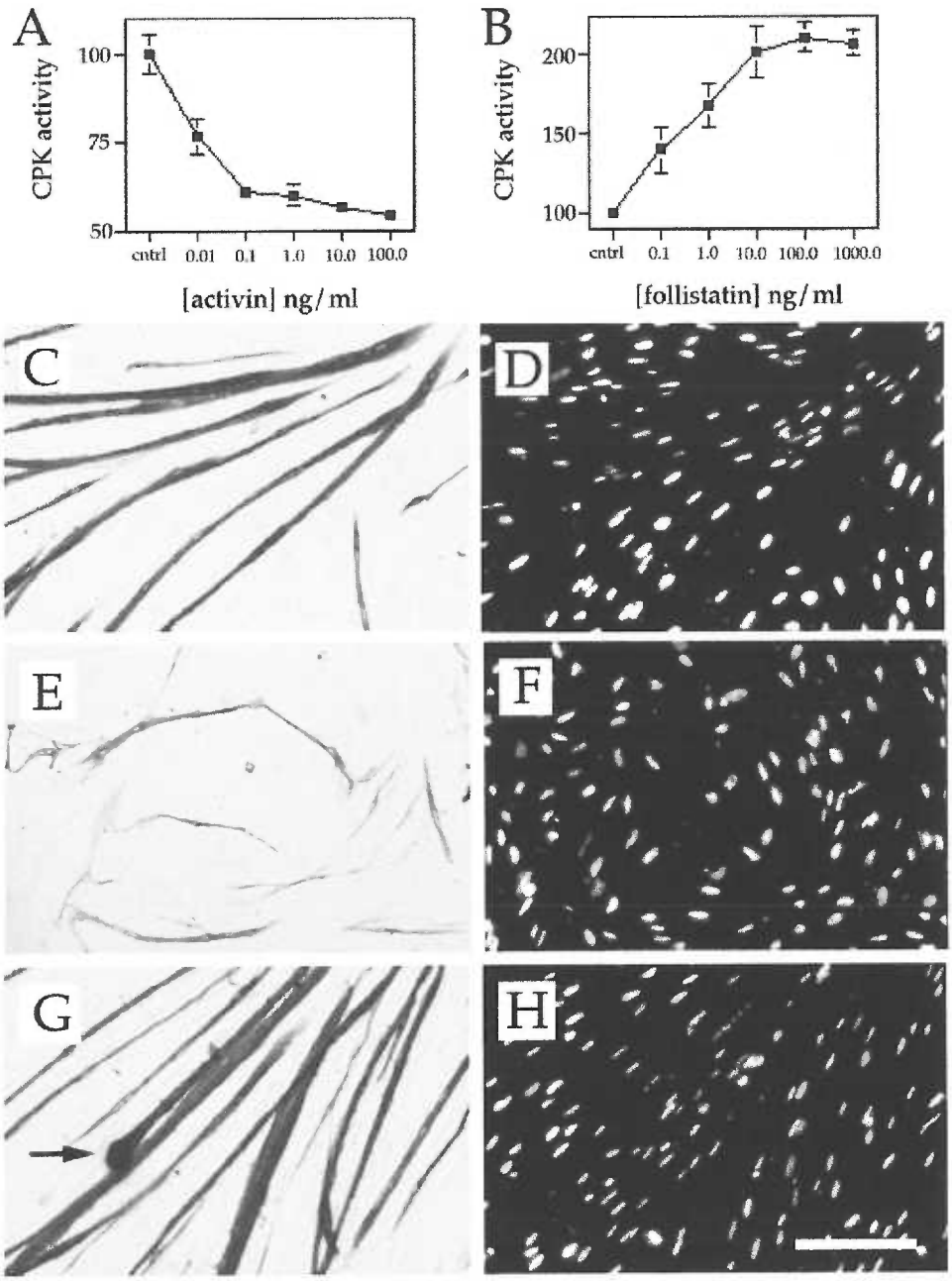


FIG. 4. Effects of activin and follistatin on muscle development. CPK activity from E11 pectoral muscle cultures treated with varying concentrations of either activin (A) or follistatin (B) for 4 days. CPK activity for each condition is graphed as the percentage of CPK for each experimental condition from one representative experiment. MyHC IR (C, E, G) and acridine orange nuclear staining (D, F, H) from 4-day control (C and D), 5 ng/ml activin-treated (E and F), and 35 ng/ml follistatin (G and H). Arrow in G indicated where a myotube has pulled away from the substratum. Bar represents 100 μm.

TABLE 1

Distribution of Nuclei per Myotube[a]

Culture condition[b]	Number of MyHC+ cells			
	Myocytes (1 nucleus)	Small myotubes (2–4 nuclei)	Large myotubes (≥5 nuclei)	Total nuclei in MyHC+ cells
Control	30.7 ± 3.3	20.0 ± 4.6	28 ± 3.6	288 ± 5.2
+ Activin	32.3 ± 3.8	11.7 ± 1.2	8.0 ± 2.5*	117.7 ± 26.8*
+ Follistatin	40.3 ± 7.5	12.7 ± 3.5	32.0 ± 3.5	335.3 ± 32.2*

[a] Data obtained from 3-day E11 chick pectoral muscle cultures. Values for myocytes (1 nucleus), small myotubes (2–4 nuclei), and large myotubes (≥5 nuclei) represent number of MyHC-immunoreactive cells with the corresponding number of nuclei. Total nuclei represent the number of nuclei found within all MyHC-immunoreactive cells per condition. Data from each condition represent means ± standard error for three cultures from one representative experiment. *Significance at $P < 0.05$ versus control, Scheffe's F test, repeated measure ANOVA.

[b] Control cultures grown without cytokine addition. Activin and follistatin cultures were grown with 5 and 35 ng/ml, respectively.

Morphologically, myoblasts which differentiated in the presence of 5 ng/ml activin developed as thin myotubes with fewer nuclei (Figure 4E, Table 1). In addition, myotubes from control conditions were often aligned in parallel to each other, while myotubes from activin treated conditions lacked parallel alignment and were at random angles to one another (Figure 4C). Cultures grown with follistatin had thicker myotubes which were aligned in parallel, but often contained greater MyHC immunoreactivity compared to controls (Figure 4G). In addition, after 4 days in follistatin, many more myotubes had begun to pull off the substratum (arrow, Figure 4G). Interestingly, the changes observed with activin and follistatin treatment were affected by the plating density - increasing the starting cell number reduced the response of the cultures to activin or follistatin (data not shown).

To test wheter the effects of activin or follistatin on muscle development were sustained in cell culture, we performed time course experiments. In control cultures, CPK activity and the number of nuclei in MyHC immunoreactive cells increased until day six, indicating the maintenance of proliferating myoblasts during this period. After eight days, increases in CPK activity had leveled (Figure 5A). Exogenous activin was able to suppress both CPK activity and the number of nuclei in MyHC cells throughout the time course (Figure 5B). Total nuclei number in activin treated cultures did not differ significantly from control (Scheffe's F-test, repeated measure ANOVA) at any time point and no obvious differences in cell death were detected by acridine orange labeling of condensed nuclei. Importantly, both CPK activity and the number of nuclei within MyHC positive cells were found to increase, although at reduced levels, in the presence of activin at all time points examined. This small increase in the number of nuclei within MyHC positive cells demonstrates that some myoblasts continue to differentiate into myocytes in the presence of activin.

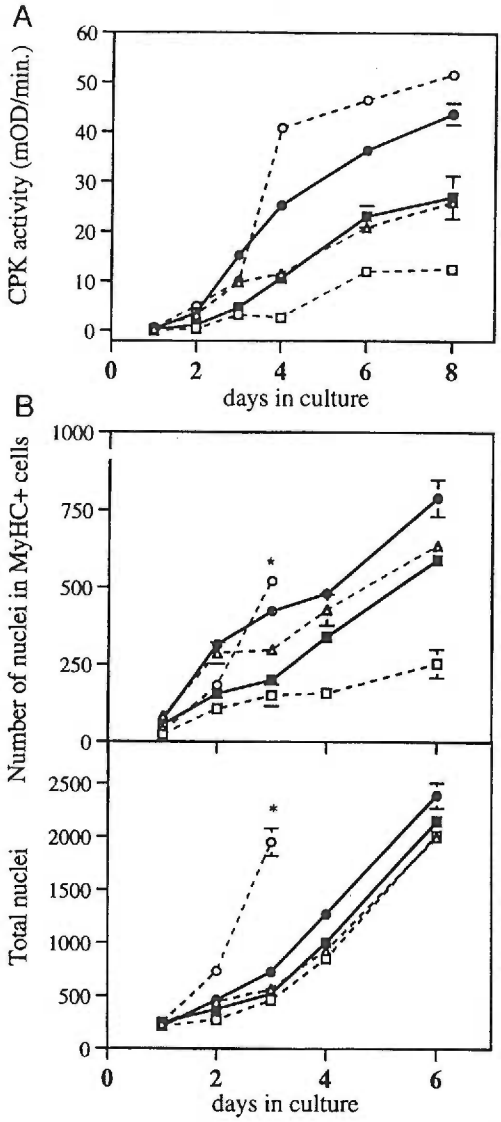


FIG. 5. Time course of muscle development in culture: comparison of activin and follistatin with FGF and TGFβ. Response of CPK activity (A), MyHC expression, as assessed by the number of nuclei found within MyHC-immunoreactive cells (B, upper), and total number of nuclei (B, lower) in E11 pectoral muscle cell cultures in control medium (open triangles), 5 ng/ml activin A (open squares), 35 ng/ml follistatin (filled circles), 5 ng/ml TGFβ1 (filled squares), or 5 ng/ml FGF 2 (open circles). Data points represent means ± standard error from three wells in one representative experiment. Data from each assay obtained from sister cultures from one representative experiment. Data from (B upper and lower) taken from the same cultures, double labeled for MyHC and acridine orange. *MyHC expression and total nuclei not determined in FGF 2-treated cultures after 3 days. Note: MyHC expression and total nuclei determined to Day 6, at which time CPK activity had leveled.

Throughout the time course, muscle cultures with added follistatin showed elevated CPK activity and increased total number of nuclei within MyHC positive cells relative to untreated cultures. In addition, the total cell number after 3 days was significantly ($p \leq 0.05$, Scheffe's F-test, repeated measure ANOVA) higher than control cultures. These effects observed with follistatin may reflect its ability to bind and inactivate endogenously secreted activin or other TGFβ superfamily members.

Inhibition of muscle development by activin is distinct from actions of FGF or TGFβ

Because FGF and TGFβ have also been shown to inhibit differentiation in muscle cell lines, we tested whether activin inhibition was qualitatively similar. Each cytokine had distinguishing characteristics in the time course assays (Figure 5A and B). In our culture conditions, FGF was found to act as a potent mitogen for pectoral myoblasts without preventing the differentiation of myotubes. In contrast to FGF, total cell number was not affected by TGFβ. Onset of differentiation was delayed with either TGFβ or FGF, but unlike activin-treated cultures, these cultures eventually reached or exceeded control CPK activity levels. Our results are in agreement with the short delays in chick muscle culture differentiation with FGF Seed and Hauschka, 1988) and TGFβ (Cussella-DeAngelis et al., 1994) reported previously.

To address the mechanisms of activin inhibition, we tested whether muscle development could recover with the removal of the cytokine. Control cultures grown in serum-free medium were compared to those exposed to activin for limited times. Two days activin exposure with subsequent washing and transfer to serum-free medium was sufficient to repress muscle culture development (Figure 6A). In order to exclude the possibility that activin may not be completely removed following washing or that exogenously added activin might up-regulate receptors for activin and sensitize the cultures to endogenously secreted activin (Stern et al., 1995b; Levin et al., 1995), excess follistatin was added after washing to bind remaining exogenous or endogenously secreted activin. CPK activity was reduced similarly in each experimental condition (Figure 6A).

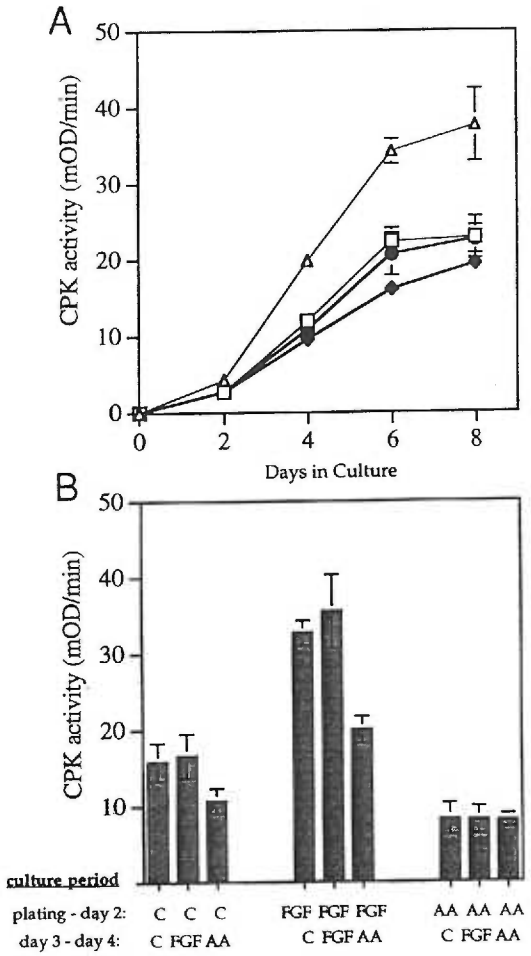


FIG. 6. Pectoral muscle cultures do not recover after activin removal. Time course of CPK activity expression (A) in control medium (open triangles), 5 ng/ml activin A for 8 days (open squares), 5 ng/ml activin A for 2 days followed by washing and replacing with control medium (filled diamonds), or 5 ng/ml activin A for 2 days followed by washing and replacing with 50 ng/ml follistatin (filled circles). CPK activity after 4-day culture period (B) following growth factor replacements after Day 2: control medium (C), 5 ng/ml FGF 2 (FGF), 5 ng/ml activin A (AA). Data points represent means ± standard error from three wells from one representative experiment.

Potentially, muscle cultures inhibited by activin could be induced to differentiate to control levels in the presence of mitogens. However, the addition of FGF following the 2 day activin treatment did not rescue CPK activity (compare AA->FGF to AA->C, Figure 6B). Reciprocally, when pectoral muscle cultures were plated in FGF and then switched to medium containing activin, inhibition was found to be proportional to the measured increases with FGF (compare FGF->AA to FGF->C, Figure 6B). Interestingly, we found that cultures plated in control medium and then exposed to FGF on day 3 could not be distinguished from cultures continually grown in control medium (compare C->FGF to C->C, Figure 6B). This loss of responsiveness to FGF supports studies that have shown a down regulation of FGF receptors with myotube formation (Olwin and Hauschka, 1990; Marcelle et al., 1995; Itoh et al., 1996).

Another distinction between activin inhibition and TGFβ inhibition was found through their interactions with FGF. When pectoral muscle cells were cultured in the presence of both activin and FGF, CPK development after four days was lowered proportionally, as compared to FGF alone, over a range of FGF concentrations (Figure 7A). In contrast, when pectoral muscle cells were cultured in the presence of both TGFβ and a range of FGF concentrations, a synergistic reduction in CPK activity was observed (Figure 7B). Similar results were obtained using activin and TGFβ concentrations of 0.5, 5.0, and 50.0 ng/ml. The MyHC immunoreactive cells present in activin+FGF were morphologically different from those differentiated in TGFβ+FGF, in that the latter were often mono-nucleated (compare 7F to 7H). These interactions with FGF could be mimicked by replacing FGF with 10% fetal calf serum.

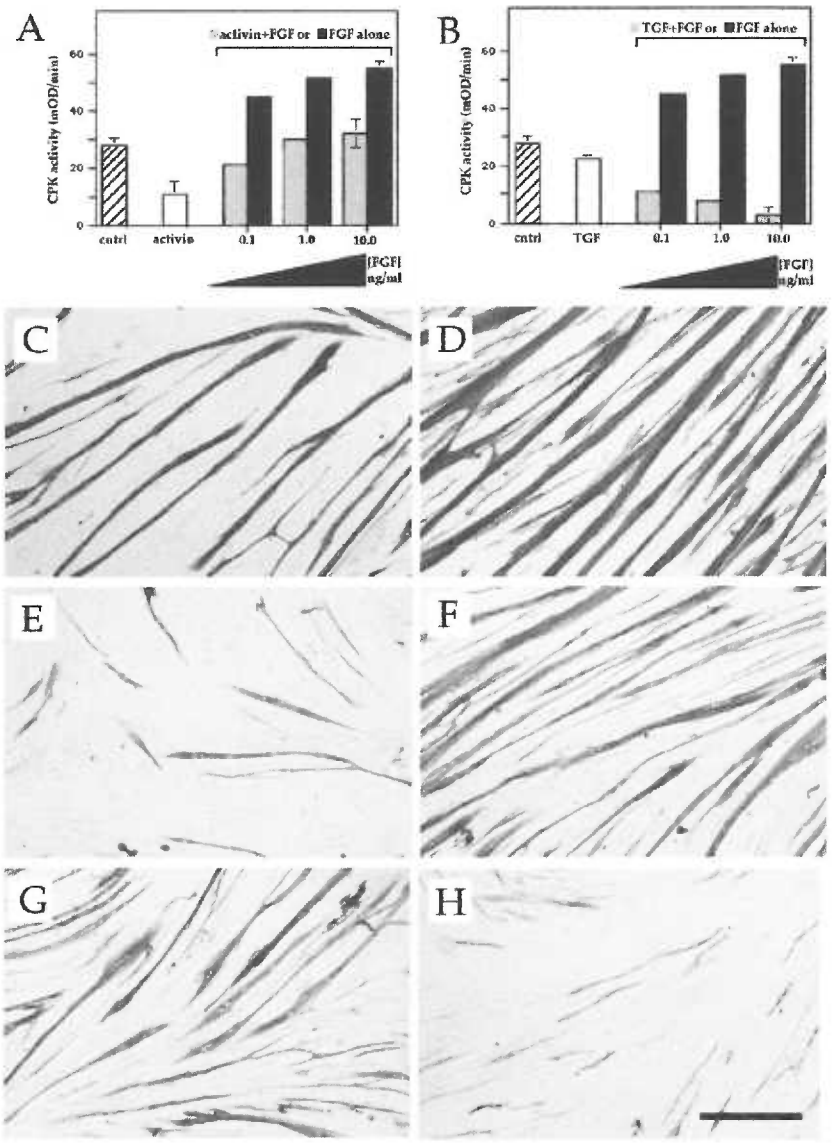


FIG. 7. Comparison of activin and TGFβ effects on muscle development in the presence of FGF. CPK activity from E11 pectoral muscle cultures after 4 days in activin, FGF, or activin+FGF (A) and TGFβ1, FGF, or TGFβ1+FGF (B). Control medium (hatched bars), 5 ng/ml activin A or 5 ng/ml TGFβ1 alone (open bars), 5 ng/ml activin A or 5 ng/ml TGFβ1 + 0.1, 1.0 or 10.0 ng/ml FGF2 (grey bars), and 0.1, 1.0 or 10.0 ng/ml FGF2 alone. Data points represent mean ± standard error from 3 wells from one representative experiment. MyHC immunoreactivity (C through H) in sister cultures to (A) and (B). Cutures differentiated in control medium (C), 1.0 ng/ml FGF 2 (D), 5 ng/ml activin A (E), 5 ng/ml activin A + 1.0 ng/ml FGF2 (F), 5 ng/ml TGFβ1 (G), 5 ng/ml TGFβ1+1.0 ng/ml FGF 2 (H). Bar represents 100 μm.

MyoD and Myf5 mRNA levels are altered by activin and follistatin

Because the experiments presented thus far have used markers of post-mitotic myocyte and myotube maturation, we tested whether activin and follistatin could affect the transition of myoblasts to myocytes. Accordingly, we used competitive RT-PCR in 1.5 day cultures to compare transcript levels for the myogenic regulatory factors myoD and myf5, which are up-regulated during the differentiation of myoblasts to myocytes (Emerson, 1993). Activin and follistatin treated cultures were compared to untreated cultures. Complementary DNA (cDNA) from untreated cultures was used to determine the concentrations of competitive templates needed to equally compete with myoD or myf5 for oligonucleotide primers (Figure 8A, i and ii). MyoD and myf5 levels in activin treated cultures were consistently lower than untreated controls (Figure 8B). Interestingly, with follistatin treatment, at 1.5 days we repeatedly observed a decrease in myoD levels and an increase or no change in myf5 levels as compared to control (Figure 8B).

DISCUSSION

Because activin and its inhibitor, follistatin, are expressed in multiple stages during muscle determination and maturation, we hypothesised that these cytokines modulate muscle development. In order to test this hypothesis, we confirmed the expression of activin and follistatin in vivo during chick pectoral muscle development and in cultures derived from this muscle group. We also showed that activin signaling inhibits committed muscle cells from differentiating, while follistatin stimulates muscle differentiation, and that the effects of activin can be distinguished from the related protein, TGFβ.

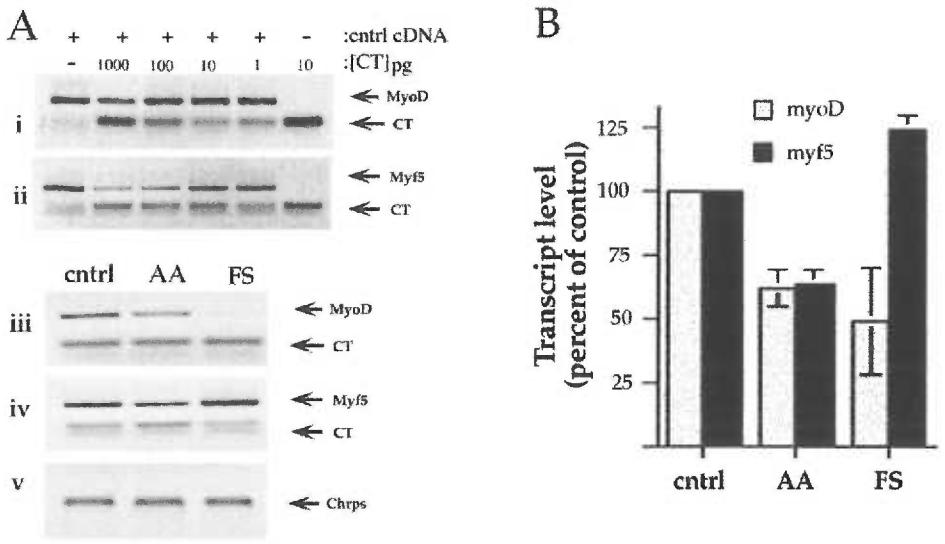


FIG. 8. Expression of myoD and myf5 in E11 pectoral muscle cultures. Dilution range in pg of competitve template plasmid DNA (CT) for myoD (A,i) or myf5 (A, ii) using constant amounts of control culture cDNA (0.2 µl for myoD and 1.0 µl for myf5). MyoD and myf5 expression (A, iii and iv, respectively) in cultures with control medium (cntrl), 5.0 ng/ml activin A (AA), or 35 ng/ml follistatin (FS). MyoD CT (100 pg, Aiii) or myf5 CT (10 pg, Aiv) were present with each experimental cDNA amplification to facilitate band intensity comparisons between experimental conditions. Chrps amplification (A, v) of each cDNA to assay reverse transcription efficiency. Histogram of transcript levels (pixel density) displayed as a pecentage of control culture levels (B). Data represents mean ± standard error obtained from three independent experiments (separate cDNAs).

Markers for three different stages of muscle differentiation were assesed in examining the effects of activin and follistatin: myotube maturation (CPK activity), terminal differentiation (quantitation of nuclei in MyHC immunoreacitve cells), and the initiation of the myoblast to myocyte transition (myoD and myf5 transcript levels). During peripheral muscle (body wall and limb) development, cells committed to the myogenic lineage migrate from the lateral somite (Ordahl and Le Douarin, 1992) to populate and divide at the premuscle mass. The onset of myoD and myf5 has been shown in vivo and in vitro to signal the onset of myoblast differentiation (Montarras et al., 1991; Pownall and Emerson, 1992; Tajbakhsh and Buckingham, 1994). Following cell cycle withdrawl, terminally differentiated myocytes rapidly express MyHC and other contractile proteins (Lin et al., 1994). Activity for the skeletal muscle-specific metabolic enzyme, CPK, is then up-regulated with myocyte fusion and myotube maturation (Shainberg et al., 1971; Olson et al., 1983).

Activin was found to repress markers for all three stages of muscle differentiation. The decreases in CPK activity with activin could be caused by limiting the number of myocytes generated or preventing the increase in myotube size. Activin was found to affect both without changing the total number of nuclei in the culture. The reductions in myoD and myf5 transcript levels with activin are consistent with activin inhibition of later stages of muscle development. We conclude that activin inhibition of skeletal muscle development acts by repressing the differentiation of committed myoblasts to post-mitotic, fusion-competent myocytes.

Surprisingly, differences in myoD and myf5 transcipt levels were found with follistatin treatment: myoD was reduced, while myf5 was elevated. This suggests that the actions of follistatin are not simply reciprocal to those of activin. In agreement with this, follistatin has been recently suggested to interact with other TGFβ superfamily members, including those of the bone morphogenic protein (BMP) family. Interestingly, the initial differential expression of myf5 and myoD in dorsomedial and dorsolateral cells of newly formed somites have been shown to be independently regulated by factors from adjacent tissues (Cossu et al., 1996). Potentially, follistatin modulates

endogenous factors which either directly regulate myoD and myf5 transcript levels or affect the survival or proliferation of myoblast sub-populations that express predominately one or the other MRF.

Our results with activin and follistatin are another example of the importance of cytokines in paracrine and autocrine signaling in muscle development. De Angelis et al., (1992) have previously noted soluble factors secreted from muscle cells that potentiate muscle differentiation. In addition transcripts for many molecules that can affect muscle development, such as the FGFs, IGFs, and TGFβs are found within muscle cells (Joseph-Silverstein et al., 1989; Tollefsen et al., 1989; Pelton et al., 1991; Niswander and Martin, 1992; deLapeyriere et al., 1993). We have established that activin and follistatin are expressed in developing pectoral muscle in vivo and in vitro. In addition, our observation that the responsiveness of pectoral muscle cultures to activin and follistatin was decreased with increased plating density suggest that endogenously available cytokines mask the effects of exogenously added molecules.

The short delays in muscle differentiation by FGF and TGFβ in our culture conditions are consistent with reports studying these cytokines in primary avian muscle cultures (Seed and Hauschka, 1988; Cusella-DeAngelis, et al., 1994). This is in contrast to continued inhibition observed in muscle cell lines derived from adult rodents (Florini et al., 1986; Vaidya et al., 1989; Li et al., 1992). The clonal nature of the transformed cell lines may explain these differences. Potentially, cytokines may differentially regulate discrete myoblast sub-populations. Cusella-DeAngelis et al., 1994 have decribed differential reponces between embryonic and fetal myoblasts to TGFβ while Seed and Hauschka, 1988 have identified myoblast sub-populations within the chick wing bud that are either FGF dependent of independent for myogenic differentiation.

Several of the experiments presented here show that the effects of activin and TGFβ on muscle development are distinct. When muscle differentiation was measured during a time course, TGFβ treated cultures showed an initial repression of markers, but eventually reached control levels. In contrast to TGFβ, the repression of muscle development with activin was maintained

throughout the culture period. Another difference between TGFβ and activin was their effects on muscle culture development in the presence of FGF: activin inhibition was proportional with the amount of FGF, while TGFβ inhibition was synergistic with the mitogen. This synergistic reduction of CPK activity with TGFβ+FGF supports the hypothesis put forward by Zentella and Massagué (1992) that TGFβ in the presence of FGF rapidly promotes terminal differentiation of myoblasts. Under such conditions, non-myogenic cells would continue to proliferate, lowering the proportion of cells expressing muscle markers within the culture and resulting in decreased CPK activity as compared to control. In agreement with this interpretation is evidence for the induced muscle differentiation in a rat skeletal myoblast cell line by TGFβ in the presence of mitogens (Zentella and Massagué, 1992) and the recently described synergistic interactions between TGFβ and FGF on paraxial mesoderm myogenic differentiation in chick (Stern and Hauschka, 1997).

The inability of muscle cultures to fully differentiate with the removal of activin may be due to the following mechanisms. 1). Activin may affect the survival or proliferation of myoblasts. We did observe consistent, but small reductions in total cell numbers at early time points, however final cell numbers between activin treated and control cultures were indistinguishable. Furthermore, we did not observe any changes in the number of condensed nuclei, a marker for apoptotic cell death, as assessed by acridine orange labeling. 2). Activin may alter the fate of undifferentiated cells to another mesodermal cell-type lineage. However, changes in the expression of typeII collagen, a marker for chondroblasts (George-Weinstein et al., 1994), was not detected (data not shown). 3). Activin could permanently reduce the increased expression of muscle markers by promoting the rapid terminal differentiation of the myoblasts initially plated. We do not believe this occurs, as no increases in any of the muscle markers assayed (myoD, myf5, MyHC, or CPK) were induced with activin at early time points. While each of the listed mechanisms of activin action can not be excluded, we feel the cumulative evidence presented in this paper suggest such events are unlikely. The physiological significance of a permanent repression is difficult to reconcile. Myoblasts isolated at earlier embryonic ages may have the ability to recover from activin treatment or molecules not tested in our studies

could induce myogenic differentiation in activin-repressed myoblasts at their proper time in vivo.

In conclusion, the results presented here support a model where activin inhibits the differentiation of myoblasts, while follistatin acts as a local modulator to prevent this myogenic repression. The effects of activin on pectoral muscle cultures were found to be distinct from those of TGFβ. These results, coupled with work from others, emphasize the integration and importance of multiple cytokine signaling cues during myogenic differentiation and maturation. The physiological significance of these findings are supported by the in vivo expression patterns of activin, activin receptors, and follistatin at sites of myogenesis and muscle differentiation. The proposed functions of activin and follistatin in muscle development may have implications in coordinating the timing of myofiber formation from myoblasts which populate the embryonic structures that will eventually give rise to functional skeletal muscle groups. In addition, the described myogenic inhibitory actions of activin aid in understanding the muscle phenotype in follistatin deficient mice, which display decreased muscle mass of the diaphragm, pectoral, and intercostal muscles (Matzuk et al., 1995c). In such animals, an increase in activin signaling due to the absence of follistatin could permanently repress myogenic differentiation in activin-sensitive myoblasts.

ACKNOWLEDGMENTS

We thank Dr. Jan Christian, Diane Darland, Tristan Darland, and Dr. Matt Thayer for invaluable discussions and for critical reading of this manuscript. We also thank Ellie Margolis for help with cell counts. We gratefully acknowledge the following for generously providing reagents: Dr. Felix Eckenstein, recombinant FGF-2; Dr. Patricia Johnson, full-length activin β_A cDNA; Dr. Shunichi Shimasaki, anti-follistatin antiserum; Dr. Wylie Vale, anti-activin A antiserum. Finally, we are appreciative to Dr. Steven Hauschka and Dr. Howard Stern for sharing results prior to publication. This work was supported by grants 5T32EY07123 (B.L.) and 5RO1NS25767 (R.N.).

CHAPTER III

Development of the Avian Iris and Ciliary Body: Mechanisms of Cellular Differentiation During the Smooth-to-striated muscle transition.

Brian A. Link and Rae Nishi*

Department of Cell and Developmental Biology, L215
Oregon Health Sciences University
3181 SW Sam Jackson Park Road
Portland, Oregon 97201

*To whom correspondence should be addressed.

Abstract

During development of the avian eye, the muscles of the iris undergo a transition from smooth to striated type muscle. Smooth muscle cells arise from the epithelium at the margin of the optic cup and migrate into the overlying iris stroma and establish the slow contracting, single nucleated smooth muscle of the early developing iris. This initial phenotype is gradually supplanted by fast contracting, multi-nucleated striated muscle. We have studied the cellular mechanisms underlying this non-conventional mode of muscle differentiation. To address the role of the local environment in regulating this transition we have established dissociated cell and explant culture models. The cellular relationships between the smooth and striated muscle were assessed through a novel method of analysis which permitted us to follow the differentiation of single cells in isolation or in heterochronic environments. We found co-localization within the iris of various smooth and striated muscle specific markers including contractile proteins, acetylcholine receptor sub-types, and transcriptions factors both in vitro and in vivo. Using these markers we also observed a smooth-to-striated muscle transition in the ciliary body. Our data argue against a multipotent stem cell for smooth and striated muscle cells of the ocular anterior segment, but support a role for transdifferentiation of smooth-to-striated muscle during iris development. Cumulatively these results, coupled with those from others, suggest that both smooth muscle and migratory myoblasts contribute to the development of myotubes in the avian iris and that this process is regulated in a non-cell autonomous fashion by locally generated signals.

Introduction

Muscle cells can be grouped into three general categories: cardiac, striated, and smooth muscle. Cardiac muscle is similar to striated in that the intracellular contractile apparatus appears cross-banded, but it is composed of mono-nucleated cells that are tightly associated and linked by gap junctions. Striated muscle, also referred to as skeletal muscle, is multi-nucleated and also displays a well organized contractile apparatus. Smooth muscle cells are more diverse in function and morphology, are mono-nucleated, and do not show banded striations. The three muscle types arise from distinct mesodermal precursors following gastrulation. Cardiac muscle develops from cells in the anterior lateral plate mesoderm; skeletal muscle arises from specific regions of somitic and head mesoderm; and most smooth muscle is derived from splanchnic lateral plate mesoderm. One exception is a population of vascular smooth muscle cells that contribute to the aortic arteries, which descend from the neural crest and are therefore ectodermal in origin (LeLievre and LeDouarin, 1975; Kirby and Waldo, 1990).

Although the three types of muscle are viewed as being derived from distinct lineages, the initial specification of muscle fate is believed to be regulated by a a family of transcription factors termed myocyte enhancer factors (MEFs), that are shared by cardiac, smooth, and striated muscle (reviewed in Olson et al., 1995). The divergence of the three types of muscle, however, occurs early in development and is thought to be controlled by different sets of transcription factors specific for each muscle lineage. The best understood are those responsible for striated muscle development, such as myoD, myf 5, myogenin, and mrf4 (reviewed in Emerson, 1993).

In addition to an early separation of developmental lineages among muscle types, examples of transdifferentiation, the conversion of one differentiated cell type into another, have been documented. For example in the jellyfish when striated muscle is isolated it can transdifferentiate into smooth muscle (Alser and Schmid, 1987; Reber-Müller et al., 1994). In the mouse, esophageal cells undergo a conversion from smooth to striated muscle as a part of normal development (Patapoutian et al., 1995). Subpopulations of striated

muscle cells in fish convert into electrocytes that then form the electric organ (Patterson and Zakon, 1997). Co-localization of cell-type specific markers has been used as the primary criterion to demonstrate these examples of transdifferentiation.

The avian iris develops from cells of diverse embryonic origins and during development undergoes a smooth-to-striated muscle transition that has been characterized morphologically, immunohistochemically, and electrophysiologically (Pilar et al., 1987; Scapolo et al., 1988; Volpe et al., 1993). The iris and ciliary body form from ectodermal cells at the anterior margin of the optic cup as well as migratory mesenchymal cells derived from the embryonic head region (Johnston et al., 1979; Wachtler and Jacob, 1986). Morphologically, the iris and ciliary body are first apparent at stage 27 (embryonic day 5 (E5), Hamburger and Hamilton, 1951) as a prominent pigmented domain surrounding the pupil. This domain continues to darken and a border, the ora serrata, is evident between the edge of the ciliary body and the neural retina. At stage 29 (E6.5), epithelial cells at the margin of the pupil invaginate into the mesenchymal iris stroma and differentiate into the smooth muscle cells (Lewis, 1903; Ferrari and Koch, 1984). The muscle transition is apparent at E10, when striated muscle emerges midway along the length of the iris and within close proximity to the smooth muscle cells (Lewis, 1903; Gabella and Clarke, 1983; Ferrari and Koch, 1984). Differentiation of striated muscle continues through hatching, while the smooth muscle cells stop growing at E15, and are eventually confined to the pupillary margin. (Gabella and Clarke, 1983; Pilar et al., 1987).

The origin of striated muscle in the iris is controversial. Based on morphological criteria and the observation that isolated cultures of iris epithelium can develop into striated muscle, Ferrari and Koch (1984) concluded that both smooth and striated muscle are derived from the invaginating epithelial cells, but that smooth muscle cells do not contribute to the striated myotubes. In contrast, Volpe et al. (1993) have suggested that smooth muscle transdifferentiates into striated muscle because smooth- and striated-specific muscle markers co-localize in single cells. Other studies utilizing the chick-quail transplant technique suggest that some, if not all irideal striated muscle is derived from head mesoderm and cranial neural

crest and is therefore separate in lineage from the smooth muscle (Nakano and Nakamura, 1985; Yamashita and Sohal, 1986; Yamashita and Sohal, 1987). These transplant studies also confirmed Johnston et al.'s (1979) observation that the ciliary muscles are derived from migratory mesenchymal cells. Thus, while it is established that the final phenotype of the ciliary muscles is striated, it is not clear whether this muscle group progresses through a transient smooth muscle phenotype.

Cumulatively, these results suggest three possible models for the cellular mechanisms of the iris muscle transition. 1. Multi-potent stem cells derived from either the epithelium of the optic cup or from the neural crest reside in the embryonic iris and give rise to both smooth and striated muscle precursors that differentiate at different, but overlapping times. 2. Smooth muscle cells transdifferentiate into fusion competent myocytes that then form multinucleated, striated myotubes. 3. Smooth and striated muscle precursor cells are of separate lineages and, as suggested for a multi-potent stem cell mode of development, are promoted to differentiate at different, but overlapping times. To test the hypothesis that a multi-potent stem cell acts as a precursor for smooth and striated muscle, we have performed clonal analyses in vitro to test for pluripotency or lineage restrictions in cells from the undifferentiated iris and ciliary body. In addition, we have tested the hypothesis that transdifferentiation can contribute to the development of striated muscle in these structures by investigating the co-localization of multiple markers specific for smooth or striated muscle. These experiments explore possible cellular mechanisms underlying the smooth-to-striated muscle transition in the anterior segment of the chick eye.

Results

Smooth-to-striated muscle transition

The smooth-to-striated muscle transition in the developing chick iris was followed using antibodies specific for alpha-smooth muscle specific actin (αSMA) and striated muscle specific myosin heavy chain (MyHC). Immunolocalization in vivo at E8, E11, and E16 confirmed previous morphological assessments of smooth and striated muscle differentiation. At E8 invaginating epithelial cells at the pupillary margin were αSMA immunoreactive. In addition, migrating neural crest cells that form the corneal endothelium transiently expressed αSMA as described previously by Beebe et al. (1997) (arrows, Figure 1). In adjacent sections at E8, no MyHC expression was detectable. Epithelial cells continued to invaginate, differentiate into smooth muscle, and migrate peripherally as observed in sections of E11 iris (Figure 1C). At this age, MyHC-expressing striated muscle was observed to be interspersed with smooth muscle cells in an area midway between the pupilary margin and the peripheral edge of the iris (Figure 1D). We were not able to discern whether there was co-localization of αSMA and MyHC in single cells using adjacent sections; however, previous studies have reported co-localization of smooth muscle specific IP_3 receptors with MyHC immunoreactive cells (Volpe et. al., 1993). At later stages, a majority of the cells in the iris stroma expressed MyHC, while the proportion that express αSMA is reduced. By hatching, only the cells at the pupillary margin and those lining blood vessels express markers for smooth muscle.

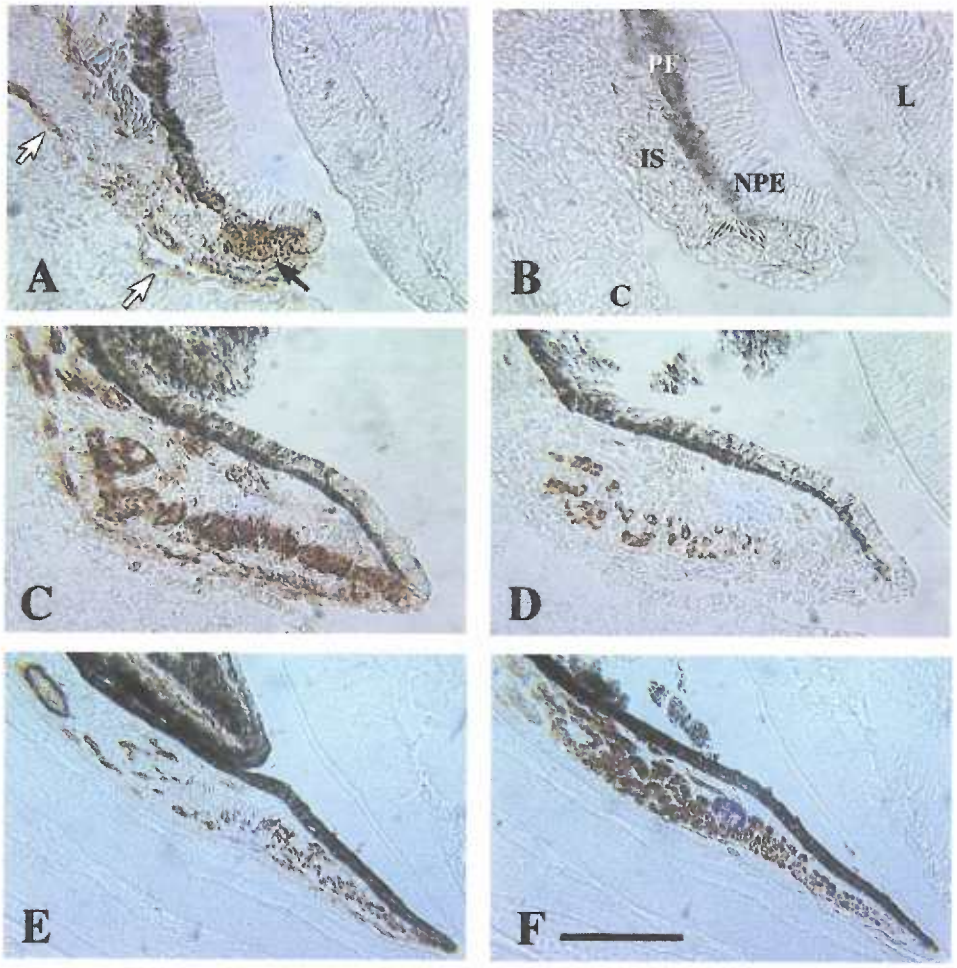


Figure 1. Smooth to striated muscle transition in the developing iris. αSMA (A, C, E) and MyHC (B, D, F) immunoreactivity in adjacent 8μm cryostat sections at E8 (A, B), E11 (C, D), and E16 (E, F). In (A), αSMA immunoreactivity (brown) can be seen in the invaginating epithelium (black arrow) as well as in the developing corneal endothelium (white arrows). C, cornea; IS, iris stroma; L, lens; NPE, non-pigmented epithelium; PE, pigmented epithelium. Bar equals 400 μm (A, B), 500 μm (C, D), 1 mm (E, F).

Transient smooth muscle phenotype in the ciliary body

At E8 in the developing ciliary muscles, we observed numerous cells that expressed αSMA, but no cells which expressed MyHC. In addition to αSMA, we tested whether other markers specific for smooth muscle are expressed by the cells in the developing ciliary muscles. Smooth muscle specific myosin light chain kinase (MLCK), a Ca^{2+}/calmodulin depedent kinase that regulates smooth muscle contraction, and calponin (Clpn), a calmodulin and F-actin binding protein, are expressed in fully differentiated smooth muscle cells (Olson et al., 1990; Frid et al., 1992). Immunoreactivity for these proteins was observed at E8 in the developing ciliary muscles, but in a more restricted domain than αSMA. Corneal endothelial cells, while immunoreactive for αSMA, were negative for both MLCK and Clpn. None of the smooth muscle markers were expressed in the developing extraocular muscles. Confirming the smooth muscle phenotype in the iris, MLCK and Clpn were also expressed within αSMA positive cells, including the invaginating epithelial buds at E8. By E11, cells in the ciliary muscles expressed the striated muscle marker MyHC and, in adjacent sections, cells expressing αSMA, MLCK, and Clpn were observed (Figure 2 A, C, E, G). By E16, striated myotubes, which expressed MyHC, were visible by phase-contrast microscopy. At this time MLCK, and Clpn were absent from the ciliary muscles, and αSMA immunoreactivity was dramatically reduced (Figure 2 B, D, F, H). These results demonstrate that the ciliary muscles, like the iris muscles, undergo a developmental smooth-to-striated muscle transition.

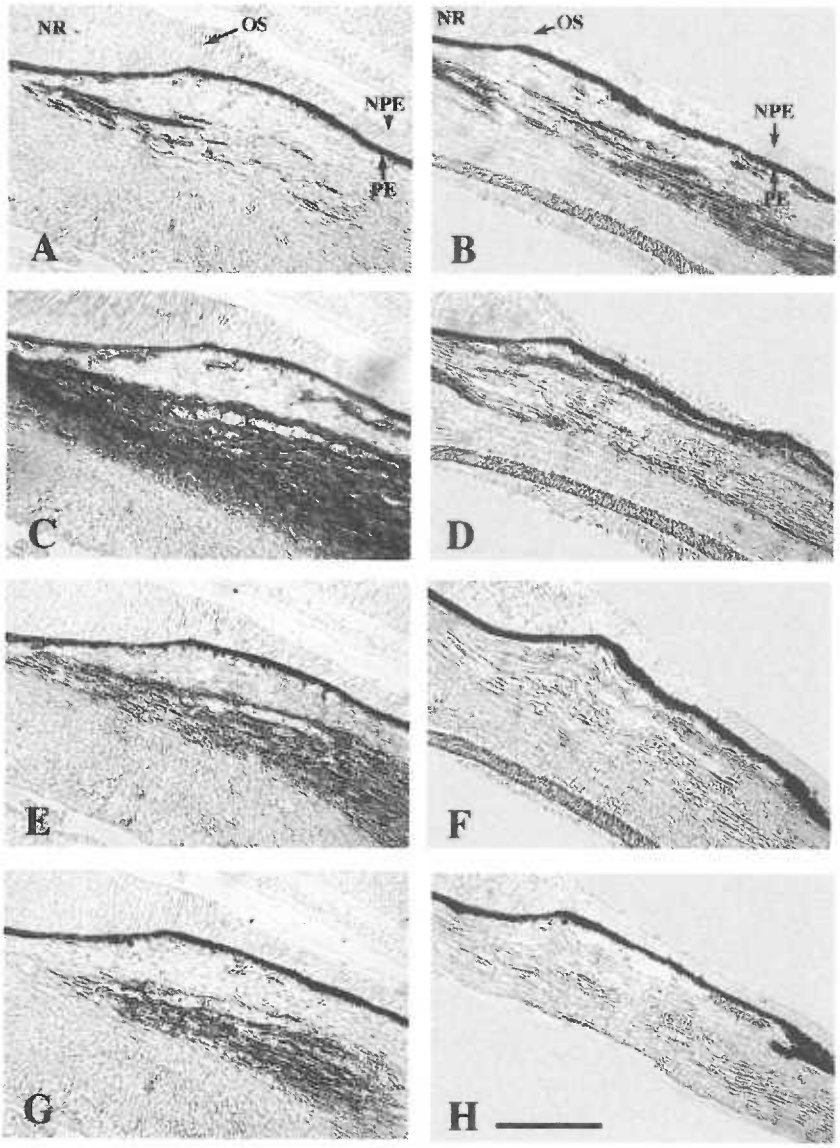


Figure 2. Smooth-to-striated muscle transition in the developing ciliary body. Immunoreactivity for multiple smooth muscle markers in adjacent 8 μm cryostat sections of the ciliary body: αSMA (A, B), MLCK (C, D), Calponin (E, F); and striated muscle specific MyHC (G, H) in E11 (A, C, E, G) and E16 (B, D, F, H) ciliary body. NPE, non-pigmented epithelium; NR, neural retina; OS, ora serrata; PE, pigmented epithelium (delineated with arrows). Bar equals 500 μm (A, C, E, G), 1 mm (B, D, F, H).

Developmental changes in the ability of iris and ciliary body to form striated muscle in vitro

In order to address the cellular mechanisms which control the smooth-to-striated muscle transition in the iris and ciliary body we established a culture model for iris-ciliary body differentiation. To validate this model we isolated cells and tested whether myogenesis in vitro was comparable to that in vivo. Equivalent numbers of dissociated cells were plated from different stages of development. The number of nuclei in cells immunoreactive for MyHC was found to increase rapidly from E7 until E11, the age at which the striated myogenic potential was greatest (Figure 3A). The amount of striated muscle developing from dissociated iris-ciliary muscle slowly decreased after E11, possibly reflecting a depletion of the pool of striated muscle precursor cells that had incorporated into terminally differentiated myotubes. Although myogenic potential was low at E7, MyHC immunoreactive cells were observed after 4 days in culture. These results raise the following questions. Is the observed increase in myogenesis in tissues isolated from older iris-ciliary body due to immigration of fusion competent myoblasts into the target region? Alternatively, do striated muscle precursor cells reside within this tissue, but become fusion competent in a developmentally regulated fashion? To begin to test these possibilities, we measured creatine phosphokinase (CPK) activity, a marker of myotube formation (Shainberg et al., 1971; Olsen et al., 1983) in explant cultures of E8 iris-ciliary body. Development of CPK activity from iris-ciliary body explants, where cellular interactions remain intact, was comparable to age matched iris-ciliary body in vivo (Figure 3B). These results suggests that all of the striated muscle precursors are resident within the iris-ciliary by E8 and that differentiation is regulated locally within these ocular structures. This regulation could be controlled temporally by either intrinsic mechanisms acting cell autonomously or through extrinsic signals derived from outside the cell. We therefore performed two types of clonal analyses to address extrinsic regulation of cell fate or differentiation, as well as the pluripotency of muscle precursors.

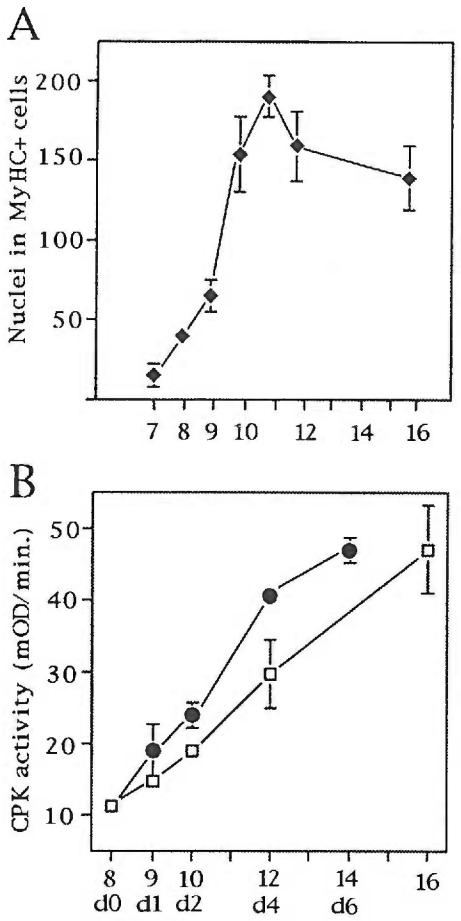


Figure 3. Striated muscle development in dissociated and explant cultures of iris and ciliary body. (A) Dissociated cultures: iris-ciliary body cells were isolated, dissociated, and plated at $5x10^4$ cells per ml for each age shown. The number of nuclei in MyHC immunoreactive cells were counted after 4 days in culture. Data points represent means ± standard error from four wells from one representative experiment. (B) Explant cultures: E8 iris-ciliary body were explanted onto collagen/agarose cushions and allowed to develop for 1, 2, 4, or 6 days. CPK activity from explants (filled circles) was compared to age matched iris-ciliary body that had developed in vivo (open squares). Data points represent means ± standard error from three iris-ciliary bodies from one representative experiment.

Clonal analysis of iris cells: assessing the pluripotency of single cells.

In order to test whether young (E7/8) iris contains pluripotent precursors of muscle, we cloned quail iris cells and examined their differentiation alone or in the presence of actively fusing striated muscle myocytes (Figure 4A). Quail embryos were used as a source of iris tissue for all the cloning experiments because quail cells are more readily cloned and can be distinguished from chicken cells in co-culture by their immunoreactivity to QCPN antigen. Single cells from E7 iris were grown in rich medium to a colony size of ~100 cells, then each clone was split and reseeded into two different culture conditions. Half of each clone was allowed to differentiate in serum-free medium and then assayed for αSMA immunoreactivity or for the ability to incorporate into multi-nucleated myotubes. The other half of the clone was plated in the presence of actively fusing E11 chick iris-ciliary body cells to test whether cells giving rise to smooth or striated muscle could be induced to incorporate into myotubes. Although we observed clones containing all smooth or all striated muscle cells, clones containing both smooth and striated muscle cells were not observed in the 71 clones that were analyzed (Table 1). In addition, clones giving rise to smooth muscle cells could not be induced to incorporate into myotubes when placed in co-culture with actively fusing myocytes. These results argue against the existence of a multi-potent muscle stem cell. Cloning efficiency in this experiment was approximately 50 percent. While this may reflect a general low survival rate following the initial dissociation process, it is possible that multi-potent precursor cells were selected against or that a multipotent stem cell became committed to a single cellular lineage under these conditions.

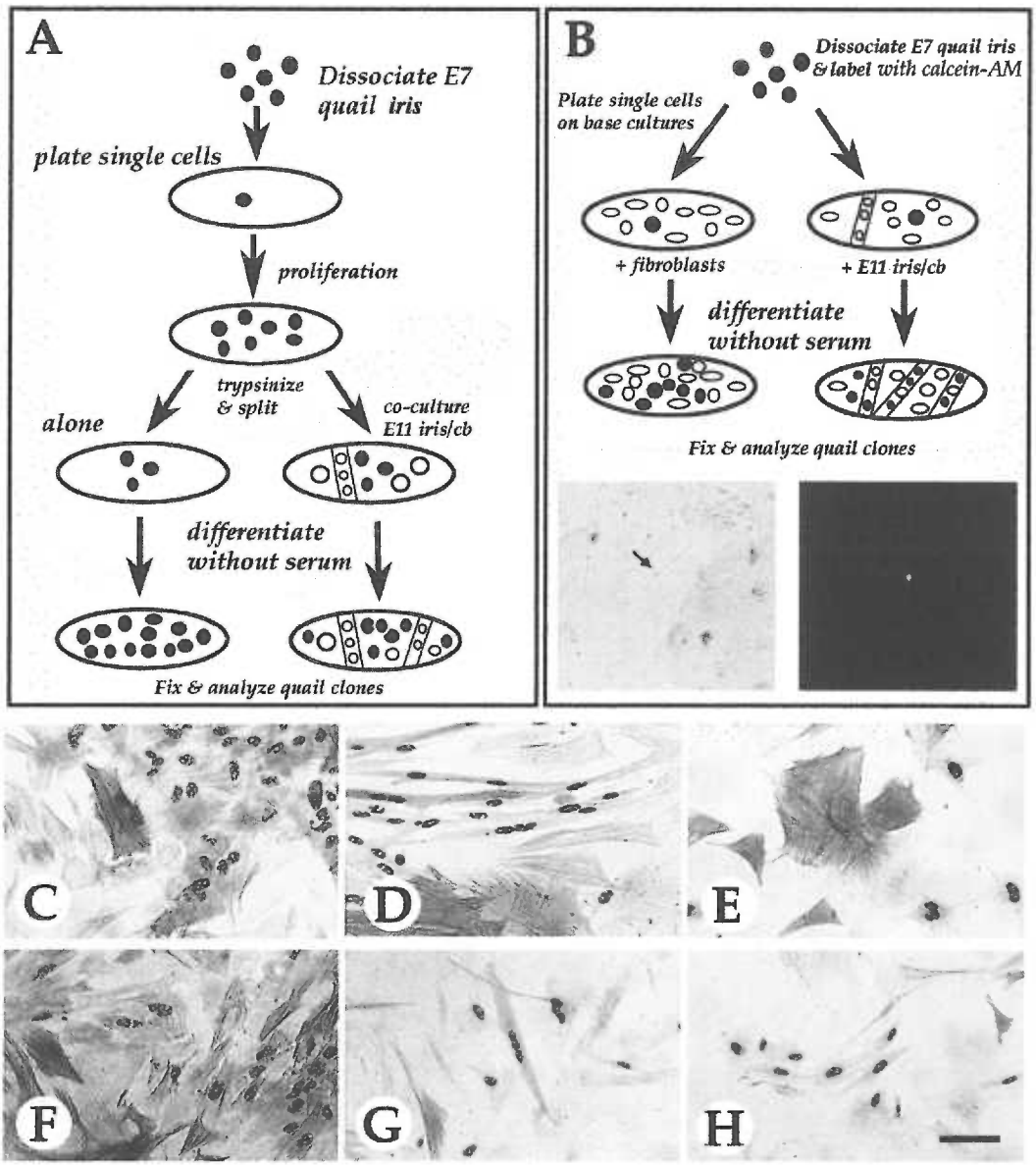


Figure 4. Clonal analyses: experimental design and clone morphology. Single cell cloning with serum rich medium (A) or with serum free medium in co-culture with other cells (B). In (A), cells isolated from undifferentiated E7 quail irises were cloned by limited dilution and allowed to proliferate. When colonies reached ~100 cells they were trypsinized, divided into two suspensions, and re-seeded in serum free (differentiation promoting) medium alone or with chick E11 iris-ciliary body cultures. Clonal progeny were identified with a quail specific antibody and analyzed for muscle phenotypes after 4 days in the new environment. In (B), dissociated E7 quail iris cells were labeled with the vital dye calcein-AM and cloned by limited dilution with either embryonic fibroblasts or E11 iris cells. Wells that contained a single fluorescent quail cell (B, bottom), were allowed to develop for 4 days and then analyzed for muscle marker expression. Examples of colonies from (B) processed for QCNP and myotube incorporation (C-H): (C, D, E) Clones with E11 iris-ciliary body cells, (F, G, H) Clones with embryonic fibroblasts. Clonal types included smooth muscle (C, F), striated muscle (D, G), differentiated/other cells (E, H). Bar equals 75 μm.

Single cell cloning directly in co-culture

A second cloning experiment was designed to test whether selective processes present in the developmental environment can influence early cell fate decisions (Figure 4B). Embryonic day 7 quail iris was dissociated, prelabeled with the vital dye calcien-AM, and plated at a clonal density in the presence of chick embryonic fibroblasts or E11 iris-ciliary body cells. Chick embryonic fibroblasts were found to promote survival of E7 quail iris cells without altering the differentiation of striated muscle precursors (Link and Nishi, submitted). Culture wells containing one labeled quail cell were identified and marked shortly after plating. The resulting clonal colonies were identified with the quail nuclei specific QCPN marker and the composition of smooth (αSMA immunoreactive) and striated (myotube incorporation) muscle was assessed (Table 2). Unlike the previous cloning experiment, the survival rates of colonies derived from a single E7 quail cell were high, 98.1% and 98.2% respectively when cultured with fibroblasts or E11 iris-ciliary body cells. As before, all were classified as one of the three types observed previously and no clonal colonies contained both smooth and striated muscle (Table 2). These high, initial survival rates and the ability to culture both clonal colonies composed entirely of smooth or entirely of striated muscle in either culture condition argue against the selective elimination of a multipotent stem cell.

Interestingly, the proportions of clone types observed were different in fibroblast and E11 iris co-cultures. The percentage of striated muscle clones was higher in co-culture with E11 iris, while the percentage of undifferentiated/other clones was lower by a similar amount. The percentage of smooth muscle clones was not significantly altered. Not only were the clonal cell-type compositions different, but the number of cells in smooth and striated muscle cell colonies was approximately two-fold greater when cultured with E11 iris, while undifferentiated/other clone sizes were not altered (Table 2).

TABLE 1

Phenotypic analysis of E7 iris cell clones

Composition	αSMA/myotube incorporation	n
Smooth muscle	+/-	45
Striated muscle	-/+	10
Undifferentiated/ Other	-/-	16
Mixed clones	+/+	0

E7 quail iris cells were cloned as diagramed in Fig 4A. "+" indicates clones with one or more cells that expressed the listed muscle phenotype. "-" indicates no cells within the clone expressed the muscle phenotype. n indicates the number of colonies scored for each phenotype. Data are pooled results from 4 independent cloning experiments.

TABLE 2

Clonal Analysis of E7 Iris Cells in Co-culture: survival, phenotype frequency, and clone size

Co-culture:	E11 iris and Ciliary body	Embryonic Fibroblasts	
total clones	159	110	
clone survival	156, (98.1%)	108, (98.2%)	
phenotype:	***n, (frequency)*** ***ave clone size***		X^2***-test*** ***t-test***
Smooth muscle	26, (16.7%)	16, (14.8%)	n.s.d.
	31.3±27	18.4±14.5	p≤.096
Striated muscle	60, (38.5%)	29, (26.9%)	p≤.049
	14.6±11.0	7.3±5.2	p≤.001
Undifferen-tiated/other	70, (44.8%)	63, (58.3%)	p≤.032
	18.3±13.1	19.9±15.7	n.s.d.

E7 quail iris cells were cloned as diagramed in Fig. 4B. n (frequency) indicates the number and percentage of surviving clonal colonies for each phenotype, as defined in Table 1. Chi-squared analyses were performed to estimate significance of change in clone phenotype distributions. Two-tailed t-tests were performed to estimate significance of change in average clone sizes. Statistics were calculated with data from the surviving clones. n.s.d., no significant difference. Data are pooled results from 5 independent cloning experiments. Clone phenotype distributions were similar in each experiment.

These data suggest that extrinsic influences provided by the local environment act in regulating the smooth-to-striated muscle transition: the ability of an undifferentiated iris cell to form striated muscle is potentiated by signals provided by cells within the maturing muscle.

Expression of myoD and mrf4 in the developing iris

The results of our clonal analyses argue against a multi-potent stem cell for smooth and striated muscle of the iris; however, they do not exclude the possibility of direct transdifferentiation of smooth muscle cells to striated muscle precursors. To investigate the contribution of transdifferentiation to the smooth-to-striated muscle transition, we analyzed the pattern of expression of myogenic regulatory factors specific for skeletal muscle and tested for co-localization of these factors with smooth muscle markers in vivo and in isolated cells. Myogenic regulatory factors (MRFs) are basic helix-loop-helix type transcription factors specific for skeletal, striated-type muscle and regulate determination and differentiation of this cell type (reviewed in Sasson, 1993). The expression patterns of myoD and mrf4, two MRFs that are expressed during early differentiation, or in maturing myotubes, respectively, were investigated in the iris and ciliary body. Surprisingly, during early stages of iris and ciliary body formation (E7.5), myoD immunoreactivity was localized to epithelial cells anterior to the ora serrata (Figure 5A and 5B). Nuclear localization was evident in numerous cells. Interestingly, when E7.5 iris was explanted onto collagen-agarose cushions, some epithelial cells were induced to express MyHC (Figure 5D, arrows). MyHC was never observed in the iris epithelium or ciliary epithelium in vivo. By E9, the domain of myoD expression was confined to nuclei within the irideal epithelium at the pupilary margin (Figure 6A). Mrf4, however, was never expressed in these epithelial cells (Figure 5C). At later stages of development (E16), myoD and mrf4 were expressed in muscle cells of the peripheral stroma of the iris and ciliary body (Figure 5E and 5F).

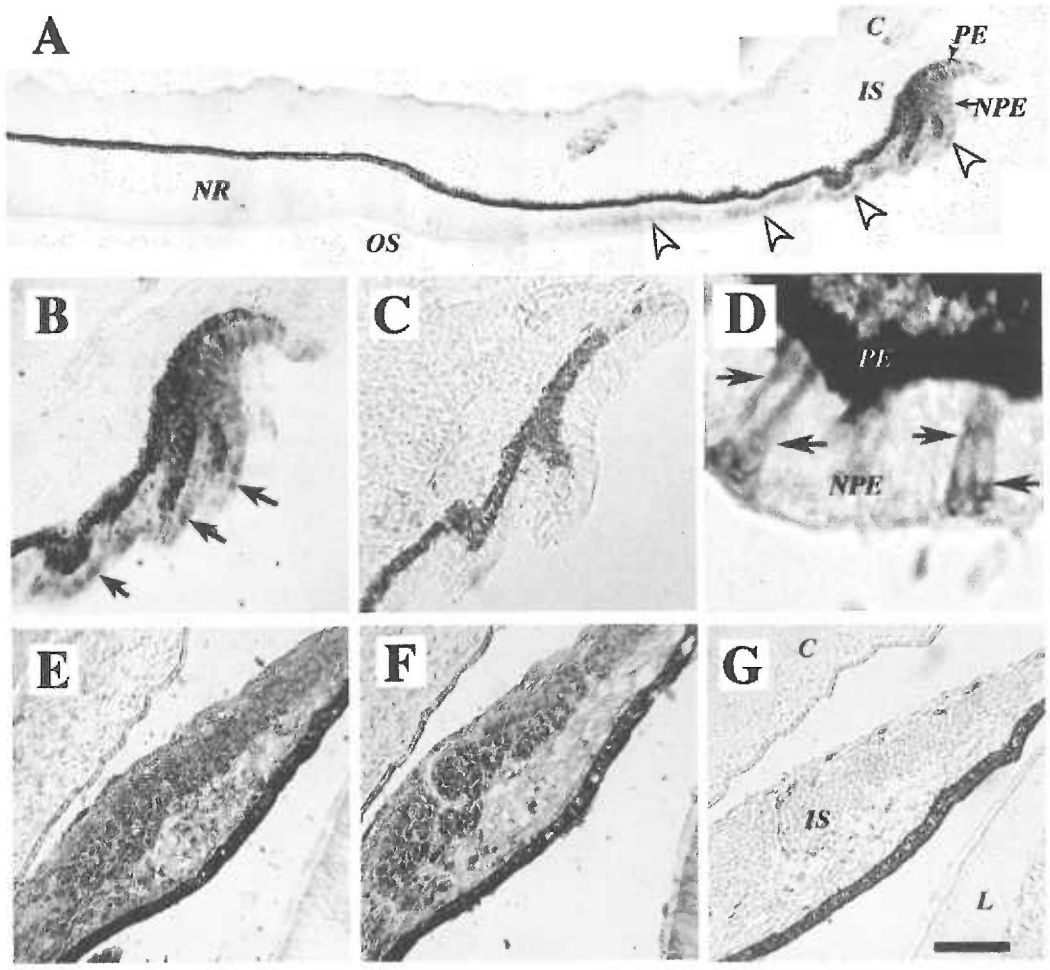


Figure 5. Muscle regulatory factor expression in the developing iris. (A) MyoD immunoreactivity in E7 chick iris and ciliary body epithelium (arrowheads). Note the absence of myoD immunoreactivity in the prospective neural retina and stroma of the iris and ciliary body. (B) Higher magnification shows many cells of the non-pigmented iris epithelium with nuclear myoD immunoreactivity (arrows). (C) Mrf4 was not expressed in the iris at this stage in adjacent sections. (D) Epithelial cells of E7 iris and ciliary body expressed MyHC when explanted on collagen-agarose (arrows), but not in vivo (compare to Figure 1B). Immunoreactivity for both myoD (E) and mrf4 (F) was detectable in stromal cells of the iris and ciliary body in adjacent sections at E16. Normal rabbit serum staining was included as a control (G). Bar equals 1600 μm (A), 100 μm (B, C), 25 μm (D), 125 μm (E-G). C, cornea; NPE, non-pigmented epithelium; NR, prospective neural retina; OS, prospective ora serrata; PE, pigmented epithelium.

At E9, the myoD expression domain overlapped with invaginating epithelial cells that expressed smooth muscle markers. In a few instances, cells co-expressing myoD and αSMA were observed (Figure 6A-C, arrows). As individual cells are difficult to distinguish in vivo, we addressed co-localization in dissociated iris cell cultures where cell spacing is greater. Co-localization was observed on a low, but consistent basis in cultures fixed after 24 hours in vitro (Figure 6D-F). By 72 hours, no co-localization was observed, although both myoD and αSMA immunoreactive cells were present (data not shown). Two independent antisera, one against recombinant chicken myoD and another against human myoD, were used and showed similar staining patterns.

Co-expression of muscarinic and nicotinic acetylcholine receptors in myotubes derived from the iris

Two other markers for smooth and striated muscle were examined for co-localization within single iris cells. The expression of smooth (muscarinic) and striated (nicotinic) type acetylcholine receptors (AChRs) was characterized pharmacologically on myotubes derived from either E10 iris and ciliary body or embryonic pectoralis. Pilar et al. (1987) previously observed that intracellular recordings of iris myotubes showed both alpha-bungarotoxin (αBTX, an irreversible nicotinic AChR antagonist) sensitive and insensitive conductances in response to acetylcholine. Extending this observation, we found that muscarine evoked voltage changes in myotubes derived from the iris and ciliary body, but not from myotubes derived from embryonic pectoral muscle (Figure 7A and B). The muscarinic response could be blocked by the addition of the muscarinic antagonist atropine. In addition, the duration of depolarization with acetylcholine, which activates both muscarnic and nicotinic receptors, was longer in iris-ciliary body myotubes as compared to pectoral myotubes, consistent with the former expressing muscarinic receptors, which display slower kinetics. Using cRNA probes or antisera for chick muscarinic AChR sub-types, we found that both mRNA and protein for mAChR type2 were expressed in cultures from E10 iris and ciliary body, including within patches of single nucleated mesenchymal cells (typical of smooth muscle) and within multi-nucleated myotubes (Figure 7C-F).

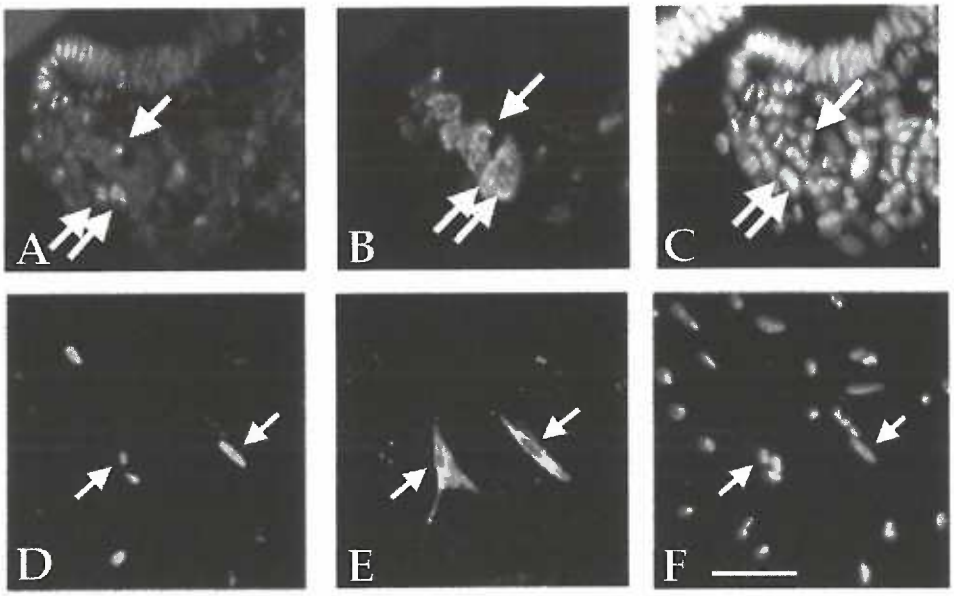


Figure 6. MyoD and smooth muscle marker co-localization. MyoD (A, D) and αSMA (B, E) expression in E9 iris in vivo (A, B, C) and in 24 hour dissociated cultures of E9 iris (D, E, F). Total nuclei were labeled with Hoechst dye (C, F). Bar equals 100 μm.

The other sub-types investigated, mAChR 3 and 4, were not detectable by either in situ hybridization or immunocytochemistry, suggesting that both smooth muscle and striated muscle of the iris and ciliary body express the same muscarinic sub-type. In accordance with the electrophysiological results, none of the muscarinic sub-types were expressed on pectoral myotubes. To investigate whether nicotinic receptors are expressed on iris-ciliary body derived myotubes at this age, we co-labeled cultures with rhodaminated-αBTX and MyHC (Figure 7G and H). Myotubes from iris-ciliary body were found to cluster nicotinic acetylcholine receptors as described previously for myotubes from other tissue sources (Anderson et al., 1977).

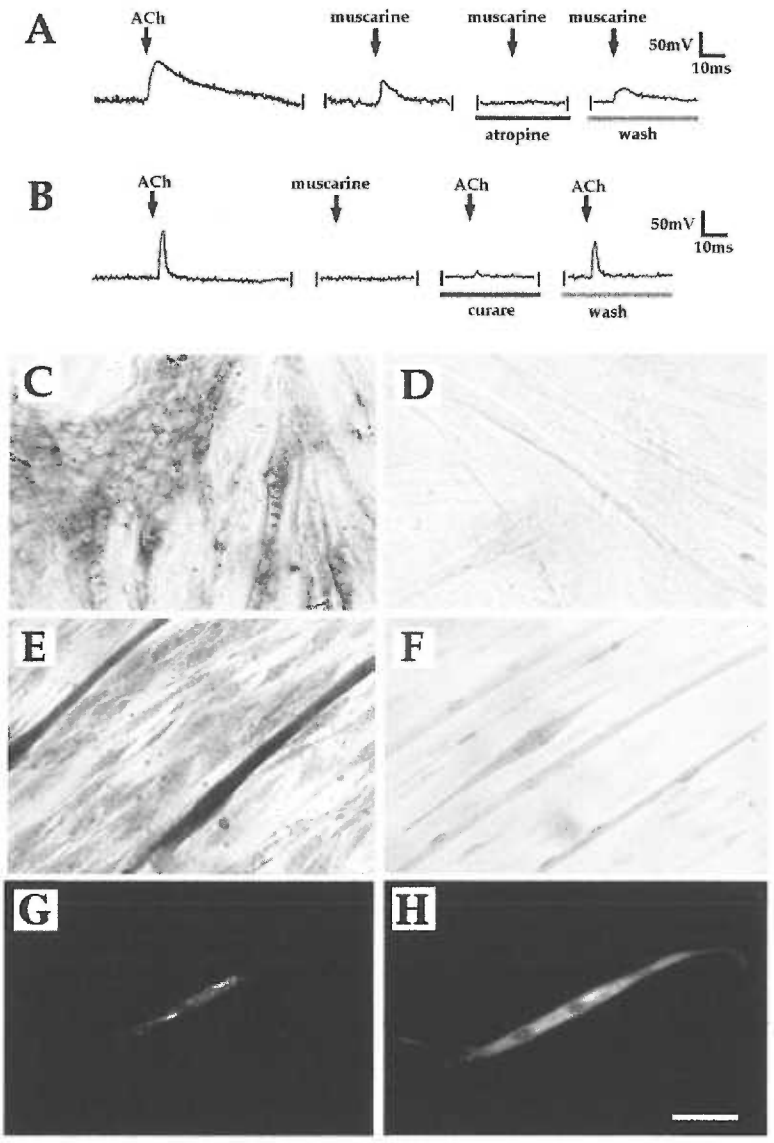


Figure 7. Iris and ciliary body myotubes express nicotinic and muscarinic ACh receptors. Electrophysiological characterization of iris-ciliary body (A) and pectoral (B) myotubes. Data shown are from continuous (~20-25 minutes), representative recordings. Acetylcholine (1 μM) or muscarine (1 μM) were used to activate AChRs. Atropine was used to antagonize muscarinic receptors (5 minute perfusion) and curare (10 μM) was used to antagonize nicotinic receptors. Recovery was assessed after 5 min. reperfusion. In situ RNA hybridization (C, D) and immunoreactivity (E, F) for muscarinic type 2 receptors in cultures of iris-ciliary body (C, E) and pectoral muscle (D, F). Nicotinic receptor clusters (α-bungarotoxin labeling) (G) on an iris derived myotube immunoreactive for MyHC (H). Bar, 75 μm.

Discussion

We have performed studies to examine the smooth-to-striated muscle transition during development of the avian iris and ciliary body. Here, we describe the maturation of iris and ciliary body muscle cells in vivo and investigate the lineage relationships and mechanisms of their development in vitro. At early developmental stages (E8, pre-transition), cells resident within the chick iris and ciliary body possess the capacity to form the mature striated muscle of these structures. When iris and ciliary body are removed from E8 embryos and explanted in vitro, myotube development is comparable to that in vivo. Our developmental studies of the capacity of the iris and ciliary body to from striated muscle indicate that either the competence of striated muscle precursors to form myotubes increases or that signals necessary for induction of the striated muscle phenotype are upregulated with developmental age. Using a novel method of analysis which permitted the identification and characterization of clones derived from single cells when placed in ectopic environments, we show that factors produced by cells within the iris and ciliary body potentiated striated muscle differentiation. We also found that multiple markers for smooth and striated muscle are co-expressed within cells during the transition. Our data argue against differentiation of smooth and striated muscle from a multi-potent muscle stem cell, but suggest that both migratory striated myoblasts as well as transdifferentiating smooth muscle contribute to iris myotubes.

Our results are consistent with chick-quail chimera studies suggesting an independent lineage for iris smooth and striated muscle cells (Nakano and Nakamura, 1985; Yamashita and Sohal, 1986; Yamashita and Sohal, 1987). In these experiments chick head mesoderm and neural crest were replaced with equivalent quail tissues prior to the onset of migration of these cells and the chimeric embryos were then allowed to develop. These authors observed many quail nuclei in iris myotubes, while the smooth muscle was composed entirely of host chick nuclei. We found that colonies arising from individual E8 iris cells contained only a single muscle cell type in a variety of environments. High survival rates of the clones (greater that 98%) and the presence of smooth muscle, striated muscle, and non-muscle colonies in each

condition strongly suggest that selection against a stem cell did not occur. While these results are consistent with separate lineages for many of the iris muscle cell precursors and argue against a multi-potent muscle stem cell mechanism of differentiation, they cannot exclude a role for transdifferentiation.

How are the clonal results consistent with transdifferentiation? Our observations are consistent with direct and rapid transdifferentiation of single smooth muscle cells into dividing, fusion competent myoblasts as co-localization of αSMA and myoD was observed in acutely isolated iris cells, but not in cells grown for 3 days in vitro. The contribution of transdifferentiation to the smooth-to-striated muscle transition is also supported by co-localization of other markers specific for each muscle-type. For example, Pilar et al. (1987) have shown that iris myotubes in vivo show both αBTX sensitive and insensitive depolarizations to acetylcholine. In iris myotubes isolated during the muscle transition, we show depolarization in response to muscarine and demonstrate that these myotubes express both message and protein for muscarinic sub-type 2 acetylcholine receptor - the same sub-type expressed in iridial smooth muscle. Furthermore, Volpe et al. (1993) showed that smooth and striated myosin heavy chains are co-expressed in iris cells during the transition of muscle types. Cumulatively these studies showing co-localization of multiple markers for each differentiated muscle cell-type support a role for transdifferentiation during the development of the iris muscles.

Several observations indicate that transdifferentiation is not the sole mechanism acting during the transition. The first comes from the chick-quail studies which definitively showed that prechordal mesoderm and cranial neural crest cells migrate to the prospective iris and ciliary body stroma and incorporate into differentiating myotubes. A second observation from our clonal studies suggests that not all the smooth muscle transdifferentiates into fusion competent myocytes. If transdifferentiation occurred in all of the smooth muscle, then all of the muscle clones would eventually form myotubes. However, in each of our clonal analyses stable smooth muscle colonies were formed. This result is consistent with the continued presence

into adulthood of smooth muscle within the pupillary domain of the iris constrictor muscle (Scapolo et al., 1988).

Transdifferentiation is uncommon, but more frequent among ocular tissues than elsewhere. The first clear demonstration of transdifferentiation was by Eguchi and Okada (1973) who showed through clonal analysis of chicken retinal pigmented epithelial cells that they convert to lens cells in vitro. Other examples of ocular plasticity include the switching of retinal pigmented epithelium to neural retina (Coulombre and Coulombre, 1965; Reh et al., 1987; Park and Hollenberg, 1991; Pittack et al., 1991), the conversion of dorsal iris cells into neural retina (Cioni et al., 1990), and corneal transdifferentiation to lens (Bosco, 1988). Each of these examples demonstrates the capacity of ocular cells to transdifferentiate after experimental manipulation. Transdifferentiation of cells during normal development has been shown for several contractile cell types. Patapoutian et al. (1995) provided evidence for a developmentally programmed transdifferentiation of smooth to striated muscle during mouse esophageal muscle differentiation and recently, the striated muscle in the electric fish, *Sternopygus macrurus*, was found to transdifferentiate into electrocytes which then form the electric organ (Patterson and Zakon, 1997).

Our observation that myoD immunoreactivity, but not mrf4, is found in the neuroepithelium of the optic cup was surprising, but not without precedence. In the mouse another bHLH muscle regulatory factor - myf 5, is expressed in the neural tube and later in subdomains of the brain (Tajbakhsh et al., 1994; Tajbakhsh and Buckingham, 1995). None of the other MRFs are expressed in these neuroepithelial cells and myogenesis is suppressed in vivo. However, when these cells are cultured in vitro, they go on to express other striated muscle markers. Interestingly, myf5 is the first MRF to be expressed in the mouse somite, whereas myoD is the first in the chick (Emerson, 1993). Other examples have been documented of neuroectodermally derived cells that can give rise to striated muscle in vitro, such as pituitary and brain stem (Brunner and Tschank, 1982; De Vitry et al., 1994). The significance of myoD expression in the prospective iris and ciliary epithelium is not understood. However, the subsequent sculpting of this expression to the margin of the iris and the co-localization with differentiating smooth muscle cells is consistent with

transdifferentiation. How myoD is repressed from initiating striated myogenesis is unknown, but inhibition of myoD function by phosphorylation and protein association has been demonstrated in other cells (reviewed in Ludolph and Konieczny, 1995).

The results presented in this paper, coupled with data from others, suggest a model for the differentiation of the chick iris and ciliary body. Head mesoderm and cranial neural crest migrate over the neuroepithelium of the optic cup and form, among other structures, the prospective stroma of the iris and ciliary body. This stroma is composed of specified, but undifferentiated cells, including those of the striated muscle cell lineage. Within the iris, neuroectodermal cells at the pupillary margin invaginate into the overlying stroma and differentiate into smooth muscle cells. Whether mesodermally derived cells also express smooth muscle characteristics, either stably or transiently, has not been investigated. This possibility seems likely within the ciliary body, as no invaginating epithelium has been described in this region. We propose that striated muscle differentiation of the avian iris and ciliary body is then initiated in cells derived from multiple embryonic origins including head mesoderm (Yamashita and Sohal, 1986; Yamashita and Sohal, 1987), neural crest (Johnson et al., 1979; Nakano and Nakamura, 1985), and the neuroectoderm via transdifferentiation of smooth muscle cells (Volpe et al., 1993; this study). We favor this model as it is consistent with published observations from others, as well as with the clonal analysis and co-localization studies described here.

Acknowledgements

We thank Dr. Jan Christian, Dr. Abbie Jensen, Dr. Gary Reiness, and members of the Nishi Lab for invaluable discussions and critical reading of this manuscript. We also thank the following: Dr. Steve Matsumoto for help with electrophysiological recordings, in whose lab those experiments were performed; Diane Darland for suggestions of smooth muscle markers; and Shannon Carey and Marc Carey for help with statistical analysis of the clonal experiments. We gratefully acknowledge Dr. Neil Nathanson (antiserum and riboprobe constructs for chicken muscarinic receptors) and Dr. Bruce Paterson (chicken myoD atiserum) for providing reagents. Finally, we thank Dr. David Beebe for sharing results prior to publication. This work was supported by grants 5T32EY07123 (B.L.) and 2RO1NS25767 (R.N.).

CHAPTER IV

Development of the Avian Iris and Ciliary Body: The Role of Activin and Follistatin in Coordination of the Smooth-to-striated muscle transition.

Brian A. Link and Rae Nishi*

Department of Cell and Developmental Biology, L215
Oregon Health Sciences University
3181 SW Sam Jackson Park Road
Portland, Oregon 97201

*To whom correspondence should be addressed.

Abstract

Although general principles have been established in the regulation of vetebrate organogenesis, the specific molecules responsible for such signaling are just being identified. We have studied differentiation in the avian iris and ciliary body which undergoes a transition from smooth to striated muscle. Using heterochronic co-cultures, we have found that striated muscle differentiation in pre-transition (E8) cells is induced by mid-transition (E11) cells through a secreted and soluble activity. In addition, contact-mediated mechanisms among pre-transition cells prevented precocious striated muscle differentiation. We have tested the role of activin and its antagonist follistatin, as candidate regulators of this muscle transition. Activin induced smooth muscle differentiation while repressing striated muscle development. Conversely, follistatin promoted the emergence of striated muscle, while inhibiting smooth muscle differentiation. Significantly, secreted follistatin activity was found to increase during the smooth-to-striated muscle transition. Moreover, the striated muscle inducing activity from mid-transition iris and ciliary body cell conditioned medium was depleted with an activin-affinity column which binds follistatin. These results suggest that activin and follistatin coordinate differentiation in the avian iris and ciliary body.

Introduction

Secreted signaling molecules play an important role in mediating inductive interactions during embryogenesis. For example, dorsoventral pattern within both ectodermal and mesodermal germ layers is specified by signals originating from the embryonic organizer during gastrulation. Further refinement of the body plan occurs during organogenesis, where cells from multiple embryonic origins differentiate in a spatiotemporally controlled fashion. The identity of the molecules that coordinate these events have only recently begun to be elucidated. Examples of local signaling events that have been well studied include limb formation (reviewed in Johnson and Tabin, 1997) and kidney development (reviewed in Vainio and Müller, 1997).

The structures in the anterior chamber of the eye arise from diverse embryonic origins and develop under the influence of the local signaling environment. Specification of the prospective iris and ciliary body epithelium is directed by signals from the lens (Genis-Galvez, 1966; Stroeva, 1967; Beebe et al., submitted). However, lens transplant and extirpation experiments in the chick embryo suggest that the signals responsible for specification of the iris and ciliary body are distinct from signals that govern differentiation within these structures (Dhawan et al., submitted). Differentiation of these tissues proceeds in a centripetal fashion dorsally and then temporally from the edge of the optic fissure. This wave of differentiation is consistent with controlled, inductive signaling.

Our studies have focused on the smooth-to-striated muscle transition in the iris and ciliary body. Cell culture experiments have shown that iris and ciliary body explants possess the capacity for self-differentiation, demonstrating that the signals that regulate differentiation in these structures are generated locally (Ferrari and Kock, 1984; Link and Nishi, submitted). Furthermore, clonal analysis of undifferentiated iris cells co-cultured in various environments suggests that extrinsic factors can regulate differentiation: the number of clones that differentiate as striated muscle increase in the presence of mature, differentiated iris-ciliary body cells (Link and Nishi, submitted).

Activin A, a TGFβ superfamily member, and its antagonist follistatin, are candidates for regulating the smooth-to-striated muscle transition based on expression patterns and previously described bioactivities. The TGFβ superfamily encompasses a large group of secreted proteins with conserved primary and secondary structures (reviewed in Wall and Hogan, 1994; Moses and Serra, 1996). The efficacy of these proteins can be regulated at multiple levels including activation by proteases, limiting availability through matrix sequestration, and reducing bioactivity through interaction with secreted binding proteins. Follistatin, which can bind activin and prevent activin signaling, has recently been shown to antagonize other TGFβ superfamily members including BMP-2, 4, and 7 (Nakamura et al., 1990; Yamashita et al., 1995; Fainsod et al., 1997). Activin and follistatin are expressed in the periocular mesenchyme during ocular morphogenesis (Feijen et al., 1994; Verschueren et al., 1995) and in the chick iris and ciliary body during the smooth-to-striated muscle transition (Darland et. al., 1995). These proteins have been proposed as target derived factors that modulate neuropeptide phenotype in the neurons that innervate the iris and ciliary body (reviewed in Coulombe and Kos, 1997). Functionally, activin and follistatin modulate skeletal muscle differentiation in culture: activin represses differentiation, while follistatin, presumably through interaction with activin or related molecules, potentiates muscle differentiaton (Link and Nishi, 1997; Shiozuka et al., 1997). The present study addresses the regulation of the smooth-to-striated muscle transition in the iris and ciliary body and tests the hypothesis that activin and follistatin function to coordinate this process.

Results

E11 iris-ciliary body cells secrete a muscle inducing activity

We performed heterochronic and heterotypic co-culture experiments under various conditions to investigate the environmental influences that regulate the emergence of striated muscle from the iris and ciliary body. In the first experiment, cells from E7 quail iris and ciliary body were dissociated and cultured alone or in the presence of chicken cells including embryonic fibroblasts, other undifferentiated (E8) iris cells, E11 iris-ciliary body cells, or pectoral striated muscle cells. Previous studies have shown that E11 is the age which gives the greatest proportion of myotubes in dissociated iris and ciliary body cultures (Link and Nishi, submitted). We found that co-culture with E11 iris-ciliary body or pectoral myoblasts resulted in increases in the proportion of E7 quail cells that expressed MyHC as compared to equal numbers of cells cultured alone. Co-culture with other undifferentiated iris-ciliary body cells repressed MyHC expression, while co-culture with embryonic fibroblasts did not alter the expression of this striated muscle marker (Figure 1A). Inhibition of MyHC expression in cultures of E7 quail iris-ciliary body cells alone was also found to be cell density dependent (data not shown).

To test whether the increased number of nuclei within MyHC expressing cells in co-cultures was due to non-specific cell fusion or whether this was due to a secreted activity from the surrounding chick cells, we performed trans-filter experiments. Experiments were carried out as before, except undifferentiated E7 quail cells were grown alone or 300 μm above the other cell types on a porous substrate that allowed free diffusion of macromolecules, but maintained the isolation of the two cultures. As before, culture with E11 iris-ciliary body cells resulted in a greater proportion of quail cells that expressed MyHC (Fig. 1B). Interestingly, the inhibition observed with co-culture with other undifferented iris-ciliary body cells was not maintained in the transwell assay suggesting that this inhibition was contact-mediated.

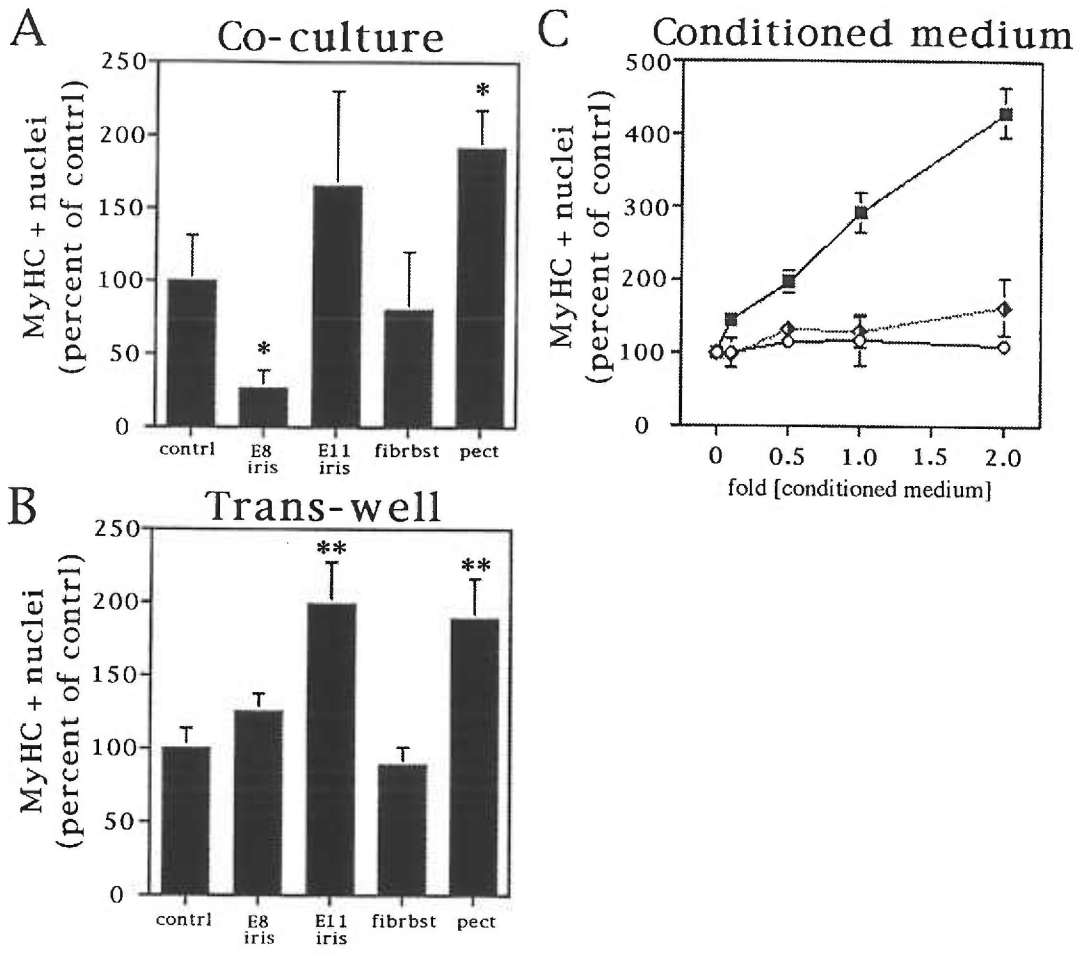


Figure 1. Older iris-ciliary body cells secrete a soluble activity that can potentiate striated muscle formation in younger iris-ciliary body cells. (A) Undifferentiated E7 quail iris-ciliary body cells were co-cultured with the following chick cells: other undifferentiated iris-ciliary body cells (E8 iris), older iris-ciliary body cells (E11 iris), embryonic fibroblasts, and pectoral striated myoblasts. The number of nuclei in MyHC immunoreactive cells was scored after 4 days in culture (n=3). Quail: chick cells were plated in a ratio of 1:5 ($1x10^4$ and $5x10^4$ cells per ml, respectively). (B) Plating and culture conditions were preformed as in co-cultures, except quail cells were physically separated by 300 µm using trans-well polyamide inserts (n=4). (C) Control medium (open circles), embryonic fibroblast conditioned medium (half-filled diamonds), and E11 iris-ciliary body conditioned medium (closed squares) were concentrated 10 fold and added to E7 quail iris-ciliary body cells at 0.1-2.0 fold final concentrations. Medium was replaced daily until day 4, when MyHC immunoreactivity was scored (n=4). *$p \leq 0.05$; **$p \leq 0.01$ as compared to controls (2-tailed t-test).

Fibroblasts again provided a neutral environment with respect to striated muscle development. When concentrated E11 iris-ciliary body conditioned medium was applied to E7 quail iris-ciliary body cells a dose-dependent increase in striated muscle development was observed (Fig. 1C). Fibroblast conditioned medium at high concentrations only slightly potentiated striated muscle development in undifferentiated iris cells. These results suggest that within the iris and ciliary body, undifferentiated (E7) cells are competent to respond to temporal changes (E7 vs. E11) in secreted, soluble signals that can regulate the muscle differentiation.

Follistatin increases with development in the iris and ciliary body

Because follistatin regulates striated muscle development in other systems (Matzuk et al., 1995; Amthor et al., 1996; Link and Nishi, 1997; Shiozuka et al., 1997), and mRNA and protein for this molecule is expressed by the chick iris and ciliary body (Darland et al., 1995) and in pectoral muscle cultures (Link and Nishi, 1997), we tested whether follistatin-like bioactivity was present during the smooth-to-striated muscle transition. Follistatin is a binding protein and an antagonist for specific members of the TGFβ superfamily. Among the TGFβ superfamily members that interact with this molecule, follistatin shows greatest affinity for the activins (Yamashita et al., 1995). Activin A mRNA and protein are also expressed within the developing chick iris and ciliary body; thus, the relative concentration of these molecules dictate bioactivity (Darland et al., 1995). We employed an activin-dependent erythrocyte differentiation assay (Schwall and Lai, 1991) to test the ability of iris-ciliary body conditioned medium to either potentiate (activin-like bioactivity) or inhibit (follistatin-like bioactivity) the synthesis of hemoglobin in these cells (Figure 2). We found that concentrated conditioned medium from either E8 or E11 iris-ciliary body was able to inhibit activin induced-hemoglobin synthesis, but not hemin-induced differentiation, in K562 cells. Dose-response experiments indicated that the levels of follistatin-like bioactivity increased from E8 to E11, the time when striated myogenesis occurs in these structures in vivo (Figure 3).

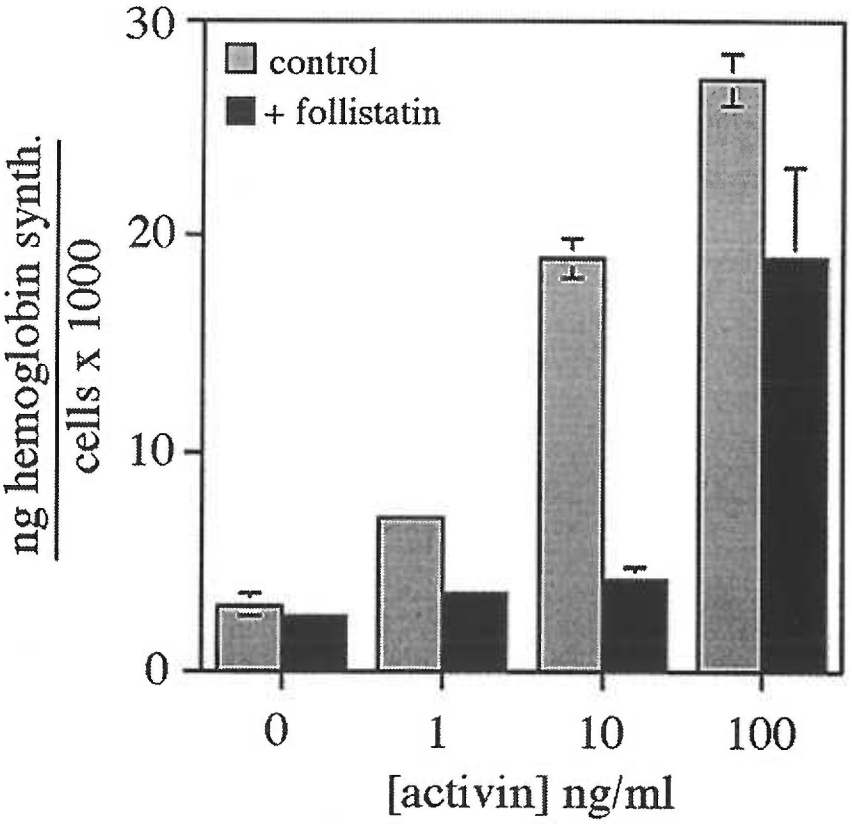


Figure 2. Bioassay for activin and follistatin-like bioactivity. Response of K562 ethrythrocytes (hemoglobin synthesized) to increasing concentrations of activin in control medium (gray bars) or with 100 ng/ml of follistatin (black bars). Data represents mean ± standard error (n=3) from one representative experiment.

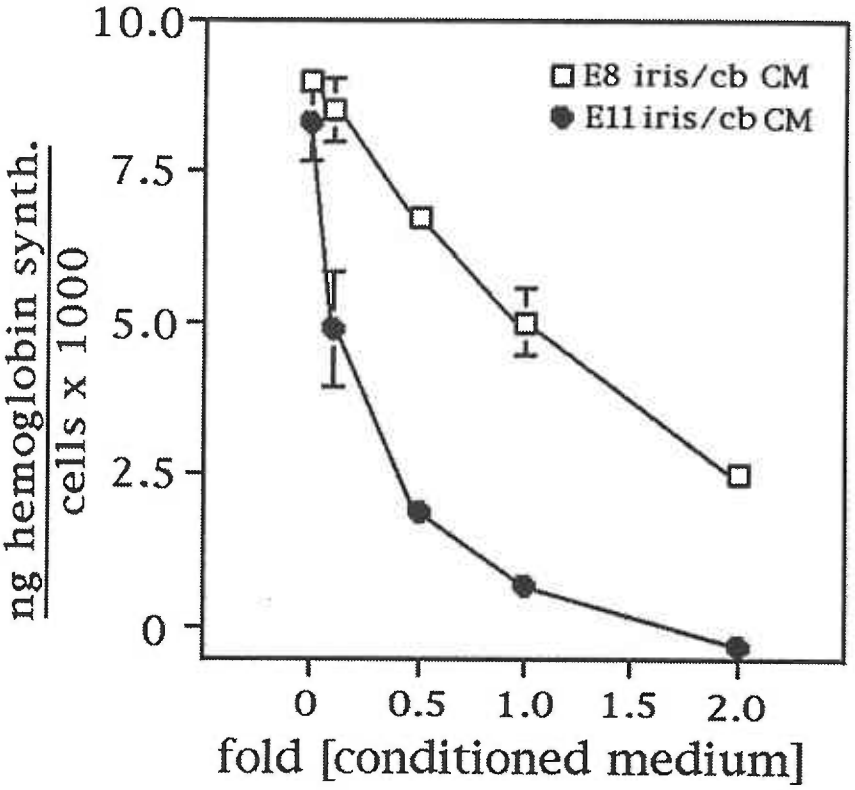


Figure 3. Follistatin-like bioactivity increases from E8 to E11: comparison of E8 vs E11 iris-ciliary body conditioned medium. Measurements of hemoglobin synthesis in K562 cells cultured with 4 ng/ml activin with increasing concentrations, 0.1-2.0 fold, of either E8 iris-ciliary body conditioned medium (open squares) or E11 iris-ciliary body conditioned medium (closed circles). Data represents mean ± standard error (n=3) from one representative experiment.

Activin and follistatin regulate muscle differentiation

We next addressed whether recombinant activin and follistatin could modulate the development of either smooth or striated muscle from cultures of undifferentiated iris-ciliary body. Activin A increased the proportion of cells that expressed smooth muscle actin, while inhibiting the expression of striated MyHC. Exogenous follistatin had the opposite effects: smooth muscle development was repressed, while striated muscle development was potentiated (Figure 4). Total cell numbers and the proportion of BrdU labeled cells, an index of mitosis, were not dramatically altered with either treatment (Figure 4B). The secretion of follistatin-like bioactivity and the ability of exogenous follistatin to potentiate striated muscle development in cultures of undifferentiated iris and ciliary body suggest that follistatin is a component of the muscle inducing activity found in conditioned medium from the maturing iris and ciliary body.

To test whether follistatin is a constituent of the E11 iris-ciliary body muscle inducing activity, we constructed an activin A affinity column to remove activin-binding proteins from conditioned medium. E11 iris-ciliary body conditioned medium retained the abiltiy to stimulate striated muscle development in undifferentiated iris-ciliary body cells when passed through a control column (ethanolamine linked agarose). However, passage through the column packed with activin A linked agarose depleted the ability of E11 iris-ciliary body conditioned medium to stimulate striated muscle development (Figure 5). As observed before, fibroblast conditioned medium did not potentiate striated muscle development.

Because the local environment is altered with cell dissociation, we tested whether activin and follistatin could regulate striated muscle development in anterior chamber explants, where three-dimensional cellular contacts are maintained. Intact E8 chick iris, ciliary body, and associated lens were cultured on collagen-agarose (Figure 6A) with exogenous activin, follistatin, or in control medium.

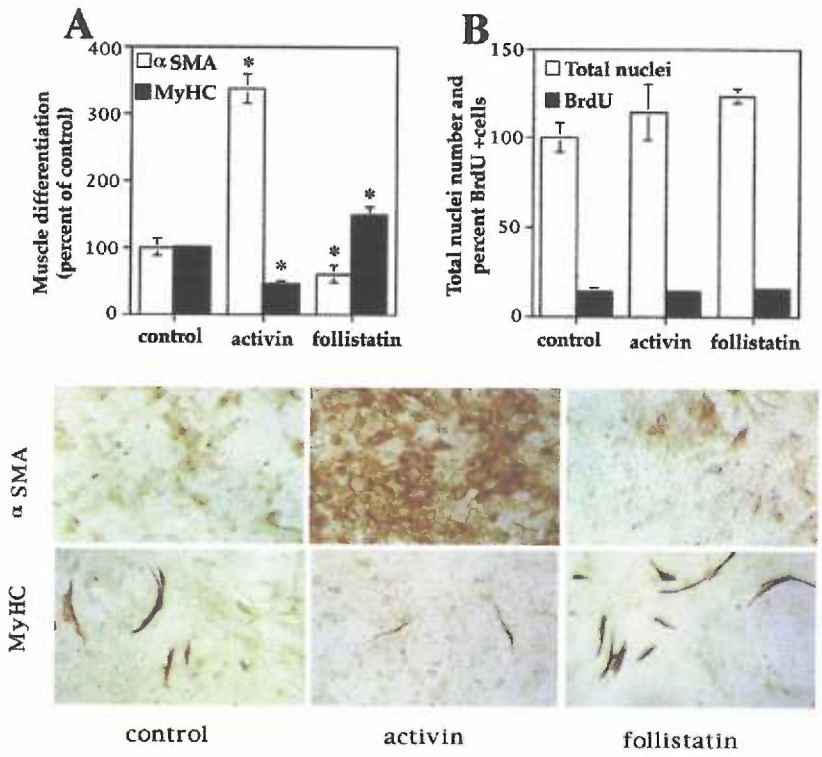


Figure 4. Effects of activin and follistatin on undifferentiated iris-ciliary body cells. (A) Comparison of the number nuclei in αSMA (open bars) or MyHC (closed bars) immunoreactive cells in sister cultures of dissociated E8 iris-ciliary body after 4 days. (B) Comparison of total nuclei (open bars) and BrdU incorporation (closed bars) in cultures of dissociated E8 iris-ciliary body after 4 days. Total nuclei are graphed as percentage of control conditions, while BrdU incoporation respresents percent of cells undergoing DNA synthesis in each culture. (C-H) Morphology of αSMA immunoreactive (C, D, E) and MyHC immunoreactive (F, G, H) cells in control medium (C, F), with 10 ng/ml activin (B, G), or 35 ng/ml follistatin (E, H) (n=4). *$p \leq 0.005$ as compared to controls (2-tailed t-test).

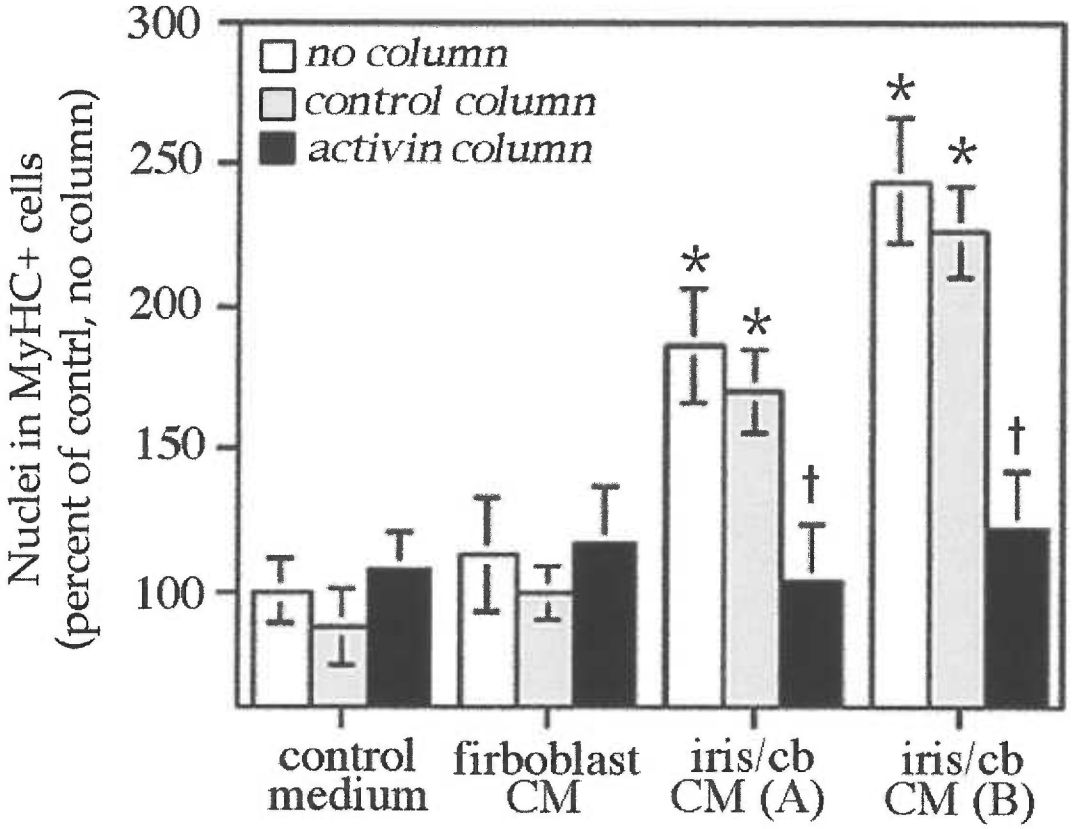


Figure 5. Depletion of activin-binding proteins: effects on striated muscle differentiation. Dissociated E8 iris-ciliary body cells were cultured with conditioned medium from control medium, fibroblast conditioned medium, or one of two batches of E11 iris-ciliary body conditioned medium (A and B) that were added directly (open bars), or run through either a control column (ethanolamine linked to agarose, gray bars) or an activin column (activin linked to agarose, black bars). The number of nuclei in MyHC immunoreactive cells were scored after 4 days in culture. Data represents mean ± standard error (n=3). *$p \leq 0.001$ as compared to control medium, no column; †$p \leq .003$ as compared to equivalent conditioned mediums, no column.

After 4 days, explants were analyzed either immunohistochemically for MyHC expression or for creatine phoshphokinase (CPK) activity, a marker for striated muscle differentiation. Immunoreactivity for MyHC suggested that activin repressed, while follistatin promoted striated muscle development (Fig 6C-E). Quantitation of CPK activity from explant homogenates confirmed these observations (Fig 6B).

Discussion

The focus of the present study was to identify local influences that regulate the transition from smooth-to-striated muscle in the avian iris and ciliary body. We have found that secreted, soluble signals present during the transition promote striated muscle differentiation. In addition, our data are consistent with contact-mediated inhibition of striated muscle development within the undifferentiated iris and ciliary body prior to the transition. Removal of activin-binding proteins blocked muscle induction by conditioned medium from cultures of maturing (E11) iris and ciliary body. We have previously shown that both activin and its antagonist - follistatin, an activin-binding protein, are expressed throughout the transition from smooth-to-striated muscle (Darland et al., 1995). Here, we measured follistatin bioactivity and found that it is upregulated during differentiation in the iris and ciliary body. In cultures of undifferentiated iris and ciliary body cells, activin promoted smooth muscle, while inhibiting striated muscle, differentiation. Follistatin displayed the reverse effect: striated muscle differentiation was increased, while smooth muscle differentiation was repressed. These results suggest that activin and follistatin are key local modulators of muscle morphogenesis in the anterior chamber of the avian eye.

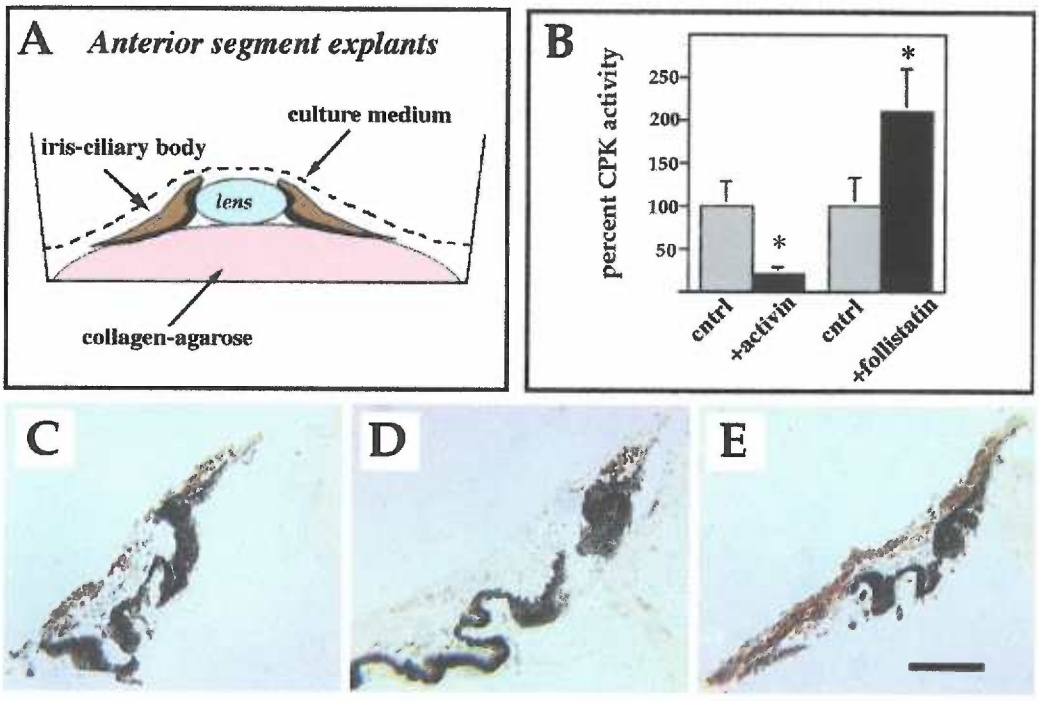


Figure 6. Effects of activin and follistatin on striated muscle development in anterior segment explants. (A) Diagram of explant experiments. Collagen/agarose cushions were equilibrated with control or activin- and follistatin-containing medium prior to explanting. A low volume of medium was used to form a thin layer over the explant. This prevented drying, but provided adequate oxygen exchange. (B) Striated muscle development (CPK activity) in the iris-ciliary body after 4 day treatments. For either activin or follistatin treated explants, control explants were removed from the same embryo and placed into serum free control conditions. Data represents mean ± standard error (n=6). *$p \leq 0.01$ as compared to control. (C-E) MyHC expression (brown immunoreactivity) in 8 μm sections through respresentative explants of treated with control medium (C), 20 ng/ml activin (D), or 100 ng/ml follistatin (E). In each the collagen/agarose cushion is down and the lens is situated to the right. Bar respresents 900 μm.

Contact-mediated repression of striated muscle

Prior to the initiation of the smooth-to-striated muscle transition, inhibitory signals, in addition to that of activin, may prevent precocious differentiation of striated muscle. We found that cells derived from the pre-transition iris and ciliary body were able to reduce striated muscle differentiation in E7 quail iris cells. This effect was only observed when cell contact was maintained: no repression was observed when the cells were physically separated by trans-wells or when conditioned medium from these young iris-ciliary body cells was applied. This suggests that contact-mediated and/or non-diffusible signals can prevent striated muscle differentiation at early stages of iris and ciliary body development. In addition, this observation lends insight into why striated myogenesis is not initiated by E8 iris-ciliary body cultures, which show follistatin bio-activity (albeit at low levels). Candidate molecules for contact-mediated repression include members of the Notch/lin-12 family. In mammals, notch and its ligand, jagged, are expressed in the developing ciliary body (Bao and Cepko, 1997). Activation of these transmembrane molecules has been shown to inhibit striated myogenesis in cultured cells and in Xenopus embryos (Kopan et al., 1994; Nye et al., 1994). The muscle induction observed in co-cultures with older iris-ciliary body cells suggest that contact-mediated repression is either down regulated with age, or induction by soluble factors exceeds a threshold necessary to overcome this inhibition.

Striated muscle regulation by follistatin

Several observations suggest that secreted follistatin is the primary signal for promoting striated muscle differentiation during the transition from smooth-to-striated muscle. Two results from this study support this view. First, the increase in striated muscle development within E7 iris-ciliary body cells was similar when cellular contact was allowed (co-culture experiments) and when only secreted, soluble signals could pass between the two cell types (trans-well experiments). This similarity in proportion of muscle induction was not due to saturated differentiation of striated muscle cell precursors. Addition of concentrated conditioned medium pushed a greater number of cells to differentiate than either of the co-culture assays. These data underline the importance of secreted signals from E11 iris-ciliary body cells that were

isolated at the peak of striated myogenesis. Secondly, nearly all of this induction could be abolished by the removal of activin-binding proteins, demonstrating activin-antagonism as the chief component of the inducing activity.

Follistatin, a secreted activin-antagonist is known to be up-regulated during the muscle transition (Darland et al., 1995); however, other activin-binding proteins may also be present at this time and inhibit the biological effects of activin. Potentially, the recently identified follistatin-like protein (Flik) is one such molecule. Flik protein is highly homologous to follistatin in the growth factor-binding domain and is likely a secreted glycoprotein (Patel et al., 1996). During development, Flik is often co-expressed with follistatin, including sites of muscle differentiation (Amthor et al., 1996); however, there is no evidence that Flik binds or inhibits activin. Follistatin or related proteins may also modulate the bioactivity of signaling molecules other than activin. Recent experiments indicate that follistatin can bind and antagonize bone morphogenetic proteins (BMPs) 2, 4, and 7. Interestingly, these molecules have also been shown to inhibit striated myogenesis (Yamaguchi et al., 1991; Murray, 1993; Duprez, 1996; Pourquie, 1996) and, in undifferentiated neural crest cells, BMP-2 can promote smooth muscle differentiation (Shah et al., 1996). Furthermore, targeted gene disruption of follistatin and members of the TGFβ superfamily, demonstrate an essential role for these proteins in myogenesis (Matzuk et al., 1995; McPherron et al., 1997) and ocular morphogenesis (Dudley et al., 1995; Luo et al., 1995; Jena et al., 1997; Sanford et al., 1997).

Previous experiments testing the effects of follistatin or activin on homogeneous cultures of skeletal muscle myoblasts showed that these proteins act during the early phases of differentiation, where activin was inhibitory and follistatin was stimulatory. Proliferation and survival of myoblasts were not affected by either molecule (Link and Nishi, 1997; Shiozuka et al., 1997). Moreover, we have shown E11 iris-ciliary body cells stimulate myogenesis by promoting the differentiation of determined, resident striated muscle precursors (Link and Nishi, submitted). Analysis of colonies derived from single undifferentiated iris cells grown in the presence of either Ell iris-ciliary body cells or a non-inducing cell type, demonstrated

that striated muscle differentiation occurs primarily at the expense of undifferentiated/non-muscle clones.

Smooth muscle diffferentiation

Our results from experiments investigating the effects of activin and follistatin on smooth and striated muscle development suggest that the differentiation of the two muscle types may be coordinately regulated in the iris and ciliary body. Activin specifically promotes smooth muscle differentiation in E7/8 iris cultures, while follistatin inhibits differentiation of this muscle type. Conversely, the differentiation of striated muscle in sister cultures was reduced in responce to activin, while follistatin promoted myotube formation. In accordance with this, the relative concentration of follistatin:activin, as measured in a bioassay, was found to increase during the transition. However, cells derived from E7/8 iris and ciliary body secrete activin inhibiting activity during the time that smooth muscle differentiation is initiated in vivo. Several plausible explanations exist for this observation. First, regionally elevated concentrations of activin may be present at this age to facilitate smooth muscle differentiation. Indeed, in the iris, smooth muscle differentiation is confined to the pupillary margin where invaginating epithelial cells undergo an epithelial to muscle cell transition. In addition, other signaling molecules, that are not antagonized by follistatin, may regulate smooth muscle differentiation. One such candidate, TGFβ, is known to promote smooth muscle development, while inhibiting striated muscle, in multiple cell types (reviewed in Moses and Serra, 1996), including undifferentied iris and ciliary body cells (Link and Nishi, unpublished observations). In mammals, TGFβ1 and β2 are secreted by resident cells of the iris and cilliary body (Knisely et al., 1991; Pasquale et al., 1993), but the expression of these molecules during development of the avian iris and ciliary body is unkown.

Follistatin as a multifunctional coordinator of differentiation

This study was focused on understanding the role of the local environment in regulating avian iris and ciliary body muscle differentiation, and in particular, on understanding the emergence of multi-nucleated striated

muscle. By identifying follistatin as a regulator of the smooth-to-striated muscle transition, we have confirmed previous studies that suggested cellular differentiation in the iris and ciliary body is controlled by signals generated within these tissues (Ferrari and Koch, 1984; Link and Nishi, accompanying paper; Dhawan et al., submitted). Specification of this tissue, however, is accomplished by signals derived from the lens epithelium (Genis-Galvez, 1966; Stroeva, 1967; Beebe et al., submitted). Following specification of the iris and ciliary body, epithelial cells at the pupillary margin of the iris invaginate into the overlying stroma and differentiate into smooth muscle. We have previously proposed that the fusion competent myocytes which form the multi-nucleated striated muscle of the avian iris and ciliary body are derived from smooth muscle, via transdifferentiation, together with migratory mesenchymal cells from the embryonic head region. We now suggest that activin and yet to be identified signals, perhaps TGFβ, regulate the begining of this transition by promoting smooth muscle differentiation, while inhibiting striated muscle development. Locally produced activin inhibitors, including follistatin, are then upregulated and permit striated muscle differentiation.

Lastly, our results lend support to a model in which activin-antagonism serves a dual function in neuron-target maturation. In the iris and ciliary body, follistatin has previously been shown to modulate neuropepide phenotype, by repressing somatostatin induction in ciliary neurons (Coulombe et al., 1993; Darland et al., 1995; Kos and Coulombe, 1997). These functions, neuron phenotype regulation and target differentiation, suggest that follistatin produced within the iris and ciliary body, ensures temporal coordination in the differentiation of these intimately associated cell types.

Acknowledgements

We thank Dr. Jan Christian, Diane Darland, Dr. Abbie Jensen, Vivian Lee, and Dr. Gary Reiness, for critical reading of this manuscript and all the members of the Nishi and Eckenstein lab for invaluable discussions. We gratefully acknowledge Dr. Brian Drucker for providing K562 cells. Finally, we thank Dr. David Beebe for sharing results prior to publication. This work was supported by grants 5T32EY07123 (B.L.) and 2RO1NS25767 (R.N.).

DISCUSSION

Summary of experiments and results

Through the experiments presented in this dissertation, I have examined the development of the avian iris and ciliary body with respect to muscle differentiation and neuron maturation. Using multiple markers specific for smooth and striated muscle cells, I confirmed the embryonic transition from smooth to striated muscle in the iris and described a similar conversion in the ciliary body. In addition, I established and validated culture models for iris and ciliary body muscle development. To test the cell-type potential of individual undifferentiated cells, and to begin to address the role of non-cell autonomous regulation during iris and ciliary body muscle differentiation, I performed a series of clonal analyses in both heterochronic and heterotypic environments. Although smooth, striated, and non-muscle cell types were obtained in all conditions tested, progeny from single cells differentiated as one lineage. However, multiple markers for smooth and striated muscle were transiently co-expressed in individual iris cells in vivo and in vitro. Non-cell-autonomous (extrinsic) influences on differentiation were observed, as the proportion of striated muscle clones obtained from undifferentiated iris cells was potentiated in co-culture with older iris and ciliary body cells.

Activin and its antagonist, follistatin, were tested for their role in regulating the transition between smooth to striated muscle cells and modulating neuropeptide phenotype of the innervating ciliary neurons. Protein expression and bioactivities for these two molecules were described in the developing iris and ciliary body in vivo and in vitro. Analysis of conditioned medium from E11 iris and ciliary body cells indicated that secreted factors from these structures could (A) repress activin-mediated somatostatin induction in ciliary neurons and (B) potentiate striated muscle formation in undifferentiated iris and ciliary body cells. Both of these effects could be attenuated by removal of follistatin. Addition of activin to undifferentiated iris and ciliary body cultures potentiated smooth muscle development, while inhibiting striated muscle differentiation. Conversely, follistatin potentiated striated muscle development and inhibited smooth muscle differentiation.

Lastly, contact-mediated inhibition of striated muscle development was observed in isochronic cultures of undifferentiated iris-ciliary body cells. The following list contain the main conclusions drawn from these studies.

Conclusions

1. Both the avian iris and ciliary body undergo a smooth-to-striated muscle transition during development.

2. Clonal analyses results have provided evidence against the existience of a multi-potent muscle stem cell resident within the iris and ciliary body. However, muscle marker co-localization studies have provided evidence that smooth muscle cells transdifferentiate into fusion-competent striated muscle myocytes during the transition.

3. Signals intrinsic to the iris and ciliary body can regulate muscle differentiation and provide retrograde signals for ciliary neuron maturation.

4. Activin and follistatin are expressed within the developing iris and ciliary body, and follistatin bioactivity increases during the differentiation of these structures.

5. Follistatin derived from the iris and ciliary body functions to represses activin and prevent somatostatin induction in ciliary neurons.

6. During iris and ciliary body development, increasing amounts of follistatin promote striated muscle differentiation while inhibiting smooth muscle development. Precocious differentiation of striated muscle is prevented by non-diffusible signals present within the iris and ciliary body before the muscle transition.

The results from my thesis research support a model where locally produced signaling molecules, activin and follistatin, coordinate the differentiation of resident muscle cell presursors (smooth and striated muscle development) and maturation of the innervating neurons (neuropeptide phenotype). This model is discussed in more detail at the end of this chapter.

Activin and follistatin regulate determination and differentiation for multiple cell types during development

Since the original descriptions that activin and follistatin regulate the release of hormones from the pituitary, these proteins have been shown to modulate determination and differentiation of many cell types in addition to those in the anterior chamber of the eye. Both the activins and follistatin, as well as receptors for the activins, are expressed in multiple regions of the embryo throughout development (Albano et al., 1994; Feijen et al., 1994; Stern et al., 1995; Verschueren et al., 1995). The following examples demonstrate the diversity of actions ascribed to activin and follistatin.

Activin and follistatin function before gastrulation

At the onset of gastrulation, the cells of the blastula become committed to mesoderm, endoderm, and ectoderm. Numerous studies have demonstrated the importance of activin and follistatin in regulating the formation and patterning of mesoderm. Using explants of Xenopus blastula cells (animal caps) that normally will only form epidermis, Mitrani et al., 1990 and Thomsen et al., 1990 showed that exogenous activin will induce mesodermal tissues including mesenchyme, somites, and notochord. In those studies, the formation of neural tissue was also evident. Ectopic expression of activin by ventral injections of mRNA into a 32 cell Xenopus embryo elicited a second axis with dorsal mesoderm and anterior neural characteristics. Inhibition of activin signaling in developing Xenopus embryos diverted prospective ectoderm and endoderm to a neural fate (Hemmati-Brivanlou and Melton, 1994). In that study, activin inhibition was accomplished by overexpression of a truncated activin type II receptor, which was deficient in kinase activity and acted as a dominate negative. This reagent was later shown to inhibit signaling of other TGFβ superfamily members (Schulte-Merker et al., 1994) and cast doubt on the role of activin in mesoderm formation. However, a direct role for activin and activin-antagonism in early development was demonstrated using reagents that specifically inhibited activin signaling (Wittbrodt et al., 1995; Dyson and Gurdon, 1997). Furthermore, the endogenous inhibitor, follistatin, is expressed in the embryonic organizer and

notochord, two tissues with neural inducing activity, and application of follistatin to animal cap explants leads to neuralization in the absence of mesoderm (Hemmati-Brivanlou, 1994). The importance of local activin concentrations in patterning mesodermal tissue was shown by Stern et al., 1995. By explanting mid-blastula stage chick tissue (anterior-lateral and posterior margin), these authors demonstrated that specific concentrations of activin differentially induced markers of several mesodermal cell types including organizer/node cells, smooth muscle, striated muscle, and notochord.

Activin and follistatin during organogenesis

While the studies described above demonstrate the importance of activin signaling in the pre-gastrulating embryo, other studies have suggested a role for activin and follistain in regulating cellular decisions during later stages of development. For example, in chicks, activin-coated beads implanted in lateral mesoderm can establish sonic hedgehog asymmetry and regulate heart laterality (Levin et al., 1995). Consistent with a role in determining left-right pattern, the activin receptor IIA is expressed asymmetrically in the ectoderm of the primitive streak. In developing parenchymal organs such as kidney, lung, salavary glands, and pancreas, epithelial branching patterns may be defined by the actions of activin and follistatin. Both proteins are expressed in each of these tissues, and application of activin will cause branching malformations in two tissues tested, the kidney and salivary gland (Gilbert, 1996). Targeted disruption of the activins and follistatin in mice also suggest roles for these proteins in organogenesis. Activin βA disruption leads to lack of wisker formation and craniofacial development including the absence of lower incisors and defects in the secondary palate (Matzuk et al., 1995a). These mice died within 24 hours of birth because they fail to suckle. Mice with null mutations in activin βB have eyelid defects characterized by a failure of fusion at birth (Matzuk et al., 1995b). Malformation of the eyelid led to other eye defects including corneal opacification and anterior uveitis. In addition, activin βB deficient female mice manifest a reproductive impairment. The reduced fertility resulted from perinatal loss of both mutant and wild-type progeny and therefore reflects a maternal defect, although the exact nature of the defect is uncharacterized to date. Pleiotropic

effects were observed in follistatin knock-out mice (Matzuk et al., 1995c). Targeted mutation in this gene led to reduced muscle mass of the pectoral, intercostal, and diaphragm muscles, rib and palate defects, shiny taut skin, abnormal wisker and tooth development, and overall growth reduction. Gene disruption experiments have demonstrated essential roles for the activins and follistatins in multiple aspects of organogenesis. In these mice, no other ocular phenotypes were described other than the eyelid and subsequent corneal defects in the activin βB deficient mice. Unlike avians, mice do not undergo a smooth-to-striated muscle transition in the iris and ciliary body. These muscles in mice remain smooth-type. Interestingly, these genetic approaches also demonstrated that mesoderm specification and patterning are unaffected in the absence of activin or follistatin. Consistent with this observation, other cytokines can induce and/or pattern mesoderm in Xenopus animal cap assays. Germ layer specification, as well as many aspects of organogenesis, most likely requires the interactions of multiple cytokines and other signaling molecules.

The role of other signaling molecules in iris and ciliary body differentiation

My research has focused on studying the role of secreted and soluble molecules, and in particular that of activin and follistatin as regulators of differentiation; however, other signaling molecules may regulate other aspects of develop in the iris and ciliary body. Signaling molecules identified in other developing structures include multiple cytokines, cell surface associated proteins, matrix associated proteins, and retinoids. The role of other cytokines during iris and ciliary body development is discussed below.

Orchestration of proliferation and differentiation: The role of cytokines in the anterior chamber

Several secreted and soluble molecules have been identified in the developing eye that show functions in both cell proliferation and differentiation (Tripathi et al., 1991). For example, insulin-like growth factors (IGFs) function in both growth and differentiation during lens development. Lens epithelial cells show dose-responsive mitosis to IGFs I and II, while lens

fiber cells elongate and display polarity in response to IGF I (Beebe et al., 1987). Similar to follistatin's regulation of activin, IGF binding proteins, as well as receptor modulation, regulate IGF activities during lens growth and differentiation (Hyatt and Beebe, 1993). In the rat iris and ciliary body, there is a correlation between the processes of proliferation and determination. In experiments combining ^{3}[H]-thymidine labeling and cultivation of anterior eye cups with and without lens, the time of determination of the iris-ciliary body epithelium and the dependence of this determination on lens-derived signaling was evaluated. Stroeva (1967) found that the lens directed the specification of the anterior optic cup to iris-ciliary body epithelium, and this process was linked to an increase in the duration of G1-phase of proliferating cells and a concomitant decrease in the number of cells undergoing DNA synthesis. Stroeva also noted that proliferation in the overlying stroma was independent of lens influences and continued after reductions in mitosis in the epithelium. This second observation is in agreement with data indicating that the majority of growth in the avian iris and ciliary body is due to proliferation of cells within the overlying stroma (Gabella and Clarke, 1983).

Two studies from my thesis are consistent with a role for locally produced soluble factors that promote proliferation of cells in the avian iris and ciliary body. The first comes from analysis of single cells isolated from undifferentiated iris (E8) and cloned in the presence of either heterochronic, older iris and ciliary body cells or heterotypic, embryonic fibroblasts. Analysis of the average clone size indicated that both smooth and striated muscle colonies were approximately twice as large when grown with older iris and ciliary body cells. Non-muscle cell clone sizes did not change, suggesting that iris-derived mitogens act on subtypes of cells within the tissue. However, the role of factors that affect survival could not be ruled out in those experiments. In another study, soluble extracts from the developing iris and ciliary body were analyzed in assays for three biochemically distinct classes of mitogens (Appendix A). These included FGF1-like, FGF2-like, and non-FGF activities, each of which increased in the developing iris and ciliary body. FGF2-like activity represented the largest mitogenic component of the extracts, although FGF1-like and non-FGF activities were significant. The FGFs function as both mitogens and differentatiation factors in a variety of cell types, including smooth and striated muscle. The expression of specific FGFs and their

receptors has not been described in detail in the avian iris and ciliary body. However, in mice, high affinity binding sites for FGFs are expressed throughout the developing iris and ciliary body (Fayein et al., 1990). FGFs potentially underly the proliferative activities described in the clonal analyses presented in this dissertation and during growth of the iris and ciliary body in vivo.

Cytokines and neuronal survival and maturation

Many studies suggest that molecules produced within the iris and ciliary body, as well as other muscular target tissues, regulate developmental cell death in the innervating neurons (reviewed in Oppenheim, 1989). In the ciliary ganglion, cell death is regulated by chCNTF (Finn et al., in press). This molecule is expressed in the iris-ciliary body and the choroid, the other target of ciliary ganglion neurons. The level of chCNTF-like activity increases throughout development in the iris and ciliary body (non-heparin binding neurotrophic activity, appendix B). While chCNTF-like activity is the predominant neurotrophic species from soluble extracts of iris and ciliary body, both FGF1-like and FGF2-like are also present. Interestingly, all three of these molecules are secreted with low efficiency in vitro and support the survival of cultured ciliary ganglion neurons. As cell death in the ganglion is coincident with the smooth-to-striated muscle transition in the targets, it is tempting to speculate that chCNTF or other neurotrophic factors are differentially expressed or made available between the two muscle types. Thus, the transition would be requisite for appropriate regulation of neuronal cell number. However, a more detailed analysis of the cellular expression patterns and secretion of chCNTF or related molecules is necessary to evaluate this supposition.

In addition to regulating neuronal survival, iris and ciliary body development may affect other aspects of neuronal maturation. Several observations suggest that development of the cholinergic phenotype in ciliary ganglion neurons is regulated by the target tissues. In characterizing soluble extracts from the developing chick eye, Nishi and Berg (1981), described an activity of approximately 50 kDa that stimulated the activity of choline acetyltransferase (ChAT), without affecting neuronal growth or survival.

Other studies demonstrated that ciliary ganglion neurons that develop in the absence of ocular target tissues show marked reduction in acetylcholine receptor expression and sensitivity (Landmesser and Pilar, 1974; Engisch and Fischbach, 1990; Arenella et al., 1993). Postganglionic axotomy in newly hatched chicks also resulted in dramatic decreases in actetylcholine receptor expression (Jacob and Berg, 1987) and acetylcholine sensitivity (Brenner and Martin, 1976). The molecules that mediate these target effects have not been identified, nor has the regulation of these influences within the developing iris and ciliary body.

Contribution of other TGFβ superfamily members and their antagonists

Several lines of evidence suggest that other members of the TGFβ superfamily and their secreted antagonists, in addition to activin and follistatin, function in muscle differentiation and neuronal maturation. Activin belongs to the TGFβ superfamily of structurally homologous signaling molecules that often display similar bioactivities on specific cell types. Several of these, particularly the BMPs, are regulated by secreted antagonists which bind with high affinity and prevent ligand signaling in a fashion similar to follistatin and activin. Furthermore, these antagonists can interact with multiple members of the TGFβ superfamily. For example, protein interaction assays and functional bioassays have demonstrated that the antagonist noggin binds BMP2 and 4 with high affinity (K_d~20-300 pM), and BMP7 with lower affinity (K_d~1 nM), but does not interact with activin (Zimmerman et al., 1996). Chordin specificity appears limited to BMP4 (Piccolo et al., 1996). Follistatin, in addition to binding activin, can interact with BMP 2, 4, and 7 (Yamashita et al., 1995; Fainsod et al., 1997). The binding affinities of follistatin for BMPs, though, is approximately ten times less than for activin (Yamashita et al., 1995). For this reason, experiments using recombinant follistatin have the caveat that this protein may interact with additional signaling molecules (BMPs). Unlike activin and follistatin, however, the expression of BMPs, noggin, or chordin have not been explored in the avian iris and ciliary body. Therefore, the simplest interpretation of the effects that I have described with recombinant activin and follistatin, is that they reflect the activities of endogenous activin and follistatin. Nevertheless, multiple TGFβ superfamily members and their antagonists

may be important in the regulation of iris and ciliary body development, and experiments addressing their expression and function are warranted.

The role of innervation on muscle differentiation

Just as muscle targets have been demonstrated to regulate aspects of neuronal development, innervation-dependent effects have been reported for muscle differentiation and maturation. Neurons can affect target development through activity-dependent (electrical) and activity independent (neural derived signaling molecules) mechanisms. Although innervation-dependent development has not been studied in detail with respect to iris and ciliary body ontogeny, this possibility and the circumstantial evidence for such regulation is discussed here.

Neural-dependent development of muscle

While myotube formation can occur in the absence of innervation, other aspects of muscle development and maturation are dependent on neurons. For example, the functional properties of nicotinic acetylcholine receptors (AChRs) in muscle cells are dependent on functional innervation. During normal developement, AChR channel properties change from small conductances and slow gating kinetics to larger conductances and faster gating kinetics (Fischbach and Schuetze, 1980). This change can be prevented with axotomy or agents that inhibit electrical signaling between the nerve and muscle (reviewed in Scheutze and Role, 1987). Underlying these functional changes in channel properties is a switch in the subunit composition of the AChR: during embryogenesis, ε subunits are replaced with γ subunits (Kurosaki et al., 1987). Experiments in cell culture later demonstrated that the transcription of γ subunits was dependent on electrical activity but not neural-derived signaling proteins. This effect is conferred by a regulatory sequence in the 5' untranslated region of the γ subunit gene (Dutton et al., 1993).

Not all of the effects of neurons on muscle development are dependent on electrical activity. For example the redistribution of existing AChRs to clusters beneath points of nerve-muscle contact is regulated by agrin, a neural derived protein (Ruegg et al., 1992; Gautam et al., 1996). Other examples of

neural regulation of muscle development include the expression of contractile protein isoforms (reviewed in Jolesz and Sreter, 1981), expression of regulatory transcription factors (Hughes et al., 1993; Dutton et al., 1993; Leferovich et al., 1995), responsiveness to extracellular ATP (Wells et al., 1995), and the production of neurodifferentiation factors (cholinergic differentiation factor, Habecker et al., 1995) and neurotrophic factors (neurotrophin-4, Funakoshi et al., 1995).

Innervation and iris-ciliary muscle development

Results from several experiments are suggestive of a role for innervation in iris-ciliary muscle development. Pilar et al. (1980) performed axotomies of ciliary nerve branch three, which innervates the dorsal regions of the iris and ciliary muscles. The nerve branch was cut at E7, prior to smooth muscle differentiation and innervation in the target region. Although embryo survival was low (10-15%) following axotomy, two irises were studied in detail with respect to muscle differentiation. These authors reported a 20% reduction in the ratio of differentiated muscle to precursor cells and a 20% reduction in muscle thickness at E14. With serial reconstruction montages of electron micrographs, only smooth muscle cells were observed. From this study, however, it is not clear whether differentiation or maintenance of muscle was affected by the axotomy.

Two studies investigating muscle differentiation in iris explants, where functional innervation is disrupted, have been preformed. In the first, Ferrari and Koch (1984) scored the percentage of explants with striated muscle immunoreactivity after 8 days in culture. They found developmental changes in the proportion of explants that contained striated muscle. After 8 days in vitro, 33% of the irises removed at E7 contained striated muscle. The proportions of explants with striated muscle from older ages were 78% for E7.5; 81% for E8; and 97% for E9. Although the culture conditions were not optimal for striated muscle differentiation (agar substratum and high serum medium-10% fetal calf serum + 3% chicken embryo extract was used), the sharp increase from E7 to E8 in the number of explants that contained striated muscle is suggestive that innervation affects muscle development. E8 is

when the smooth muscle of the iris begins to differentiate and becomes innervated.

Using conditions more optimal for striated muscle differentiation (collagen containing substratum and serum free medium), I measured myogenic development with an enzyme marker (CPK activity) in explants of iris-ciliary body taken at E8. CPK activity was measured in explants taken after various times in culture and compared to age-matched iris-ciliary body tissue that had developed in vivo. Under these conditions, striated muscle differentiation in iris-ciliary body explants, void of functional innervation, paralleled or slighlty exceeded that in vivo. Although I did not investigate differentiation in younger iris-ciliary body explants, these results suggest that if innervation is important for striated muscle differentiation, ciliary neurons exert influences prior to E8. Further studies are necessary to understand the role of innervation on muscle differentiation (including smooth and striated) or other aspects of muscle development and maturation, such as regulation of AChR function, contractile protein expression, or the regulation of neurotrophic factor expression and availability.

Regulation of transdifferentiation

The role of transdifferentiation in the development of the iris muscle raises several questions. One important question is what controls the abilty of a fully differentiated cell to switch molecular and functional phenotypes. Development through transdifferentiation is rare, but has been described during the normal ontogeny of several vertebrate tissues including the avian iris and the mouse esophagus. In addition, cells from several tissue sources, including many from the eye, can be induced to trandifferentiate under experimental conditions. Of clinical relevence, cancer cells often undergo phenotypic diversification following transformation. While the control of transdifferentiation in most instances is largely unknown, several general modes of transdifferentiation have been described.

Mechanisms of transdifferentiation

Interconversion of cell types can be classified as direct transdifferentiation or as indirect transdifferentiation. Direct transdifferentiation is the switch in the expression of genes encoding proteins for one mature cell type to another mature cell type. Indirect transdifferentiation occurs when a cell de-differentiates and then usually re-enters the cell cycle to produce progeny of a different cell type. The conversion of smooth muscle to striated muscle in the mouse esophagus or the avian iris is direct transdifferentiation. This can be shown by the transient co-expression of markers for mature smooth and striated muscle within the same cell (Patapoutian et al., 1995; chapter 3, this thesis). An example of indirect transdifferentiation occurs under experimental manipulation of the jellyfish, *Podocoryne carnea*. Isolated striated muscle of this jellyfish can be induced to re-enter mitosis and produce smooth muscle and neurons at a ratio of 1:1. The neurons stop dividing, whereas the smooth muscle cells continue to divide and produce more smooth muscle cells and neurons (Alder et al., 1987).

Cell-matrix interactions and cytokines can regulate transdifferentiation

Although no studies have been conducted to investigate regulation of vertebrate transdifferentiation that occurs as a part of normal development, insights into this process have been gained by studying experimentally induced transdifferentiation. While the specific molecules regulating experimentally induced transdifferentiation vary with the model being studied, the role of the extracellular matrix and/or secreted, soluble factors appears key. For example, retinal pigmented epithelium (RPE) will transdifferentiate into neural retina under the influence of FGFs and the appropriate substrate. In vitro, this process is faciliated by laminin (Reh et al., 1987) and inhibited by collagen (Yasuda, 1979). When RPE is grown on a gelatinous substrate containing basement membrane proteins isolated from the lamina adjacent to the RPE, stratification and neuron sub-type differentiation occurs (Opas et al., 1994). An essential role of the extracellular matrix during transdifferentiation of striated muscle in the jellyfish has been

demonstrated by antibody treatments that disrupt interactions between the muscle cell and specific extracellular matrix glycoproteins. In addition, destabilization of cell-matrix interactions with proteases such as collagenase, hyaluronidase or pronase also affect transdifferentiation in this system (Reber-Müller et al., 1994).

Regulation of smooth to stratied muscle transdifferentiation during iris development

Initial characterizations of smooth-to-striated muscle transdifferentation has been carried out in the mouse esophagus and avian iris. Although none of the studies directly investigated the regulation of this type of transdifferentiation, results from several experiments suggest directions for such studies. In both the esophagus and iris, the smooth-to-striated muscle transition progresses in a spacially-ordered fashion. Early in development, the muscular layers are composed entirely of smooth muscle. In the mouse esophagus, striated muscle proteins are first evident in the outermost, peripheral cells at the rostral end of the cylindrical structure. Later, differentiation progresses both caudally along the esophagus and centrally within it. In the avian iris, striated muscle appears midway between the pupillary margin and the peripheral edge. Differentiation progresses both centrally and peripherally with time. Similar to the rostral to caudal gradient of development in the esophagus, differentiation in the iris progresses in a centripetal fashion from the edge of the ocular fissure. These observations are consistent with a role of local cell-cell propagated signaling. In the avian iris, however, it is unclear whether differentiation of mesenchymal striated myoblasts and transdifferentiation of smooth muscle is regulated by identical signals. In addition, it is not known whether these two striated muscle precursors are spatially separated. Therefore, my studies with activin and follistatin simply indicate that these factors are involved in the regulation of the overall transition. In the mouse esophagus, the embryonic origin of the smooth muscle precursors have not been mapped. A dual origin for the striated muscle in that structure remains a possibility.

For both the mouse esophagus and the avian iris, myoD and related transcription factors are expressed in smooth muscle cells; however, the timing of expression appears different. In the esophagus, smooth muscle markers precede the expression of myoD and myogenin. In the iris, the neuroectodermal cells that differentiate into the smooth muscle cells express myoD (but not mrf4) prior to expression of smooth muscle markers. MyoD is co-expressed with α-smooth muscle actin in ectodermal cells that have just migrated into the overlying stroma, but it is then down-regulated as the smooth muscle migrates peripherally. Both myoD and mrf4 are (re)expressed when striated muscle differentiation is later initiated. Again, a role for local cell-cell interactions is suggested by explant experiments which demonstrated that the neuroectodermal cells will precociously express differentiated striated muscle markers (myosin heavy chain) when removed from the anterior chamber and cultured on collagen. Together, these marker expression studies and explant manipulations suggest that striated muscle differentiation is actively repressed in vivo until temporally appropriate. Furthermore, results from isochronic co-culture studies demonstrated that a contact-mediated signal represses striated muscle differentiation in the pre-transition iris cells.

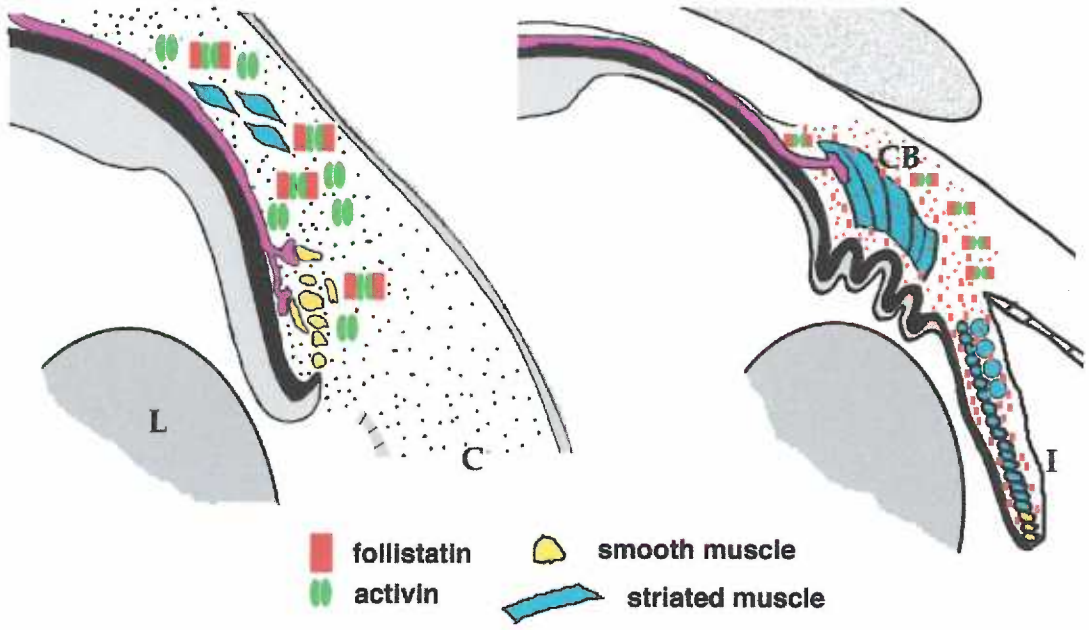
Experiments addressing the expression and function of candidate cell-cell and cell-matrix signaling molecules will be informative in understanding the regulation of these transitions. Additional cell culture experiments with mouse esophageal and avian iris cells can be designed to confirm candidate cell-cell and cell-matrix signaling molecules. For the esophagus, the availablity of mice with targeted disruptions in canidate signaling genes will prove useful in addressing their function in vivo. For the iris, retroviral transgene-delivery technology can be used to alter the expression of candidate signaling molecules and better understand the regulation of transdifferentiation.

A model for iris and ciliary body development

The results presented in this thesis support a model whereby the interaction of activin and follistatin coordinate the differentiation and maturation of multiple cell types within the developing iris and ciliary body. These cell types include precursor cells for both the smooth and the striated muscle as

well as the innervating ciliary neurons. All of these cells, including axons of ciliary neurons, are resident within the undifferentiated (E8), iris and ciliary body. Both activin and follistatin are expressed at this early developmental time and throughout iris and ciliary body development. Follistatin bioactivity prevails in conditioned medium from iris and ciliary body cells and prevents activin-mediated somatostatin induction in the ciliary neurons. This is in contrast to the choroid layer, where activin bioactivity is secreted and somatostatin is induced in choroid neurons.

Differentiation of the masculature is also regulated by these factors. Neuroectodermal cells invaginate into the irideal stroma and differentiate into smooth muscle cells under the influence of local concentrations of activin, as well as other signaling molecules. Precocious striated muscle differentation is prevented by non-soluble signaling molecules. As development progresses, follistatin bioactivity increases and induces striated muscle differentiation from myoblasts derived from cranial neural crest, head mesoderm, and transdifferentiating smooth muscle cells. Myotube fusion continues until hatching, when the final muscle phenotype is achieved. Thus, with this model, myotubes of the iris and ciliary body are derived from multiple embryonic sources and the smooth-to-striated muscle transition and neuronal maturation of the innervating neurons are coordinated by the key signaling molecules activin and follistatin.



Model Figure. Differentiation during iris and ciliary body development. A developing, pre-transition iris and ciliary body (E8) is respresented on the left and a mature, post-transition iris and ciliary body (E16 and older) is respresented on the right. Both activin and follistatin are expressed in these structures throughout development (represented in green ovals and red rectangles, respectively). As development progresses, follistatin in up-regulated and promotes differentiation of striated muscle from multiple cell types including smooth muscle cells (orange cells) and undifferentiated mesenchymal cells (spindle-shaped blue cells). Ciliary neurons (purple axons) innervate both the smooth and striated muscle, and through the actions of follistatin, are prevented from expressing somatostatin. (C) cornea, (L) lens, (CB) ciliary body, (I) iris.

Appendix A

Characterization of soluble extracts of iris and ciliary body

As a first step in exploring the role of local soluble factors in iris development, mitogenic and neurotrophic assays were utlilyzed to characterize extracts of iris and ciliary bodies from different ages. Soluble extracts from E8 to E19 were prepared in a cocktail of buffered protease inhibitors and applied to a heparin-agarose column. Heparin binding and non-heparin binding fractions were tested for ^{3}[H]-thymidine incorporation in murine AKR-2B cells (Shipley, 1986) or for the ability to support survival of chick ciliary ganglion neurons (Nishi and Berg, 1979). The heparin binding fraction was tested in the presence or absence of exogenous heparin. In these assays, heparin-binding activities potentiated with heparin represents FGF2-like bioactivity, where as the heparin binding activity measured without exogenous heparin represents FGF1-like bioactivity (Eckenstein et al., 1991). In the neurotrophic assay, non-heparin binding activity represents chCNTF-like bioactivity (Finn and Nishi, 1996). Choline acetyltransferase (ChAT) activity was also measured in unfractioned supernatants and served as a control for the integrity of the extracts. In the iris and ciliary body ChAT activity increases through embryonic development (Chiappinelli, 1976). Activities were calculated per individual iris-ciliary body and per μg total protein in order to correlate activity with growth and changes in cellular composition within the tissues.

Mitogenic factors were found to increase with development of the iris and ciliary body (A and B). The majority of mitogenic activity measured in this assay was heparin-binding and dependent on exogenous heparin (FGF2-like). Similar to the mitogenic assay, neurotrophic activity was found to increase with development (C and D). In contrast to results with the mitogenic assay, however, the majority of neurotrophic activiy was measured from the non-heparin binding fractions. Interestingly, chCNTF-like neurotrophic activity was found to sharply increase from E14 to E16, the time when the majority of striated myotubes are formed. Activity for ChAT, which is synthesized in cholinergic neurons, increased throughout development with a sharp increase at E16 as has been previously described (E and F).

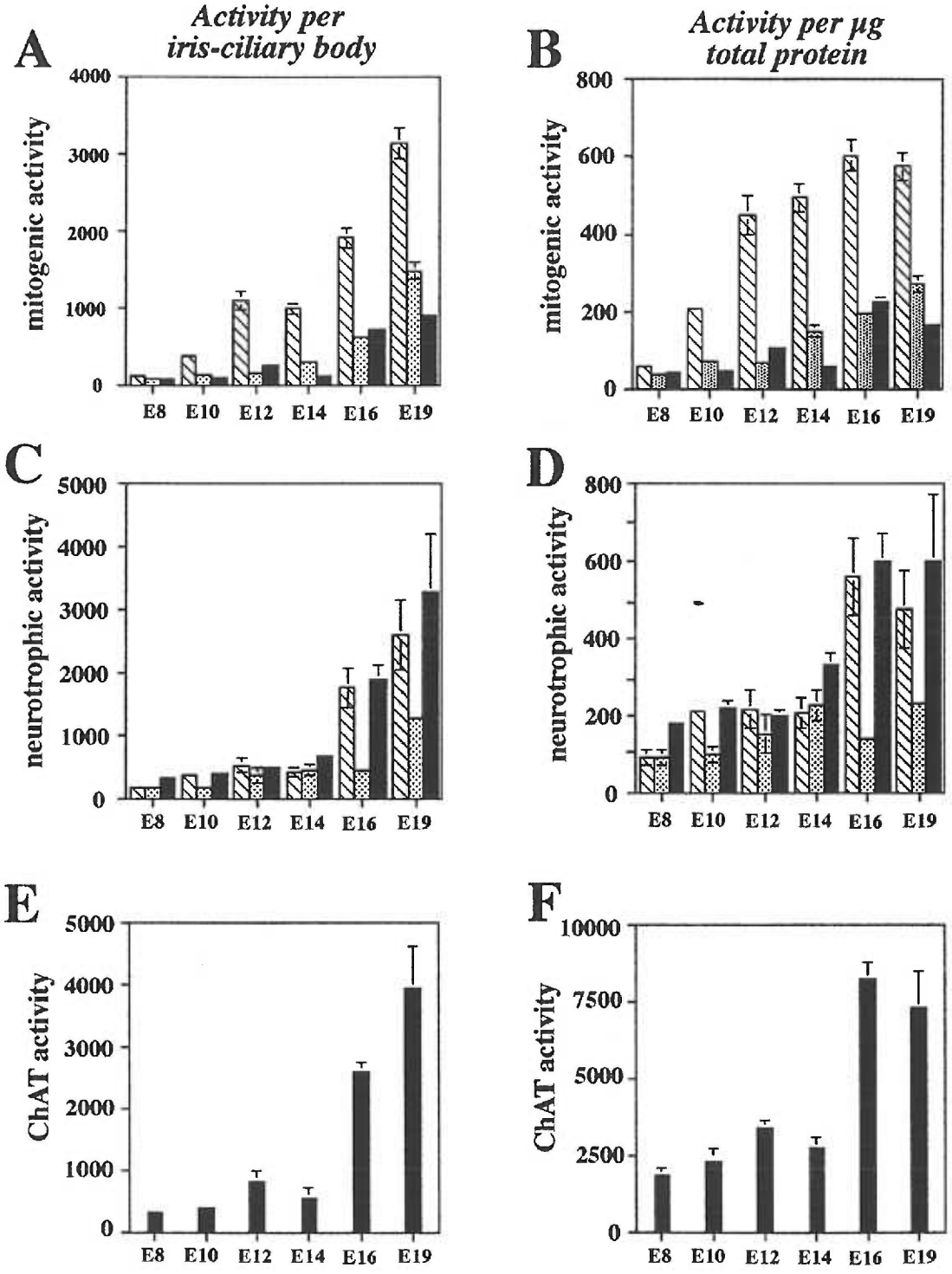
Appendix B

Characterization of soluble extract of ciliary ganglion

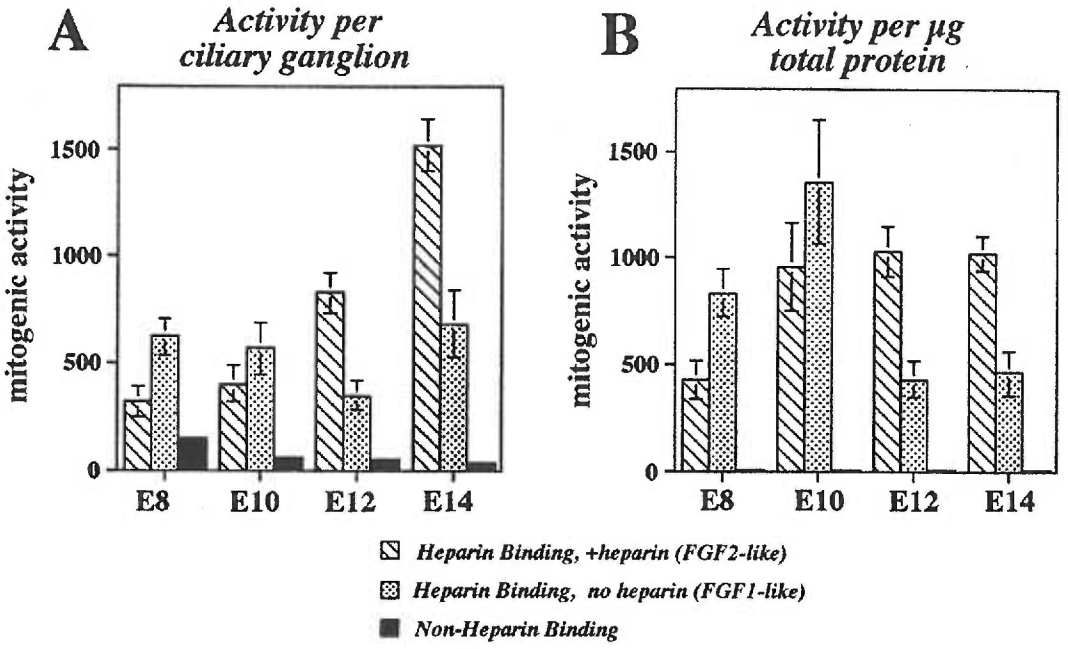
The AKR-2B mitogenic assay used to characterize FGF1-like and FGF2-like bioactivity present in the ciliary ganglion during the period of developmental cell death. Both FGF1-like and FGF2-like bioactivities were detectable in ciliary ganglions from E8 to E14 (A and B). As with the target tissues, activity was calculated per individual ganglion and per μg total protein in order to correlate activity with growth and changes in cellular composition. When considering total activities per ganglion, FGF1-like activity remained constant, whereas FGF2-like activity increased from E8 to E14 (A). When normalizing for growth of the ganglion, FGF1-like activity is more dynamic. The highest FGF1-like activities per μg of protein was measured in the younger ages with a peak at E10.

The activities described from iris-ciliary body and ciliary ganglion extracts are indicative of the presence of specific molecules (FGF1, FGF2, and chCNTF). However, the identity of these molecules was not demonstrated with these studies and the extracts are undoubtedly a complex mix of signaling molecules. Indeed, the FGFs are a large family of homologous proteins with at least 10 members that often share similar biological activities. This initial characterization of soluble extracts provide basis for future studies directed at identifying the presence, function, and regulation of specific mitogenic, neurotrophic, and differentiation factors in these tissues.

Appendix A


A
Activity per iris-ciliary body
mitogenic activity
4000
3000
2000
1000
0
E8 E10 E12 E14 E16 E19
B
Activity per µg total protein
mitogenic activity
800
600
400
200
0
E8 E10 E12 E14 E16 E19
C
neurotrophic activity
5000
4000
3000
2000
1000
0
E8 E10 E12 E14 E16 E19
D
neurotrophic activity
800
600
400
200
0
E8 E10 E12 E14 E16 E19
E
ChAT activity
5000
4000
3000
2000
1000
0
E8 E10 E12 E14 E16 E19
F
ChAT activity
10000
7500
5000
2500
0
E8 E10 E12 E14 E16 E19
Heparin Binding, +heparin (FGF2-like)
Heparin Binding, no heparin (FGF1-like)
Non-Heparin Binding, (cCNTF-like)

Appendix B



(above)
Appendix B. Mitogenic activity in heparin-binding (with added heparin, hatched or without added heparin, stippled) and non-heparin binding extracts (black) from E8-E14 ciliary ganglion. Activity was calculated per ciliary ganglion (A) or normalized to total protein (B).

(previous page)
Appendix A. Mitogenic (A-B), neurotrophic (C-D), and ChAT (E-F) activities in heparin-binding (with added heparin, hatched or without added heparin, stippled) and non-heparin binding extracts (black) from E8-E19 iris-ciliary body were calculated per iris-ciliary body (A, C, E) or normalized to total protein (B, D, F). Mitogenic activity was determined by measuring cpm ^{3}[H]-thymidine incorporated in acid precipitated DNA from quiescent AKR-2B fibroblasts. Neurotrophic activity was determined by counting the number of surviving ciliary ganglion neurons after 4 days in culture (5 fields of view were totaled per well and three wells were averaged for each condition). ChAT activity was determined by assaying the amount of ^{3}[H] acetyl-coenzyme A transferred to choline. Labeled acetylcholine was then extracted with an organic solvent and cpms were measured.

REFERENCES

Albano, R. M., Arkell, R., Beddington, R. S. P., and Smith, J. C. (1994). Expression of inhibin subunits and follistatin during postimplantation mouse development: decidual expression of activin and expression of follistatin in primative streak, somites and hindbrain. *Development* **120,** 803-813.

Alder, H., and Schmid, V. (1987). Cell cycles and in vitro transdifferentiation and regeneration of isolated, striated muscle of jellyfish. *Dev. Biol.* **124,** 358-369.

Amthor, H., Connolly, D., Patel, K., Brand-Saberi, B., Wilkinson, D. G., Cooke, J., and Christ, B. (1996). The expression and regulation of *follistatin* and *follistatin-like* gene during avian somite compartmentalization and myogenesis. *Dev. Biol.* **178,** 343-362.

Anderson, M. J., Cohen, M. W., and Zorychta, E. (1977). Effects of innervation on the distribution of acetylcholine receptors on cultured muscle cells. *J. Physiol.* **268,** 731-756.

Arenella, L. S., Oliva, J. M., and Jacob, M. H. (1993). Reduced levels of acetylcholine receptor expression in chick ciliary ganglion neurons developing in the absence of innervation. *J. Neurosci.* **13,** 4525-4537.

Aziazu, N., and Frasch, M. (1993). *tinman and bagpipe*: two homeobox genes that determine cell fates in the dorsal mesoderm of Drosophila. *Genes & Dev.* **7,** 1325-1340.

Bao, Z. Z., and Cepko, C. L. (1997). The expression and function of *Notch* pathway genes in the developing rat eye. *J. Neurosci.* **17,** 1425-1434.

Beebe, D. C., Silver, M. H., Belcher, K. S., Van Wyk, J. J., Svoboda, M. E., and Zelenka, P. S. (1987). Lentropin, a protein that controls lens fiber formation, is related functionally and immunologically to the insulin-like growth factors. *Proc. Natl. Acad. Sci. USA* **84,** 2137-2330.

Benezra, R., Davis, R. L., Lockshon, D., Turner, D. L., and Weintraub, H. (1990). The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. *Cell* **61,** 49-59.

Bodmer, R. (1993). The gene *tinman* is required for specification of the heart and visceral muscles in *Drosophila*. *Development* **118,** 719-729.

Bosco, L. (1988). Transdifferentiation of ocular tissues in larval *Xenopus laevis*. *Differentiation* **39,** 4-15.

Brenner, H. R., and Martin, A. R. (1976). Reduction in acetylcholine sensitivity of axotomized ciliary ganglion cells. *J. Physiol.* **260,** 159-176.

Brunner, G., and Tschank, G. (1982). Contracting striated muscle fibers differentiated from pituitary cultures. *Cell Tissue Res.* **244,** 665-662.

Burt, A. M., and Narayanan, C. H. (1976). Choline acetyltransferase, choline kinase, and acetylcholinesterase activities during the development of the chick ciliary ganglion. *Exp. Neurol.* **53,** 703-713.

Cepko, C. L., Austin, C. P., Yang, X., Alexiades, M., and Ezzeddine, D. (1996). Cell fate determination in the vertebrate retina. *Proc. Natl. Acad. Sci. USA* **93,** 589-595.

Chen, C.-M. A., Kraut, N., Groudine, M., and Weintraub, H. (1996). I-mf, a novel myogenic repressor, interacts with members of the myoD family. *Cell* **86,** 731-741.

Chiappinelli, V., Giacobini, E., Pilar, G., and Uchimura, H. (1976). Induction of cholinergic enzymes in chick ciliary ganglion and iris muscle cells during synapse formation. *J. Physiol.* **257,** 749-766.

Choi, D., and Fischbach, G. D. (1981). GABA conductance of chick spinal cord and dorsal root ganglion neurons in cell culture. *J. Neurophysiol.* **45,** 605-620.

Chomczynski, P., and Sacchi, N. (1987). Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162,** 156-159.

Cioni, C., Bosco, L., and Filoni, S. (1990). Transdifferentiation of larval *Xenopus laevis* iris under the influence of the pituitary. *Experientia* **46,** 1078-1080.

Coleman, M. E., DeMayo, F., Yin, K. C., Lee, H. M., Geske, R., Montgomery, C., and Schwartz, R. J. (1995). Myogenic vector expression of insulin-like growth factor I stimulates muscle cell differentiation and myofiber hypertrophy in transgenic mice. *J. Biol. Chem.* **270,** 12109-12116.

Cornell, R. A., and Kimelman, D. (1994). Activin-mediated mesoderm induction requires FGF. *Development* **120,** 453-462.

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.

Cossu, G., Tajbakhsh, S., and Buckingham, M. (1996a). How is myogenesis initiated in the embryo? *Trends in Genetics* **12,** 218-223.

Coulombe, J., and Bronner, F. M. (1990). Development of cholinergic traits in the quail ciliary ganglion: expression of choline acetyltransferase-like immunoreactivity. *Neuroscience* **37,** 259-70.

Coulombe, J. N., and Kos, K. (1997). Target tissue influence on somatostatin expression in the avian ciliary ganglion. *Annl. N.Y. Acad. Sci.* **814,** 209-225.

Coulombe, J. N., and Nishi, R. (1991). Stimulation of somatostatin expression in developing ciliary ganglion neurons by cells of the choroid layer. *J. Neurosci.* **11,** 553-562.

Coulombe, J. N., Schwall, R., Parent, A. S., Eckenstein, F. P., and Nishi, R. (1993). Induction of somatostatin immunoreactivity in cultured ciliary ganglion neurons by activin in choroid-cell conditioned medium. *Neuron* **10,** 899-906.

Coulombre, A. J. (1965). Regulation of ocular morphogenesis. *Invest. Ophthal.* **8,** 25-31.

Coulombre, J. L., and Coulombre, A. J. (1965). Regeneration of neural retina from the pigmented epithelium in the chick embryo. *Dev. Biol.* **12,** 79-92.

Cusella-De Angelis, M. G., Lyons, G., Sonnino, C., De Angelis, L., Vivarelli, E., Farmer, K., Wright, W. E., Molinaro, M., Bouche, M., Buckingham, M., and et al. (1992). MyoD, myogenin independent differentiation of primordial myoblasts in mouse somites. *J. Cell Biol.* **116,** 1243-55.

Cusella-De Angelis, M. G., Molinari, S., Le Donne, A., Coletta, M., Vivarelli, E., Bouche, M., Molinaro, M., Ferrari, S., and Cossu, G. (1994). Differential response of embryonic and fetal myoblasts to TGF beta: a possible regulatory mechanism of skeletal muscle histogenesis. *Development* **120,** 925-33.

Darland, D. C., Link, B. A., and Nishi, R. (1995). Activin A and follistatin expression in developing targets of ciliary ganglion neurons suggests a role in regulating neurotransmitter phenotype. *Neuron* **15,** 857-866.

Davis, R. L., Weintraub, H., and Lassar, A. B. (1987). Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell* **51,** 987-1000.

De Angelis, L., Cusella-De Angelis, M. G., Boucher, M., Vivarelli, E., Boitani, C., Molinaro, M., and Cossu, G. (1992). ACTH-like peptides in post-

implantation mouse embryos: a possible role in myoblast proliferation and muscle histogenesis. *Dev. Biol.* **151,** 446-458.

De Vitry, F., Hillion, J., Catelon, J., and Gros, F. (1994). Myogenic cells in the rat embryonic brain stem. *Comp. Rend. Acad. Sciences* **317,** 332-40.

deLapeyriere, O., Ollendorff, V., Planche, J., Ott, M. O., Pizette, S., Coulier, F., and Birnbaum, D. (1993). Expression of the Fgf6 gene is restricted to developing skeletal muscle in the mouse embryo. *Development* **118,** 601-11.

DeStefano, M. E., Luzzatto, A. C., and Mugnaini, E. (1993). Neuronal ultrastructure and somatostatin immunolocalization in the ciliary ganglion of chicken and quail. *J. Neurocytol.* **22,** 868-892.

Dryer, S. E. (1994). Functional development of the parasympathetic neurons of the avian ciliary ganglion: A classic model system for the study of neuronal differentiation and development. *Prog. Neurobiol.* **43,** 281.

Dudley, A. T., Lyons, K. M., and Robertson, E. J. (1995). A requirement for bone morphogenetic protein-7 during development of the mammalian kidney and eye. *Genes & Dev.* **9,** 2795-2807.

Duprez, D. M., Coltey, M., Amthor, H., Brickell, P. M., and Tickle, C. (1996). Bone morhogenic protein-2 (BMP-2) inhibits muscle development and promotes cartilage formation in chick limb bud cultures. *Dev. Biol.* **174,** 448-452.

Dutton, E. K., Simon, A. M., and Burden, S. J. (1993). Electrical activity-dependent regulation of the acetylcholine receptor γ-subunit gene, myoD, and myogenin in primary myotubes. *Proc. Natl. Acad. Sci. USA* **90,** 2040-2044.

Dyson, S., and Gurdon, J. B. (1997). Activin signalling has a necessary function in *Xenopus* early development. *Current Biology* **7,** 81-84.

Eagleson, G., Ferreiro, B., and Harris, W. A. (1995). Fate of the anterior neural ridge and the morphogenesis of the *Xenopus* forebrain. *J. Neurobiol.* **28,** 146-158.

Eckenstein, F. P., Esch, F., Holbert, T., Balcher, R. W., and Nishi, R. (1990). Purification and characterization of a trophic factor for embryonic peripheral neurons: comparison with fibroblast growth factors. *Neuron* **4,** 623-631.

Eckenstein, F. P., Shipely, G. D., and Nishi, R. (1991). Acidic and basic fibroblast growth factors in the nervous system: distribution and

differential alteration of levels after injury of central versus peripheral nerve. *J. Neurosci.* **11,** 412-419.

Eguchi, G., and Okada, T. S. (1973). Differentiation of lens tissue from the progeny of chick retinal pigment cell cultured in vitro: a demonstration of a switch of cell types in clonal cell culture. *Proc. Natl. Acad. Sci. USA* **70,** 1495-1499.

Emerson, C. P., Jr. (1993a). Embryonic signals for skeletal myogenesis: arriving at the beginning. *Curr. Op. Cell Biol.* **5,** 1057-64.

Emerson, C. P., Jr. (1993b). Skeletal myogenesis: genetics and embryology to the fore. *Curr. Op. Genet. Dev.* **3,** 265-74.

Engisch, K. L., and Fischbach, G. D. (1990). The development of ACh- and GABA-activated currents in embryonic chick ciliary ganglion neurons in the absence of innervation *in vivo*. *J. Neurosci.* **12,** 1115-1125.

Epstein, J. A., Lam, P., Jepeal, L., Maas, R. L., and Shapiro, D. N. (1995). Pax3 inhibits myogenic differentiation of cultured myoblast cells. *J. Biol. Chem.* **270,** 11719-11722.

Epstein, M. L., Davis, J. P., Gellman, L. E., Lamb, J. R., and Dahl, J. L. (1988). Cholinergic neurons of the chicken ciliary ganglion contain somatostatin. *Neuroscience* **25,** 1053-1060.

Ernsberger, U., Sendtner, M., and Rohrer, H. (1989). Proliferation and differentiation of embryonic chick sympathetic neurons: effects of ciliary neurotrophic factor. *Neuron* **2,** 1275-1284.

Fainsod, A., Deibler, K., Yelin, R., Marom, K., Epstein, M., Pillemer, G., Steinbeisser, H., and Blum, M. (1997). The dorsalizing and neural inducing gene *follistatin* is an antagonist of *BMP-4*. *Mech. Dev.* **63,** 39-50.

Fallon, J. F., Lopez, A., Ros, M. A., Savage, M. P., Olwin, B. B., and Simandl, B. K. (1994). FGF-2: apical ectodermal ridge growth signal for chick limb development. *Science* **264,** 104-107.

Fann, M.-J., and Patterson, P. H. (1994). Neuropoietic cytokines and activin A differentially regulate the phenotype of cultured sympathetic neurons. *Proc. Natl. Acad. Sci. USA* **91,** 43-47.

Fayein, N. A., Courtois, Y., and Jeanny, J. C. (1990). Ontogeny of fibroblast growth factor binding sites in mouse ocular tissues. *Exp. Cell Res.* **188,** 75-88.

Feijen, A., Goumans, M. J., and Eijn-van Raaij, A. J. M. (1994). Expression of activin subunits, activin receptors and follistatin in postimplantation

mouse embryos suggests specific developmental functions for different activins. *Development* **120,** 3621-3637.

Ferrari, P. A., and Koch, W. E. (1984). Development of the iris in the chicken embryo.I. A study of growth and histodifferentiation utilizing immunocytochemistry. *J. Embryol. Exp. Morph.* **81,** 153-167.

Finn, T. P., and Nishi, R. (1996). Expression of a chicken ciliary neurotrophic factor in targets of ciliary ganglion neurons during and after the cell-death phase. *J. Comp. Neurol.* **366,** 559-71.

Finn, T., Kim, S. and Nishi, R. (1997). Overexpression of ciliary neurotrophic factor in vivo rescues chick ciliary ganglion neurons from cell death. *J. Neurobiol.,* **in press.**

Fischbach, G. D., and Schuetze, S. M. (1980). A post-natal decrease in acetylcholine channel open time and rat end-plates. *J. Physiol.* **303,** 125-137.

Florini, J. R., Roberts, A. B., Ewton, D. Z., Falen, S. L., Flanders, K. C., and Sporn, M. B. (1986). Transforming growth factor-beta. A very potent inhibitor of myoblast differentiation, identical to the differentiation inhibitor secreted by Buffalo rat liver cells. *J. Biol. Chem.* **261,** 16509-13.

Frid, M. G., Shekhonin, B. V., Koteliansky, V. E., and Glukhova, M. A. (1992). Phenotypic changes of human smooth muscle cells during development: late expression of heavy caldesmon and calponin. *Dev. Biol.* **153,** 185-193.

Fukada, K. (1985). Purification and partial characterization of a cholinergic neuronal differentiation factor. *Proc. Natl. Acad. Sci. USA* **82,** 8795-8799.

Funakoshi, H., Belluardo, N., Arenas, E., Yamamoto, Y., Casabona, A., Persson, H., and Ibanez, C. F. (1995). Muscle-derived neurotrophin-4 as an activity-dependent trophic signal for adult motor neurons. *Science* **268,** 1495-1499.

Furshpan, E. J., Landis, S. C., Matsumonto, S. G., and Potter, D. D. (1986). Synaptic functions in rat sympathetic neurons in microcultures. I. Secretion of norepinephrine and acetylcholine. *J. Neurosci.* **6,** 1061-1079.

Furshpan, E. J., MacLeich, P. R., O'Lague, P. H., and Potter, D. D. (1976). Chemical transmission between rat sympathetic neurons and cardiac myocytes developing in microcultures: evidence for cholinergic, adrenergic, and dual-function neurons. *Proc. Natl. Acad. Sci. USA* **73,** 4225-4229.

Gabella, G., and Clarke, E. (1983). Embryonic development of the smooth and striated musculatures of the chicken iris. *Cell Tissue Res.* **229,** 31-59.

Gautam, M., Noakes, P. G., Moscoso, L., Rupp, F., Scheller, R. H., Merlie, J. P., and Sanes, J. R. (1996). Defective neuromuscular synaptogenesis in agrin-deficient mutant mice. *Cell* **85,** 525-535.

Genis-Galvez, J. M. (1966). Role of the lens in the morphogenesis of the iris and cornea. *Nature* **210,** 209-210.

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,** 263-74.

Gilbert, S. F. (1996). Cellular dialogues in organogenesis. *Birth Defects* **30,** 1-13.

Gilliland, G. S., Perrin, K., Blanchard, K., and Bunn, F. H. (1990). Analysis of cytokine mRNA and DNA: detection and quantitation by competitive polymerase chain reaction. *Proc. Natl. Acad. Sci. USA* **87,** 2725-2729.

Gilman, M. (1991). Ribonuclease protection assay. *In* "Current Protocols in Molecular Biology" (F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl, Eds.), pp. 4.7.1-4.7.8. Wiley-Interscience, New York.

Goulding, M., Lumsden, A., and Paquette, A. J. (1994). Regulation of Pax-3 expression in the dermomyotome and its role in muscle development. *Development* **120,** 957-71.

Gray, D. B., Zelazny, D., Manthay, N., and Pilar, G. (1990). Endogenous modulation of ACh release by somatostatin and the differential roles of calcium ion channels. *J. Neurosci.* **10,** 2687-2698.

Guillemin, R. (1976). Somatostatin inhibits the release of acetylcholine induced electrically in the myenteric plexus. *Endocrinology* **99,** 1653-1654.

Gurdon, J. B. (1992). The generation of diversity and pattern in animal development. *Cell* **68,** 185-99.

Habecker, B. A., and Landis, S. C. (1994). Noradrenergic regulation of cholinergic differentiation. *Science* **264,** 1602-4.

Habecker, B. A., Tresser, S. J., Rao, M. S., and Landis, S. C. (1995). Production of sweat gland cholinergic differentiation factor depends on innervation. *Dev. Biol.* **167,** 307-16.

Hamburger, V., and Hamilton, H. (1951). A series of normal stages in the development of the chick. *J. Morphol.* **88,** 49-92.

Hashimoto, M., Nakamura, T., Inoue, S., Kondo, T., Yamada, R., Eto, Y., Sugino, H., and Muramatsu, M. (1992). Follistatin is a developmentally regulated cytokine in neural differentiation. *J. Biol. Chem* **267,** 7203-7206.

Hemmati-Brevanlou, A., and Melton, D. A. (1992). A truncated activin receptor inhibits mesodermal induction and formation of axial structures in *Xenopus* embryos. *Nature* **359,** 609-614.

Hemmati-Brevanlou, A., and Melton, D. A. (1994). Inhibition of activin receptor signaling promotes neuralization in Xenopus. *Cell* **77,** 273-281.

Hemmati-Brevanlou, A., Kelly, O. G., and Melton, D. A. (1994). Follistatin, an antagonist of activin, is expressed in the Spemann organizer and displays direct neuralizing activity. *Cell* **77,** 283-295.

Henry, J. J., and Grainger, R. M. (1990). Early tissue interactions leading to embryonic lens formation in *Xenopus laevis*. *Dev. Biol.* **141,** 149-163.

Hill, R. E., Jones, P. F., Rees, A. R., Sime, C. M., Justice, M. J., Copeland, N. G., Jenkins, N. A., Graham, E., and Davidson, D. R. (1989). A new family of mouse homeobox-containing genes; molecular structure, chromosomal location, and developmental expression of Hox-7.1. *Genes &Dev* **3,** 26-37.

Holtzer, H., Marshall, J., and Finck, H. (1957). An analysis of myogenesis by use of fluorescent antimyosin. *J. Biophys. Biochem. Cytol.* **3,** 705-725.

Hughes, S. M., Taylor, J. M., Tapscott, S. J., Gurley, C. M., Carter, W. J., and Peterson, C. A. (1993). Selective accumulation of myoD and myogenin mRNAs in fast and slow adult skeletal muscle is controlled by innervation and hormones. *Development* **118,** 1137-1147.

Hyatt, G. A., and Beebe, D. C. (1993). Regulation of lens cell growth and polarity by an embryo-specific growth factor and by inhibitors of lens cell proliferation and differentiation. *Development 117* **117,** 701-719.

Inouye, S., Guo, Y., DePaolo, L., Shimonaka, M., Ling, N., and Shimasaki, S. (1991). Recombinant expression of human follistatin with 315 and 288 amino acids: chemical and biological comparison with native porcine follistatin. *Endocrinology* **129,** 815-822.

Itoh, N., Mima, T., and Mikawa, T. (1996). Loss of fibroblast growth factor receptors is necessary for terminal differentiation of embryonic limb muscle. *Development* **122,** 291-300.

Jacob, M. H., and Berg, D. K. (1987). Effects of preganglionic denervation and postganglionic axotomy on ACh receptors in the chick ciliary ganglion. *J. Cell Biol.* **105,** 1847-1854.

Jena, N., Martin-Seisdedos, C., McCue, P., and Croce, C. M. (1997). BMP7 null mutation in mice: developmental defects in skeleton, kidney, and eye. *Exp. Cell Res.* **230,** 28-37.

Johnson, R. L., and Tabin, C. J. (1997). Molecular models for vertebrate limb development. *Cell* **90,** 979-990.

Johnston, M. C., Noden, D. M., Hazelton, R. D., Coulombre, J. L., and Coulombre, A. J. (1979). Origins of avian ocular and periocular tissues. *Exp. Eye Res.* **29,** 27-43.

Jolesz, F., and Sreter, F. A. (1981). Development, innervation, and activity-pattern induced changes in skeletal muscle. *Ann. Rev. Physiol.* **43,** 531-552.

Joseph-Silverstein, J., Consigli, S. A., Lyser, K. M., and Ver Pault, C. (1989). Basic fibroblast growth factor in the chick embryo: immunolocalization to striated muscle cells and their precursors. *J. Cell Biol.* **108,** 2459-66.

Kingsley, D. M. (1994). The TGF-beta superfamily: new members, new receptors, and new genetic tests of function in different organisms. *Genes and Dev* **8,** 133-146.

Kirby, M., and Waldo, K. (1990). Role of neural crest in congenital heart disease. *Circulation* **82,** 332-340.

Knisely, T. L., Bleicher, P. A., Vibbard, C. A., and Granstein, R. D. (1991). Production of latent transforming growth factor-beta and other inhibitory factors by cultured murine iris and ciliary body cells. *Curr. Eye Res.* **10,** 761-771.

Kogawa, K., nakamura, T., Sugino, K., Takio, K., Titani, K., and Sugino, H. (1991). Activin-binding protein is present in pituitary. *Endocrinology* **128,** 1434-1440.

Kokan-Moore, N. P., Bolender, D., and Lough, J. (1991). Secretion of inhibin βA by endoderm cultured from early embryonic chicken. *Dev. Biol.* **146,** 242-245.

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,** 2385-96.

Kos, K., and Coulombe, J. N. (1997). Activin receptor mRNA expression by neurons of the avian ciliary ganglion. *J. Neurobiol.* **32,** 33-44.

Kurosaki, T., Fukuda, K., Konno, T., Mori, Y., Tanaka, K., Mishina, M., and Numa, S. (1987). Functional properties of nicotinic acetylcholine receptor subunits expressed in various combinations. *FEBS Letters* **214,** 253-258.

Labonne, C., and Whitman, M. (1994). Mesoderm induction by activin Rrequires FGF-mediated intracellular signals. *Development* **120,** 463-472.

Landis, S. C. (1990). Target regulation of neurotransmitter phenotype. *Trends in Neurosciences* **13,** 344-50.

Landis, S. C. (1994). Target determination of neurotransmitter phenotype in sympathetic neurons. *J. Neurobiol.* **25,** 620-639.

Landmesser, L., and Pilar, G. (1974). Synapse formation during embryogenesis of ganglion cells lacking a periphery. *J. Physiol.* **241,** 715-749.

Leblanc, G., and Landis, S. (1986). Development of choline acetyltransferase (CAT) in the sympathetic innervation of rat sweat glands. *J. Neurosci.* **6,** 260-5.

Leferovich, J. M., Lana, D. P., Sutrave, P., Hughes, S. H., and Kelly, A. M. (1995). Regulation of *c-ski* transgene expression in developing adn mature mice. *J. Neurosci.* **15,** 596-603.

LeLievre, C., and Le Douarin, N. (1975). Mesenchymal derivatives of the neural crest: analysis of chimeric quail and chick embryos. *J. Embryol. Exp. Morphol.* **34,** 125-154.

Leung, D. W., Parent, A. S., Cachianes, G., Esch, F., Coulombe, J. N., Nikolics, K., Eckenstein, F. P., and Nishi, R. (1992). Cloning, expression during development, and evidence for release of a trophic factor for ciliary ganglion neurons. *Neuron* **8,** 1045-1053.

Levin, M., Johnson, R. L., Stern, C. D., Kuehn, M., and Tabin, C. (1995). A molecular pathway determing left-right asymmetry in chick embryogenesis. *Cell* **82,** 803-814.

Lewis, W. H. (1903). Wandering pigmented cells arising from the epithelium of the optic cup with observation of the muscle sphincter pupillae in the chick. *Am. J. Anat.* **2,** 405-416.

Li, L., Zhou, J., James, G., Heller-Harrison, R., Czech, M. P., and Olson, E. N. (1992). FGF inactivates myogenic helix-loop-helix proteins through phosphorylation of a conserved protein kinase C site in their DNA-binding domains. *Cell* **71,** 1181-94.

Lin, Z., Lu, M.-H., Schultheiss, T., Choi, J., Holtzer, S., DiLullo, C., Fischman, D. A., and Holtzer, H. (1994). Sequential appearance of muscle-specific proteins in myoblasts as a function of time after cell division: evidence for a conserved myoblast differentiation program in skeletal muscle. *Cell Motil. Cyto.* **29,** 1-19.

Ling, N., Ying, S.-Y., Ueno, N., Esch, F., Denoroy, L., and Guillemin, R. (1985). Isolation and partial characterization of a Mr 32,000 protein with inhibin

activity from porcine follicular fluid. *Proc. Natl. Acad. Sci. USA* **82,** 7217-7221.

Link, B. A., and Nishi, R. (1997). Opposing effects of activin A and follistatin on developing skeletal muscle cells. *Exp. Cell Res.* **233,** 350-362.

Lopashov, G. V., and Stovea, O. G. (1972). Development of the eye, experimental studies. *J. Embryol. Exp. Morph.* **28,** 521-546.

Ludolph, D. C., and Konieczny, S. F. (1995). Transcription factor families: muscling in on the myogenic program. *FASEB Journal* **9,** 1595-604.

Luo, G., Hofmann, C., Bronckers, A., Sohocki, M., Bradley, A., and Karsenty, G. (1995). BMP-7 is an inducer of nephrogenesis, and is also required for eye development and skeletal patterning. *Genes & Dev.* **9,** 2808-2820.

Lyons, I., Parsons, L. M., Hartley, L., Li, R., Andrews, J. E., Robb, L., and Harvey, R. P. (1995). Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeobox gene Nkx2-5. *Genes & Dev.* **9,** 1654-66.

Macias-Silva, M., Adbollah, S., Hoodless, P. A., Pirone, R., Attisano, L., and Wrana, J. L. (1996). MADR2 is a substrate of the TGFβ receptor and its phosphorylation is required for nuclear accumulation and signaling. *Cell* **87,** 1215-1224.

Mains, R. E., and Patterson, P. H. (1973). Primary cultures of dissociated sympathetic neurons. *J. Cell Biol.* **59,** 329-345.

Manthorpe, M., Skaper, S., Adler, R., Landa, K., and Varon, S. (1980). Cholinergic neurotrophic factors: fractionation properties of an extract from selected chick embryonic eye tissues. *J. Neurochem.* **34,** 69-75.

Marcelle, C., Wolf, J., and Bronner-Fraser, M. (1995). The in vivo expression of the FGF receptor FREK mRNA in avian myoblasts suggests a role in muscle growth and differentiation. *Dev. Biol.* **172,** 100-14.

Martin, J. F., Li, L., and Olson, E. N. (1992). Repression of myogenin function by TGF-beta 1 is targeted at the basic helix-loop-helix motif and is independent of E2A products. *J. Biol. Chem.* **267,** 10956-60.

Marwitt, R., Pilar, G., and Weakly, J. N. (1971). Characterization of two ganglion cell populations in avian ciliary ganglia. *Brain Res* **25,** 317-34.

Mason, A. J., Hayflick, J. S., Ling, N., Esch, F., Ueno, N., Ying, S.-Y., Gullemin, R., Niall, H., and Seeburg, P. H. (1985). Complementary DNA sequence of ovarian follicular fluid inhibin show precursor structure and homology with transforming growth factor-beta. *Nature* **318,** 27-32.

Massagué, J. (1996). TGFβ signaling: receptors, transducers, and mad proteins. *Cell* **85,** 947-950.

Masu, Y., Wolf, E., Holtmann, B., Sendtner, M., Brem, G., and Thoenen, H. (1993). Disruption of the CNTF gene results in motor neuron degeneration. *Nature* **365,** 27-32.

Mathers, P. H., Grinberg, A., Mahon, K. A., and Jamrich, M. (1997). The *Rx* homeobox gene is essential for vertebrate eye development. *Nature* **387,** 603-607.

Matzuk, M. M., Kumar, T. R., and Bradley, A. (1995a). Different phenotypes for mice deficient in either activins or activin receptor type II. *Nature* **374,** 356-360.

Matzuk, M. M., Kumar, T. R., Vassalli, A., Bickenbach, J. R., Roop, D. R., Jaenisch, R., and Bradley, A. (1995b). Functional analysis of activins during mammalian development. *Nature* **374,** 354-356.

Matzuk, M. M., Lu, N., Vogel, H., Sellheyer, K., Roop, D. R., and Bradley, A. (1995c). Multiple defects and perinatal death in mice deficient in follistatin. *Nature* **374,** 360-363.

McDonald, N. Q., and Hendrickson, W. A. (1992). A structural superfamily of growth factors containing a cysteine knot motif. *Cell* **73,** 421-424.

McPherron, A. C., Lawler, A. M., and Lee, S. (1997). Regulation of skeletal muscle mass in mice by a new TGF-β superfamily member. *Nature* **387,** 83-90.

Michel, U., Farnworth, P., and Findlay, J. K. (1993). Follistatins: more than follicle-stimulating hormone suppressing proteins. *Mol. Cell. Endocrinology* **91,** 1-11.

Mitrani, E., Ziv, T., Thomsen, G., Shimoni, Y., Melton, D. A., and Bril, A. (1990). Activin can induce the formation of axial structures and is expressed in the hypoblast of the chick. *Cell* **63,** 495-501.

Montarras, D., Chelly, J., Bober, E., Arnold, H., Ott, M.-O., Gros, F., and Pinset, C. (1991). Developmental patterns in the expression of myf5, myoD, myogenin, and mrf4 during myogenesis. *New Biol.* **3,** 592-600.

Moses, H. L., and Serra, R. (1996). Regulation of differentiation by TGF-β. *Curr. Op. Genet. Dev.* **6,** 581-586.

Münsterberg, A. E., Kitajewski, J., Bumcrot, D. A., McMahon, A. P., and Lassar, A. B. (1995). Combinatorial signaling by Sonic hedgehog and Wnt

family members induces myogenic bHLH gene expression in the somite. *Genes & Dev.* **9,** 2911-22.

Murata, M., Eto, Y., Shibai, H., Sakai, M., and Muramatsu, M. (1988). Erythroid differentiation factor is encoded by the same gene as that of inhibin βA. *Proc. Natl. Acad. Sci. USA* **87,** 8365-8369.

Murray, S. S., Murray, E. J., Glackin, C. A., and Urist, M. R. (1993). Bone morphogenetic protein inhibits differentiation and affects expression of helix-loop-helix regulatory molecules in myoblastic cells. *Journal of Cellular Biochemistry* **53,** 51-60.

Nakamura, T., Takio, K., Eto, Y., Shinbai, H., Titani, K., and Sugino, H. (1990). Activin-binding protein from rat ovary is follistatin. *Science* **247,** 836-838.

Nakano, K. E., and Nakamura, H. (1985). Origin of the irideal striated muscle in birds. *J. Embryol. Exp. Morphol.* **88,** 1-13.

Narayanan, C. H., and Narayanan, Y. (1976). An experimental inquiry into the central source of preganglionic fibers to the chick ciliary ganglion. *J. Comp. Neurol.* **166,** 101-110.

Narayanan, C. H., and Narayanan, Y. (1978). On the origin of the ciliary ganglion in birds studied by the method of interspecific transplantation of embryonic brain regions between quail and chick. *J. Embryol. Exp. Morph.* **47,** 137-148.

Narayanan, Y., and Narayanan, C. H. (1981). Ultrastructural and histochemical observations in the developing iris musculature in the chick. *J. Embryol. Exp. Morphol.* **62,** 117-127.

Nascone, N., and Mercola, M. (1995). An inductive role of the endoderm in Xenopus cardiogenesis. *Development* **121,** 515-523.

Nieto, M. A., Patel, K., and Wilkinson, D. G. (1996). Methods in Avian embryology. *In* "Methods in Cell Biology" (M. A. Bronner-Fraser, Ed.), Vol. 51, pp. 220-235. Academic Press, Inc., San Diego.

Nishi, R. (1994). Target-derived molecules that influence the development of neurons in the avian ciliary ganglion. *J. Neurobiol.* **25,** 612-619.

Nishi, R., and Berg, D. K. (1979). Survival and development of ciliary ganglion neurones grown alone in cell culture. *Nature* **277,** 232-234.

Nishi, R., and Berg, D. (1981). Two components from eye tissue that differentially stimulate the growth and development of ciliary ganglion neurons in cell culture. *J. Neurosci.* **1,** 505-513.

Niswander, L., and Martin, G. R. (1993). FGF-4 and BMP-2 have opposite effects on limb growth. *Nature* **361,** 68-71.

Nohno, T., Noji, S., Koyama, E., Myokai, F., Ohuchi, H., Nishikawa, K., Sumitomo, S., Taniguchi, S., and Saito, T. (1993). Expression patterns of the activin receptor type IIA and IIB genes during chick limb development. *Prog. Clin. Biol. Res.* **383B,** 705-714.

Nye, J. S., Kopan, R., and Axel, R. (1994). An activated Notch suppresses neurogenesis and myogenesis but not gliogenesis in mammalian cells. *Development* **120,** 2421-30.

Ohuchi, H., Noji, S., Koyama, E., Myokai, F., Nishikawa, K., Nohno, T., Tashiro, K., Shiokawa, K., Matsuo, N., and Taniguchi, S. (1992). Expression pattern of the activin receptor type IIA gene during differentiation of chick neural tissues, muscle and skin. *FEBS Lett* **303,** 185-189.

Olson, E. N., Caldwell, K. C., Gordon, J. I., and Glaser, L. (1983). Regulation of creatine phosphokinase expression during differentiation of BC3H1 cells. *J. Biol. Chem.* **250,** 4007-4021.

Olson, E. N., Perry, M., and Schulz, R. A. (1995). Regulation of muscle differentiation by the MEF2 family of MADS box transcription factors. *Dev. Biol.* **172,** 2-14.

Olson, N. J., Pearson, R. B., Needleman, D. S., Hurwitz, M. Y., Kemp, B. E., and Means, A. R. (1990). Regulatory and structural motifs of chicken gizzard myosin light chain kinase. *Proc. Natl. Acad. Sci. USA* **87,** 2284-2288.

Olwin, B. B., and Hauschka, S. D. (1990). Fibroblast growth factor receptor levels decrease during chick embryogenesis. *J. Cell Biol.* **110,** 503-9.

Olwin, B. B., and Rapraeger, A. (1992). Repression of myogenic differentiation by aFGF, bFGF, and K-FGF is dependent on cellular heparan sulfate. *J. Cell Biol.* **118,** 631-9.

Opas, M., and Dziak, E. (1994). FGF-induced trasdifferentiation of RPE to neuronal progenitors is regulated by the mechanical properties of the substratum. *Dev. Biol.* **161,** 440-454.

Oppenheim, R. W. (1989). Cell death during the development of the nervous system. *Annl. Rev. Neurosci.* **14,** 453-501.

Ordahl, C. P., and Le Douarin, N. M. (1992). Two myogenic lineages within the developing somite. *Development* **114,** 339-353.

Owens, G. K. (1995). Regulation of differentiation of vascular smooth muscle cells. *Physiol. Rev.* **75,** 487-517.

Park, C. M., and Hollenberg, M. J. (1991). Induction of retinal regeneration in vivo by growth factors. *Dev. Biol.* **148,** 222-233.

Pasquale, L. R., Dorman-Pease, M. E., Lutty, G. A., Quigley, H. A., and Jampel, H. D. (1993). Immunolocalization of TGFb1, TGFb2, and TGFb3 in the anterior segment of the human eye. *Invest. Ophthal. Vis. Sci.* **34,** 23-30.

Patel, K., Connolly, D. J., Amthor, H., Nose, K., and Cooke, J. (1996). Cloning and early dorsal axial expression of Flik, a chick follistatin-related gene: evidence for involvement in dorsalization/neural induction. *Dev. Biol.* **178,** 327-342.

Patterson, J. M., and Zakon, H. H. (1997). Transdifferentiation of muscle to electric organ: regulation of muscle-specific proteins is independent of patterned nerve activity. *Dev. Biol.* **186,** 115-126.

Patterson, P. H., and Nawa, H. (1993). Neuronal differentiation factors/cytokines and synaptic plasticity. *Neuron* **10 (Suppl),** 123-137.

Pelton, R. W., Saxena, B., Jones, M., Moses, H. L., and Gold, L. I. (1991). Immunohistochemical localization of TGF beta 1, TGF beta 2, and TGF beta 3 in the mouse embryo: expression patterns suggest multiple roles during embryonic development. *J. Cell Biol.* **115,** 1091-1105.

Petraglia, F., Gallinelli, A., Grande, A., Florio, P., Ferrari, S., Genazzani, A. R., Ling, N., and Depaolo, L. V. (1994). Local production and action of follistatin in human placenta. *J. Clin. Endocrinol. Metab.* **78,** 205-210.

Piccolo, S., Sasai, Y., Lu, B., and De Robertis, E. M. (1996). Dorsoventral patterning in Xenopus: inhibition of ventral signals by direct binding of chordin to BMP-4. *Cell* **86,** 589-598.

Pilar, G., Landmesser, L., and Burstein, L. (1980). Competition for survival among developing ciliary ganglion cells. *J. Neurophysiol.* **43,** 233-254.

Pilar, G., Nunez, R., McLennan, I. S., and Meriney, S. D. (1987). Muscarinic and nicotinic synaptic activation of the developing chicken iris. *J. Neurosci.* **7,** 3813-26.

Pilar, G., Tuttle, J., and Vaca, K. (1981). Functional maturation of motor nerve terminals in the avian iris: ultrastructure, transmitter metabolism and synaptic reliability. *J Physiol (Lond)* **321,** 175-93.

Pittack, C., Grunwald, G. B., and Reh, T. A. (1997). Fibroblast growth factors are necessary for neural retina but not pigmented epithelium differentiation in chick embryos. *Development* **124,** 805-816.

Pittack, C., Jones, M., and Reh, T. A. (1991). Basic fibroblast growth factor induces retinal pigmented epithelium to generate neural retina in vitro. *Development* **113,** 577-588.

Pituello, F., Yamada, G., and Gruss, P. (1995). Activin A inhibits pax-6 expression and perturbs cell differentiation in the developing spinal cord in vitro. *Proc. Natl. Acad. Sci. USA* **92,** 6952-6956.

Potter, D. D., Landis, S. C., Matsumoto, S. G., and Furshpan, E. J. (1986). Synaptic functions in rat sympathetic neurons in microculture. II. Adrenergic/cholinergic dual status and plasticity. *J. Neurosci.* **6,** 1080-1098.

Pourquie, O., Fan, C.-M., Coltey, M., Hirsinger, E., Wantanabe, Y., Breant, 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.

Pownall, M. E., and Emerson, C. P. (1992). Sequential activation of three myogenic regulatory genes during somite morphogenesis in quail embryos. *Dev. Biol.* **151,** 67-78.

Qian, L., Vu, M. N., Carter, M. S., Doskow, J., and Wilkinson, M. F. (1993). T cell receptor-β mRNA splicing during thymic maturation *in vivo* and in an inducible T cell clone *in vitro*. *J. Immunol.* **151,** 6801-6814.

Rao, M. S., Sun, Y., Escary, J. L., Perreau, J., Tresser, S., Patterson, P. H., Zigmond, R. E., Brulet, P., and Landis, S. C. (1993). Leukemia inhibitory factor mediates an injury response but not a target-directed developmental transmitter switch in sympathetic neurons. *Neuron* **11,** 1175-85.

Reber-Muller, S., Ono, S., Wehrle-Haller, M., and Schmid, V. (1994). Transdifferentiation of striated muscle of jellyfish to smooth muscle and nerve cells: the role of cell-ECM interactions and carbohydrates revealed by a monoclonal antibody. *Differentiation* **57,** 77-87.

Reh, T. A. (1992). Generation of neuronal diversity in the vertebrate retina. *In* "Determinants of Neuronal Identity" (M. Shankland and E. Macagno, Eds.), pp. 433-467. Academic Press, New York.

Reh, T. A., Nagy, T., and Gretton, H. (1987). Retinal pigmented epithelial cells induced to transdifferentiate to neurons by laminin. *Nature* **330,** 68-71.

Robert, B., Sassoon, D., Jacq, B., Gehring, W., and Buckingham, M. (1991). Hox-7, a mouse homeobox gene with a novel pattern of expression during embryogenesis. *EMBO J.* **8,** 91-100.

Ruegg, M. A., Tsim, K. W. K., Horton, S. E., Kroger, S., Escher, G., Gensch, E. M., and McMahan, U. J. (1992). The agrin gene codes for a family of basal lamina proteins that differ in function and distribution. *Neuron* **8,** 691-823.

Rupp, R. A., Snider, L., and Weintraub, H. (1994). Xenopus embryos regulate the nuclear localization of XMyoD. *Genes & Dev.* **8,** 1311-23.

Saadat, S., Sendtner, M., and Rohrer, H. (1989). Ciliary neurotrophic factor induces cholinergic differentiation of rat sympathetic neurons in culture. *J. Cell Biol.* **108,** 1807-1816.

Sanford, L. P., Ormsby, I., Gittenberger-deGroot, A. C., Sariola, H., Friedman, R., Boivin, G. P., Cardell, E. L., and Doetschman, T. (1997). TGFβ2 knockout mice have multiple developmental defects that are non-overlapping with other TGFβ knockout phenotypes. *Development* **124,** 2659-2670.

Sasson, D. A. (1993). Myogenic regulatory factors: dissecting their role and regulation during vertebrate embryogenesis. *Dev. Biol.* **156,** 11-23.

Scapolo, P. A., Peirone, S. M., Filogamo, G., and Veggetti, A. (1988). Histochemical, immunohistochemical, and ultrastructural observations on the iris muscles of *Gallus gallus*. *Anat. Rec.* **221,** 687-699.

Scheutze, S. M., and Role, L. W. (1987). Developmental regulation of nicotinic acetylcholine receptors. *Ann. Rev. Neurosci.* **10,** 403-457.

Schmid, C., Steiner, T., and Froesch, E. R. (1983). Preferential enhancement of myoblast differentiation by insulin-like growth factors (IGF-I and IGF-II) in primary cultures of chicken embryonic cells. *FEBS Lett* **161,** 117-121.

Schotzinger, R. J., and Landis, S. C. (1988). Cholinergic phenotype developed by noradrenergic sympathetic neurons after innervation of a novel cholinergic target in vivo. *Nature* **335,** 637-9.

Schubert, D., Kimura, H., LaCorbiere, M., Vaughan, J., Karr, D., and Fischer, W. H. (1990). Activin is a nerve cell survival molecule. *Nature* **344,** 868-870.

Schulte-Merker, S., Smith, J. C., and Dale, L. (1994). Effects of truncated activin and FGF receptors and of follistatin on the inducing activities of Vg1 and activin: does activin play a role in mesoderm induction? *EMBO J.* **13,** 3533-3541.

Schultheiss, T. M., Burch, J. B., and Lassar, A. B. (1997). A role for bone morphogenetic proteins in the induction of cardiac myogenesis. *Genes & Dev.* **11,** 451-462.

Schultheiss, T. M., Xydas, S., and Lassar, A. B. (1995). Induction of avian cardiac myogenesis by anterior endoderm. *Development* **121,** 4203-14.

Schwall, R. H., and Lai, C. (1991). "Erythroid differentiation bioassays for activin." Academic Press, Inc, San Diego, CA.

Seed, J., and Hauschka, S. D. (1988). Clonal analysis of vertebrate myogenesis. VIII. Fibroblasts growth factor (FGF)-dependent and FGF-independent muscle colony types during chick wing development. *Dev. Biol.* **128,** 40-9.

Shah, N. M., Groves, A. K., and Anderson, D. J. (1996). Alternative neural crest cell fates are instructively promoted by TGFβ superfamily members. *Cell* **85,** 331-343.

Shainberg, A., Yagil, Y., and Yaffe, D. (1971). Alterations of enzymatic activities during muscle differentiation in vitro. *Dev. Biol.* **25,** 1-29.

Shimasaki, S., Koga, M., Buscaglia, M. L., Simmons, D. M., Bicsak, T. A., and Ling, N. (1989). Follistatin gene expression in the ovary and extragonadal tissues. *Mol. Endocrinol.* **3,** 651-659.

Shimasaki, S., Koga, M., Esch, F., Cooksey, K., Mercado, M., Koba, A., Ueno, N., Ying, S.-Y., Ling, N., and Guillemin, R. (1988a). Primary structure of the human follistatin precursor and its genomic organization. *Proc. Natl. Acad. Sci. USA* **85,** 4218-4222.

Shimasaki, S., Koga, M., Esch, F., Mercado, M., Cooksey, K., Koba, A., and Ling, N. (1988b). Porcine follistatin gene structure supports two forms of mature follistatin produced by alternative splicing. *Biochem. Biophys. Res. Comm.* **152,** 717-723.

Shiozuka, M., Momoi, T., and Kimura, I. (1997). Activin A inhibits differentiation of chick myogenic cells *in vitro*. *Zoolog. Sci.* **14,** 327-330.

Shipley, G. D. (1986). A serum-free [3H] thymidine incorporation assay for the detection of transforming growth factors. *J. Tiss. Cult. Methods* **10,** 117-123.

Smet, P. J., and Rush, R. A. (1993). Effect of ciliary neuronotrophic factor on somatostatin expression in chick ciliary ganglion neurons. *Brain Res.* **609,** 351-6.

Song, K., Wang, Y., and Sassoon, D. (1992). Expression of Hox-7.1 in myoblasts inhibits terminal differentiation and induces cell transformation. *Nature* **360,** 477-481.

Stern, C. D., Yu, R. T., Kakizuka, A., Kintner, C. R., Mathews, L. S., Vale, W. W., Evans, R. M., and Umesono, K. (1995a). Activin and its receptors

during gastrulation and the later phases of mesoderm development in the chick embryo. *Dev. Biol.* **172,** 192-205.

Stern, H. M., Brown, A. M., and Hauschka, S. D. (1995b). Myogenesis in paraxial mesoderm: preferential induction by dorsal neural tube and by cells expressing Wnt-1. *Development* **121,** 3675-86.

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.

Stevens, L. M., and Landis, S. C. (1990). Target influences on transmitter choice by sympathetic neurons developing in the anterior chamber of the eye. *Dev. Biol.* **137,** 109-24.

Stroeva, O. G. (1967). The correlation of the processes of proliferation and determination in the morphogenesis of iris and ciliary body in rats. *J. Embryol. Exp. Morph.* **18,** 269-287.

Sugawara, M., DePaolo, L., Nakatani, A., DiMarzo, S. J., and Ling, N. (1990). Radioimmunoassay of follistatin: application for in vitro fertilization procedures. *J. Clin. Endocrinol. Metab.* **71,** 1672-1674.

Sugino, K., Kurosawa, N., Nakamura, T., Takio, K., Shimasaki, S., Ling, N., Titani, K., and Sugino, H. (1993). Molecular heterogeneity of follistatin, an activin-binding protein. *J. Biol. Chem.* **268,** 15579-15587.

Sumitomo, S., Inouye, S., Lui, X. J., Ling, N., and Shimasaki, S. (1995). The heparin binding site of follistatin is involved in its interaction with activin. *Biochem. Biophys. Res. Comm.* **208,** 1-9.

Tajbakhsh, S., and Buckingham, M. E. (1994). Mouse limb muscle is determined in the absence of the earliest myogenic factor myf-5. *Poc. Natl. Acad. Sci. USA* **91,** 747-751.

Tajbakhsh, S., and Buckingham, M. E. (1995). Lineage restriction of the myogenic conversion factor myf-5 in the brain. *Development* **121,** 4077-83.

Tajbakhsh, S., Vivarelli, E., Cusella-De Angelis, G., Rocancourt, D., Buckingham, M., and Cossu, G. (1994). A population of myogenic cells derived from the mouse neural tube. *Neuron* **13,** 813-21.

Takada, S., Stark, K. L., Shea, M. J., Vassileva, G., McMahon, J. A., and McMahon, A. P. (1994). Wnt-3A regulates somite and tailbud formation in the mouse embryo. *Genes & Dev.* **8,** 174-189.

Thomsen, G., Woolf, T., Whitman, M., Sokol, S., Vaughan, J., Vale, W., and Melton, D. A. (1990). Activins are expressed early in *Xenopus*

embryogenesis and can induce axial mesoderm adn anterior structures. *Cell* **63,** 485-493.

Tollefsen, S. E., Lajara, R., McCusker, R. H., Clemmons, D. R., and Rotwein, P. (1989). Insulin-like growth factors (IGF) in muscle development. Expression of IGF-I, the IGF-I receptor, and an IGF binding protein during myoblast differentiation. *J. Biol. Chem.* **264,** 13810-7.

Tribioli, C., Frasch, M., and Lufkin, T. (1997). BAPX1 - an evolutionary conserved homologue of the *drosophila bagpipe* homeobox gene is expressed in splanchnic mesoderm and the embryonic skeleton. *Mech. Dev.* **65,** 145-162.

Tripathi, B. J., Tripathi, R. C., Livingston, A. M., and Borisuth, S. C. (1991). The role of growth factors in the embryogenesis and differentiation of the eye. *Amer. J. Anat.* **192,** 442-471.

Trueb, B., Schreier, T., Winterhalter, K. H., and Strehler, E. (1988). Sequence of a cDNA clone encoding chicken ribosomal protein S17. *Nucl. Acids Res.* **16,** 4723.

Tuttle, J. B., Vaca, K., and Pilar, G. (1983). Target influences on [3H]ACh synthesis and release by ciliary ganglion neurons *in vitro*. *Dev. Biol.* **97,** 255-263.

Tuuri, T., Eramaa, M., Hilden, K., and Ritvos, O. (1994). The tissue distribution of activin beta-A and beta-B subunit and follistatin messenger ribonucleic acids suggests multiple sites of action for the activin-follistatin system during human development. *J. Clinical Endo. and Metabol* **78,** 1521-1524.

Ueno, N., Ling, N., Ying, S. Y., Esch, F., Shimasaki, S., and Guillemin, R. (1987). Isolation and partial characterization of follistatin: a single chain Mr 35,000 monomeric protein that inhibits the release of follicle-stimulating hormone. *Proc. Natl. Acad. Sci. USA* **84,** 8282-8286.

Vacca, L. L., Abrahams, S. J., and Naftchi, N. E. (1980). A modified peroxidase antiperoxidase procedure for improved localization of tissue antigens: localization of substance P in rat spinal cord. *J Histoch Cytoch* **28,** 297- 307.

Vaidya, T. B., Rhodes, S. J., Taparowsky, E. J., and Konieczny, S. F. (1989). Fibroblast growth factor and transforming growth factor beta repress transcription of the myogenic regulatory gene MyoD1. *Mol. Cell. Biol.* **9,** 3576-9.

Vainio, S., and Muller, U. (1997). Inductive tissue interactions, cell signaling, and the control of kidney organogenesis. *Cell* **90,** 975-978.

Vale, W., Hsueh, A., Rivier, C., and Yu, J. (1991). The inhibin/activin family of hormones and growth factors. *In* "Peptide Growth Factors and Their Receptors" (A. B. Roberts and M. B. Sporn, Eds.), pp. 211-248. Springer-Verlag, New York.

Vale, W., Rivier, J., Vaughan, J., McClintock, R., Corrigan, A., Woo, W., Darr, D., and Spiess, J. (1986). Purification and characterization of an FSH-releasing protein from porcine ovarian follicular fluid. *Nature* **321,** 776-779.

Vandenburgh, H. H., Karlisch, P., Shansky, J., and Feldstein, R. (1991). Insulin and IGF-I induce pronounced hypertrophy of skeletal myofibers in tissue culture. *Am. J. Physiol* **260,** C475-C484.

Vassalli, A., Matzuk, M. M., Gardner, H., Lee, K.-F., and Jaenisch, R. (1994). Activin/inhibin beta-B subunit gene disruption leads to defects in eyelid development and female reproduction. *Genes & Dev.* **8,** 414-427.

Vaughan, J. M., Rivier, J., Corrigan, A. Z., McClintock, R., Campen, C. A., Jolley, D., Vogelmayr, J. K., Bardin, C. W., Rivier, C., and Vale, W. W. (1989). Detection and purification of inhibin using antisera generated against synthetic peptide fragments. *Meth. Enzymol.* **168,** 588-617.

Verschueren, K., Dewulf, N., Goumans, M.-J., Lonnoy, O., Feijen, A., Grimsby, S., Spiegle, K. V., Dijke, P., Moren, A., Vanscheeuwijck, P., Heldin, C.-H., Miyazono, K., Mummery, C., Eijnden-Van Raaij, J., and Huylebroeck, D. (1995). Expression of type I and type IB receptors for activin in midgestation mouse embryos suggests distinct functions in organogenesis. *Mech. Dev.* **52,** 109-123.

Volpe, P., Biral, D., Pizzo, P., Salviati, G., and Margreth, A. (1993). Ontogenesis of chick iris intrinsic muscles: evidence for a smooth-to-striated transition. *Dev. Biol.* **159,** 441-449.

Wachtler, F., and Jacob, M. (1986). Origin and development of the cranial skeletal muscles. *Biblthca Anat.* **29,** 24-46.

Wall, N. A., and Hogan, B. L. M. (1994). TGF-β related genes in development. *Curr. Op. Genet. Dev.* **4,** 517-522.

Weaver, C. D., Yoshida, C. K., de Curtis, I., and Reichardt, L. F. (1995). Expression and in vitro function of β_1-integrin laminin receptors in the developing avian ciliary ganglion. *J. Neurosci.* **15,** 5275-5285.

Weber, M. J. (1981). A diffusible factor responsible for the determination of cholinergic fuctions in cultured sympathetic neurons. *J. Biol. Chem.* **256,** 3447-3453.

Weintraub, H. (1993). The MyoD family and myogenesis: redundancy, networks, and thresholds. *Cell* **75,** 1241-4.

Wells, D. G., Zawisa, M. J., and Hume, R. I. (1995). Changes in responsiveness to extracellular ATP in chick skeletal muscle during development and upon innervation. *Dev. Biol.* **172,** 585-590.

Williams, B. A., and Ordahl, C. P. (1994). Pax-3 expression in segmental mesoderm marks early stages in myogenic cell specification. *Development* **120,** 785-796.

Wittbrodt, J., and Rosa, F. M. (1994). Disruption of mesoderm and axis formation in fish by ectopic expression of activin variants: the role of maternal activin. *Genes & Dev.* **8,** 1448-1462.

Woloshin, P., Song, K., Degnin, C., Killary, A. M., Goldhamer, D. J., Sassoon, D., and Thayer, M. J. (1995). MSX1 inhibits myoD expression in fibroblast x 10T1/2 cell hybrids. *Cell* **82,** 611-620.

Woo, K., and Fraser, S. E. (1995). Order and coherence in the fate map of the zebrafish nervous system. *Development* **121,** 2595-2609.

Wrana, J. L., Attisano, L., Wieser, R., Ventura, F., and Massague, J. (1994). Mechanism of activation of the TGFβ receptor. *Nature* **370,** 341-347.

Wright, W. E., Sassoon, D. A., and Lin, V. K. (1989). Myogenin, a factor regulating myogenesis, has a domain homologous to MyoD. *Cell* **56,** 607-17.

Xu, J., McKeehan, K., Matsuzaki, K., and McKeehan, W. L. (1995). Inhibin antagonizes inhibition of liver cell growth by activin by a dominant-negative mechanism. *J. Biol. Chem.* **270,** 6308-6313.

Yamaguchi, A., Katagiri, T., Ikeda, T., Wozney, J. M., Rosen, V., Wang, E., Kahn, A., Suda, T., and Yoshiki, S. (1991). Recombinant human bone morphogenic protein-2 stimulates osteoblastic maturation and inhibits myogenic differentiation in vitro. *J. Cell Biol.* **113,** 681-687.

Yamamori, T., Fukada, K., Abersold, R., Korsching, S., Fann, M. J., and Patterson, P. H. (1989). The cholinergic neuronal differentiation factor from heart cells is identical to leukemia inhibitory factor. *Science* **246,** 1412-1415.

Yamashita, H., ten Dijke, P., Huylebroeck, D., Kuber Sampath, T., Andries, M., Smith, J. C., Heldin, C., and Miyazono, K. (1995). Osteogenic protein-1 binds to activin type II receptors and induces certain activin-like effects. *J. Cell Biol.* **130,** 217-226.

Yamashita, T., and Sohal, G. S. (1986). Development of smooth and skeletal muscle cells in the iris of the domestic duck, chick and quail. *Cell Tiss. Res.* **244,** 121-131.

Yamashita, T., and Sohal, G. S. (1987). Embryonic origin of skeletal muscle cells in the iris of the duck and quail. *Cell Tissue Res.* **249,** 31-37.

Yasuda, K. (1979). Transdifferentiation of "lentoid" structures in cultures derived from pigmented epithelium was inhibited by collagen. *Dev. Biol.* **68,** 618-623.

Ying, S. Y. (1989). Inhibins, activins and follistatins. *J. Steroid Biochem.* **33,** 705-713.

Yu, J., Shao, L. E., Lemas, V., Yu, A. L., Vaughan, J., Rivier, J., and Vale, W. (1987). Importance of FSH-releasing protein and inhibin in erythrodifferentiation. *Nature* **330,** 765-767.

Zentella, A., and Massagué, J. (1992). Transforming growth factor beta induces myoblast differentiation in the presence of mitogens. *Proc. Natl. Acad. Sci. USA* **89,** 5176-80.

Zimmerman, L. B., De Jesus-Escobar, J. M., and Harland, R. M. (1996). The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. *Cell* **86,** 599-606.