National Library of Canada
Acquisitions and Bibliographic Services Branch
395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale du Canada
Direction des acquisitions et des services bibliographiques
395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

Canada

PROLIFERATION AND DIFFERENTIATION OF SATELLITE CELLS DERIVED FROM ISOLATED SINGLE MYOFIBRES FROM NORMAL AND DYSTROPHIC ADULT MICE

Alison Lunt

A Thesis Submitted to the School of Graduate Studies of the
University of Ottawa in Partial Fulfillment of the Requirements for the Degree of

Master of Science
in Physiology

Supervisor: Dr. David J. Parry

ISBN 0-612-00542-9

Canada

UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

DEDICATION:

I would like to dedicate this work to my family: to my mother who taught me the importance of learning and hard work; to my father who instilled in me a curiosity for the unknown and the thrills of science; to my brother, Michael, who reminded me that an optimistic attitude and an enthusiastic approach can take you a long way in life; and finally to my husband, Pierre, for his constant support and his unwavering faith in me.

ACKNOWLEDGEMENTS:

My warmest thanks to my supervisor, Dr. David Parry, who taught me the joys of research and the importance of the scientific method. This thesis would not have been completed without his support, advice and encouragement.

I would also like to thank all of those in the Department of Physiology who gave me encouragement and guidance, and who helped make my work possible. In particular I would like to thank Dr. Kenneth Marshall for putting the departmental facilities at my disposal and for the use of his computer; Dr. William Ross for allowing me to use his inverted microscope and Dr. Henry Fliss for the use of his incubator; Dr. Jean-Marc Renaud for invaluable help with my statistical analysis; Hua Franks for guiding me through all my computer mishaps.

My thanks to Dr. David Rosenblatt for his helpful advice, pleasant collaboration and friendship throughout the project.

I would like to extend a special thank you to my friend and colleague, Dr. Daniela Zardini-Zampini for sharing all of the joys and disappointments over the past few years.

Finally, thank you to Ms. Elzbieta Krzykwa and Ms. Sylvie LaChapelle for excellent technical support.

ABSTRACT:

A pure population of myogenic precursor cells was isolated by explanting single mature myofibres from adult mice together with their associated satellite cells and basal lamina into culture. The satellite cells contained within these myofibres subsequently proliferated and differentiated to form meshworks of mature myotubes given the appropriate growth conditions. This is the first report describing the isolation of a population of satellite cell progeny in a primary culture that was completely free of contaminating fibroblasts, macrophages or other non-myogenic cells. Furthermore, it is the first report whereby satellite cells, and their progeny, derived from a single myofibre have been isolated.

In the presence of media which promoted maximal cell proliferation fewer myofibres derived from old mice demonstrated satellite cell growth than myofibres derived from young mice, and those myofibres from old mice which did show satellite cell growth had fewer cells per colony. Application of basic Fibroblast Growth Factor (bFGF) to satellite cell cultures derived from young mouse myofibres resulted in a dose dependent increase in cell proliferation, however there was no difference from basal medium levels in parallel cultures derived from old mice. Despite age differences in the effect of bFGF on cell proliferation, its inhibitory effect on differentiation was present in both old and young mice.

Myofibre explants derived from old dystrophic, dy^{2J} and mdx, mice rarely resulted in satellite cell proliferation, regardless of media in which they were

cultured. Those colonies which did grow from myofibres derived from old dy^{2J} mouse, however, contained similar number of cells to those derived from age matched normal mice. Colonies derived from old mdx mouse myofibres contained fewer cells than the age matched normal mouse satellite cell colonies. Those satellite cell colonies derived from old mdx and old dy^{2J} myofibres appeared morphologically similar to those obtained from age-matched normal mice.

Satellite cells derived from myofibres of old mdx mice demonstrated a different pattern of proliferation as compared to satellite cells derived from old normal mice. Rather than detaching from the original parent myofibre and proliferating on the substratum covering the culture plate surrounding the myofibre, satellite cells derived from old mdx mice remained within the basal lamina tube of the parent myofibre where they proliferated and differentiated to form a single new myotube which replaced the parent myofibre. This was also seen more frequently in cultures derived from old dy^{2J} mice than those from old normal mice, although the difference was not statistically significant. The mean number of myonuclei in the newly formed single myotube within the basal lamina sheath was similar to the mean number of myonuclei in the adult myofibres.

The purity of adult myogenic precursor cell populations obtained by isolating single myofibre gives this method the potential for being a ready source of pure myoblasts, given the appropriate medium conditions, which could be a viable vehicle for various gene therapy strategies.

TABLE OF CONTENTS:

LIST OF FIGURES:

LIST OF TABLES:

LIST OF ABBREVIATIONS:

ACTH	adrenocorticotropin (hormone)
aFGF	acidic fibroblast growth factor
AFA	alcohol-formalin-acetic acid (fixative)
ANOVA	analysis of variance
bFGF	basic fibroblast growth factor
bHLH	basic helix-loop-helix
BM	Basal Medium
BUdR	bromodeoxyuridine
BSA	bovine serum albumin
cDNA	complementary DNA
CEE	chick embryo extract
CM	CEE Medium
CME	crushed muscle extract
CPSR-2	Controlled Process Serum Replacement type II
DMD	Duchenne muscular dystrophy
D-PBS	Dulbecco's phosphate buffered saline
e	embryonic day
ECM	extracellular matrix
EGF	epidermal growth factor
FACS	fluorescence-activated cell sorter
FDB	flexor digitorum brevis
GH	growth hormone
HRP	horseradish peroxidase
HS	horse serum
IGF	insulin-like growth factor
KGF	keratinocyte growth factor
LIF	leukemia inhibitory factor
M-CSF	macrophage colony-stimulating factor
MCT	multiple comparison test
MEM	Minimum Essential Medium
mRNA	messenger RNA
MHC	myosin heavy chain
MSH	melanocyte stimulating hormone
NCAM	neural cell adhesion molecule
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PDGF	platelet-derived growth factor
RT	room temperature
SC	satellite cell
TBS	tris-buffered saline
TH	thyroid hormone
TGF	transforming growth factor
TPA	12-0-tetradecanoylphorbol-13-acetate
[^{3}H]TdR	tritiated thymidine

CHAPTER I

INTRODUCTION

History and theories of skeletal muscle regeneration:

Skeletal muscle regeneration has long been a topic of investigation, however it was a subject largely ignored by English language texts until the early 20th century. Many of the early observations on muscle regeneration were published by German researchers. In the early and mid-nineteenth century microscopic changes in skeletal muscles following injury were observed and interpreted as being regeneration (Waldeyer, 1865; Böttcher, 1858 both as cited by Allbrook, 1981). Zenker (1864, as cited by Allbrook, 1981) quite accurately described the regeneration process as being fibre degeneration surrounded by spindle-shaped cells. He suggested that these cells gave rise to observed ribbon-like formations and eventually formed new muscle fibres. Volkmann (1893, as cited by Allbrook, 1981) further described such ideas as budding and new fibre formation from the ends of old striated myofibres, in addition to the important role of the sarcolemmal tube, and its resident cells (defined later as being satellite cells), in skeletal muscle regeneration. More recent research has confirmed many of these early observations and ideas, and disproven many others.

Observations of living tadpole muscle gave a clear picture of regeneration following injury (Speidel, 1938), as did experimental proof that muscle regeneration played a part in the reconstitution of partly devascularized skeletal muscle (LeGros-Clark, 1946), and the first electron microscopic study of regenerating muscle (Allbrook, 1962).

More recently, Studitsky (1964) studied the plasticity of skeletal muscle following injury. In his basic experiment Studitsky excised, minced and grafted a muscle back into its original bed. One month later, he observed that the minced muscle had been replaced by a regenerated muscle (smaller, yet fully functional in the best cases). Since then it has been well established that mammalian skeletal muscle has the ability to regenerate (Mauro et al., 1970; Carlson, 1973; Mauro, 1979).

It is important to note that the term skeletal muscle regeneration generally refers to the process of myogenesis in mature skeletal muscle. There are two types of regeneration: epimorphic and tissue (Carlson, 1973). Epimorphic regeneration involves the regeneration of a muscle as a component of a regenerating limb. Among the vertebrates epimorphic regeneration is confined principally to the amphibia and reptiles.

Tissue regeneration is the common mode by which injured vertebrate skeletal muscle normally repairs itself. This type of regeneration has been found in all classes of vertebrates. It is the processes underlying tissue regeneration that this thesis will address. Within the domain of tissue regeneration there are continuous (regeneration of damaged muscle fibres to repair the original fibre) and discontinuous (regeneration to form completely new muscle fibres that are distinct from the old fibres) regeneration (see Fig. 1.1). The processes of these two types of tissue regeneration may differ, although there is no evidence to support this.

Two theories have been proposed by various researchers to explain the origin of the myogenic cell responsible for regenerating damaged muscle. Initially it was proposed that new myogenic stem cells would be formed from existing myonuclei

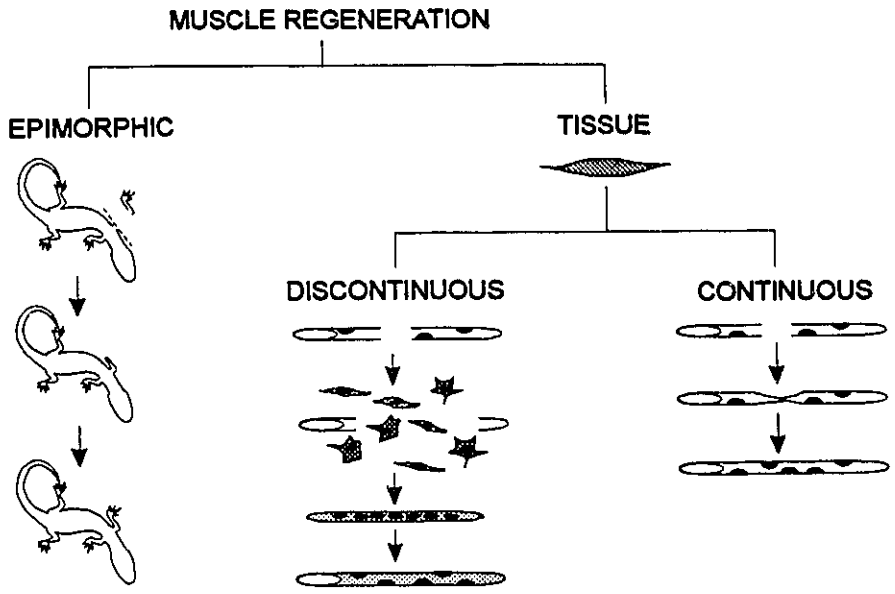


Figure 1.1: Types of muscle regeneration.

in the injured muscle fibre (Reznik, 1969; Hess and Rosner, 1970) through the formation of a plasmalemma around a single peripheral myonucleus and adjacent cytoplasm which subsequently buds off from the injured myofibre to become a regenerating mononucleated cell (Bintliff and Walker, 1960; Reznik, 1969; Kahn and Meyer, 1970; Teräväinen, 1970; Walker, 1972). However, current evidence would suggest that there exists a distinct population of reserve undifferentiated myogenic stem cells (satellite cells arrested in G_0 phase) which divide by mitosis after injury and fuse to form a new population of fibres (Carlson, 1970; Bischoff, 1975; Snow, 1979; Konigsberg, 1979; Allbrook, 1981; Stockdale and Feldman, 1990). Current research is exploring the "mitogenic signal", suspected to be one or more growth factors, that is responsible for triggering myogenesis in satellite cells, thus starting the process of skeletal muscle regeneration.

Certain diseases, such as Duchenne muscular dystrophy (DMD), involve the processes of skeletal muscle degeneration and regeneration without the trigger of trauma applied from external sources (ie. injury). DMD is characterized by persistent skeletal muscle necrosis and eventual loss of satellite cell proliferation

and differentiation which would normally form new muscle fibres. Failure of skeletal muscle regeneration is also seen in the autosomal recessive murine model of dystrophy, dy^{2J}/dy^{2J}, which is phenotypically rather similar to DMD. Another murine model, mdx, is genetically similar to the human condition, X-linked recessive, however it appears that the regenerative capability of mdx skeletal muscle is not lost. It is not known why dy^{2J} muscle has a reduced capacity for regeneration. Is it because the mitogenic signal ("regeneration signal"), normally triggering satellite cell growth following fibre damage or death, is not present, or released, by the degenerating muscle? Or, is it because the satellite cells cannot respond to the mitogenic trigger even though it is present? If the fault lies within the satellite cells then is it simply because the satellite cells have reached their maximum number of cell divisions at a very early age, as a result of ongoing regeneration?

The dy^{2J} and mdx murine models of muscular dystrophy present pathologies that are useful for studying some of the processes involved in, and factors affecting, skeletal muscle regeneration.

CHAPTER II

REVIEW OF THE LITERATURE

2.1 Myogenesis:

It is generally accepted that the growth and development process which occurs following injury in mature muscle (Jones, 1982), and which is exhibited by normal and diseased satellite cells in culture (Cossu et al., 1980), resembles the process seen during normal prenatal/neonatal growth and development, albeit on a smaller scale (see Figure 2.1.1; Campion, 1984; Alameddine et al., 1989). Therefore, a review of normal myogenesis (ie. during embryonic and fetal development), as we presently understand it, must be covered before one may study any potential abnormalities of myogenesis or muscle regeneration, as in Duchennne muscular dystrophy.

The process of myogenesis has been investigated in vivo and in vitro, proving that under these two conditions the same process of proliferation and differentiation of myoblasts to myofibres occurs (Bischoff, 1974; Konigsberg, 1979). In vitro, however, myogenesis rarely proceeds beyond the myotube phase unless

cocultured with nervous tissue (Ecob, 1983), therefore most of the evidence presented in this section has been limited to in vivo studies.

Muscle growth and development is a continuum of new fibre formation, growth and maturation of these fibres, and regeneration of damaged fibres, that occurs over a long period of time from early embryonic development through life into the aged adult (Stockdale and Feldman, 1990).

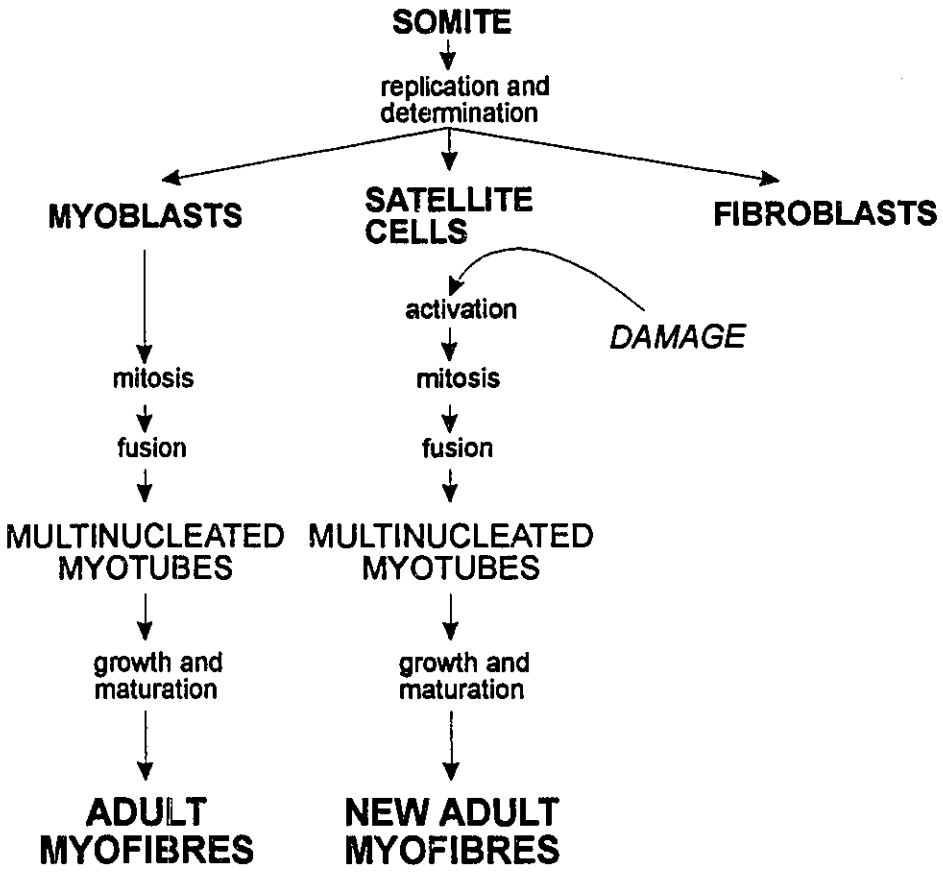


Figure 2.1.1: The processes of myogenesis and muscle regeneration.

It is well established that essentially all vertebrate muscles (striated and smooth) develop from the embryonic mesoderm, the middle of three cell layers in the embryo (See Fig. 2.1.2). The mesoderm divides to form a series of somites (well-defined masses of cells) which eventually divide to give rise to either myogenic (muscle forming) or chondrogenic (cartilage forming) lineages. The myogenic stem

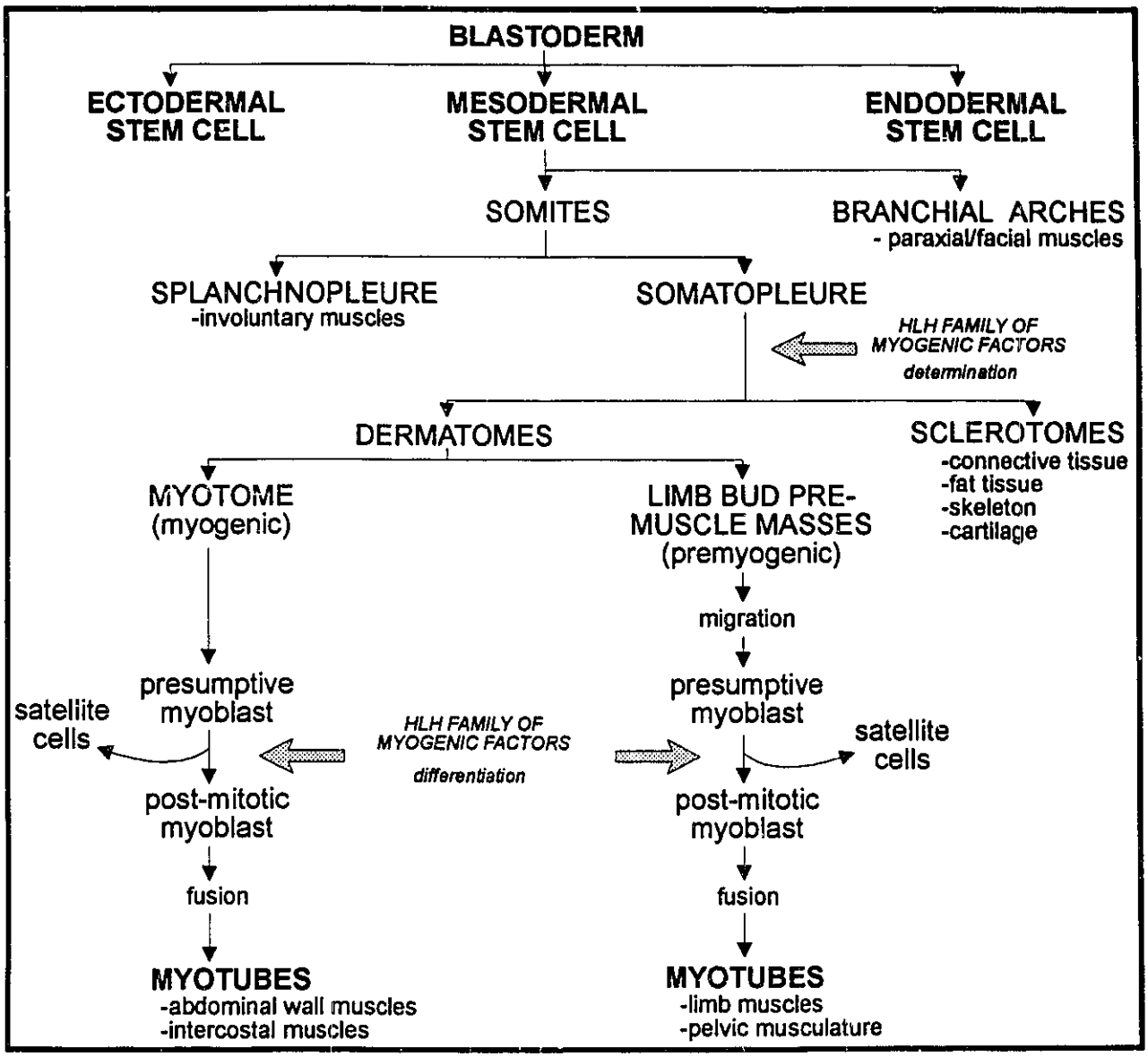


Figure 2.1.2: Developmental origin of muscle.

cell (in the dermatome) now has the potential to form either myoblasts or fibroblasts. Once determination has occurred the myogenic stem cells are called *presumptive myoblasts*, which proceed to proliferate extensively by undergoing multiple mitoses. They eventually become fusion-competent post-mitotic *myoblasts* which line up end to end, fusing to form multinucleate *myotubes* (Bischoff and Holtzer, 1969; Holtzer and Bischoff, 1970). It is generally believed that during late embryogenesis some presumptive myoblasts withdraw from the cell cycle and stay suspended in the G_0 phase; these are *muscle satellite cells* (reviewed in Cossu and Molinaro, 1987; Hartley and Yablonka-Reuveni, 1992; Stockdale, 1992; Yablonka-Reuveni, in press, as cited by Yablonka-Reuveni and Rivera, 1994).

In culture, fusion occurs only during the G_1 phase, and only after the cell has been in this phase for at least five hours (Bischoff and Holtzer, 1969). Fusion of the myoblasts only takes about 8-10 minutes and involves the breakdown of the contiguous membranes so that the nuclei are aligned along the centre of the newly formed myotube. At this point the nuclei are post-mitotic (they can no longer undergo mitosis; Stockdale and Holtzer, 1961), and they become somewhat larger and rounded. More myoblasts may fuse to this new myotube and contribute their nuclei to the existing central chain. The myotubes continue to develop; their cytoplasmic volume increases, and synthesis and assembly of myofibrillar proteins occurs. Organization and differentiation of myofilaments, sarcoplasmic reticulum and transverse tubular systems results in the formation of myofibrils with highly organized sarcomeres. By this time, the nuclei have moved from the centre of the cell to take up new positions under the sarcolemma. It must be noted that in contrast to the myonuclei in developing muscle fibres and regenerated muscle fibres in some other species (for example, the rat; Carlson et al., 1979), the myonuclei of regenerated mouse muscle fibres do not marginate but instead remain central (Schmalbruch, 1986).

The time and place of diversification of myoblasts within embryonic and fetal development is an unresolved issue. There has been, and continues to be,

much research into the questions surrounding myogenic lineage. The possibility of the existence of different myogenic cell lineages stems from the fact that in adult tissue there are multiple types of muscle fibres (in mammals: myosin isoforms type I, IIA, IIB, and IIX). Differences have also been found among myoblasts during development. In contrast to later stage fetal myoblasts (chick embryonic day (e) 8-12, or mouse e15-18), early, or embryonic myoblasts, isolated on chick e3-7 (approximately equivalent to mouse e7-13) form myotubes which express different isoforms of myosin heavy chain in culture (Miller et al., 1985); they will form myotubes, in culture, in the presence of 12-O-tetradecanoylphorbol-13-acetate (TPA; Cossu et al, 1988); they require special types of media if they are to form myotubes in culture (White et al., 1975; White and Hauschka, 1971); they respond differently to growth factors (Seed and Hauschka, 1988); they form small myotubes (2-3 nuclei) with irregular morphology (Miller and Stockdale, 1986a,b); they constitute a relatively small number of cells of the limb bud; and are a transient population (Miller and Stockdale, 1986b).

Muscle fibre formation occurs in two temporally-defined phases (primary and secondary) which are concomitant with the embryonic and fetal stages of the developing mammal or bird (Rubinstein and Kelly, 1981; Bennett, 1983; Kelly and Zachs, 1969, Ontell and Kozeka, 1984). During the first stage of myogenesis (embryonic phase, mouse e11-12) embryonic myoblasts proliferate and fuse to form primary myotubes (mouse e13-14) before morphogenesis is complete. During the subsequent fetal phase of development, proliferation of fetal myoblasts occurs (mouse e15-17) and results in the formation of secondary myotubes (mouse e16-17) which run parallel to the primary myotubes (Ontell and Kozeka, 1984; see reviews by Miller, 1992; Miller and Stockdale, 1987). Satellite cells appear during late fetal development and through the early neonatal phase. Diversity in contractile protein gene expression in developing rodent muscle is evident and has been accounted to different lineages of myoblast populations. Initially, only embryonic myoblasts are present (e9-e12) and they express slow and embryonic myosin heavy chain (MHC;

Vivarelli et al., 1988; Condon et al., 1990; Smith and Miller, 1992; Pin and Merrifield, 1993). Fetal myoblasts, appearing later (e15-e17) also coexpress slow and embryonic MHC (Vivarelli et al., 1988; Smith and Miller, 1992), however, unlike the embryonic myoblasts, a large percentage of the myocytes express perinatal MHC. Even when cultured under identical conditions, embryonic and fetal myoblasts appear to have intrinsically different abilities to express perinatal MHC upon differentiation (Smith and Miller, 1992).

Normal postnatal muscle growth is generally considered to be due to muscle cell hypertrophy, rather than to muscle cell hyperplasia as occurs in the embryo (Allen et al., 1979). Due the apparent requirement that the cytoplasmic/nuclear ratio remains constant, muscle cell hypertrophy is usually accompanied by increases in the number of nuclei contained within the myofibre, and these new nuclei are contributed by the myofibre satellite cells (Rosenblatt and Parry, 1993). Modest increases in fibre number have been reported in neonatal rats, mice and humans, although this phenomenon seems to be confined to the neonatal period of life (Goldspink, 1972).

Recent advances in the field of molecular biology have allowed muscle biologists to explore the ultimate controlling factor in myogenesis; the myogenic determination genes. Extrinsic controls, such as growth factors or innervation, merely superimpose their modulatory effects on the message dictated by the myogenic determination genes.

2.2 Myogenic determination genes:

As discussed previously, establishment of a skeletal muscle phenotype requires commitment of multipotential stem cells to the myogenic lineage, followed by proliferation of, and then differentiation of skeletal myoblasts to form terminally differentiated myotubes.

Early evidence suggested that a "master gene" could set in motion a cascade of events culminating in the formation of mature muscle fibres. These studies (Constantinides et al., 1977, 1978; Taylor and Jones, 1979, 1982; Konieczny and Emerson, 1984) demonstrated that the immortalized fibroblasts cell line C3H10T1/2 (10T1/2) could be converted to myoblasts by exposure to the demethylating agent, 5-azacytidine. The high frequency of conversion (up to 50%, as compared to less than 10^{-8} in the absence of 5-azacytidine) suggested that demethylation of one closely linked locus (gene) by 5-azacytidine exposure was responsible for generation of the myogenic phenotype (Konieczny and Emerson, 1984). Lassar and colleagues (1986) showed direct evidence that a single locus was responsible for fibroblast to myoblast conversion through a genomic transfection study. A single myoblast-specific cDNA was then identified (named MyoD1) and hypothesized to be a regulatory locus (perhaps the "master gene"), continually expressed by myoblasts, which both activates the expression of lineage-specific markers in proliferating myoblasts and makes these cells competent to express other muscle-specific genes when induced to differentiate (Davis et al, 1987).

Subsequently, three MyoD related mammalian factors were independently isolated using different isolation strategies; myogenin (Wright et al., 1989; Edmondson and Olson, 1989), myf5 (Braun et al., 1989a) and MRF4/herculin/myf6 (Rhodes and Konieczny, 1989; Miner and Wold, 1990; Braun et al., 1990) (See Table 2.2.1). Each of these factors can convert 10T1/2 fibroblasts to myoblasts and they each induce virtually all of the morphologic and biochemical aspects of myogenesis. Not only does each member of the MyoD family activate those genes "downstream" associated with myogenesis (eg. *muscle creatine kinase,*

myosin light chain-1/3, acetylcholine receptor α-subunit, myosin heavy chain and *α-skeletal actin* genes), but they also appear to autoregulate their own, and cross-

Table 2.2.1: The MyoD gene family. There is remarkable conservation of the four genes among vertebrates and each family member shares about 80% homology within a segment of approximately 70 amino acids (Murre et al., 1989a,b). (Table modified from Emerson, 1993.)

HUMAN	RODENT	AVIAN (Pownall and Emerson, 1992; Fujisawa-Sehara et al., 1992)	XENOPUS (Gurdon et al., 1992)
myf3 (Braun et al., 1989b)	MyoD (Davis et al.,1987)	QMyoD(qmf1)/CMD1	XMyoD
myf4 (Braun et al., 1989b)	myogenin (Wright et al, 1989)	Qmyogenin/Cmyogenin (qmf2)	Xmyogenin
myf5 (Braun et al., 1989a)	myf5	Qmyf5(qmf3)	Xmyf5
myf6 (Braun et al., 1990)	MRF4/herculin (Rhodes and Konieczny, 1989; Miner and Wold, 1990)	CMRF4	XMRF4

activate one anothers' expression to varying degrees (Thayer et al, 1989; Braun et al., 1989b). These genes are expressed exclusively in muscle and each has a basic amino acid rich region which form as a helix-loop-helix conformation (bHLH). The bHLH region is homologous with a domain in the *myc* family of oncogenes which regulate cell proliferation (Murre et al., 1989a,b).

Distinct, but overlapping, and differential patterns of expression (within skeletal muscle) of the various members of the MyoD gene family have been demonstrated. During mouse embryogenesis myf5 is first expressed (e8; Ott et al., 1991), then myogenin (e8.5; Wright et al., 1989) and MyoD (e10.5; Sassoon et al.,

1989). MRF4 is only expressed very briefly following reexpression of myogenin at 14.5 embryonic days (Emerson, 1993; D. Sassoon, personal communication cited from Li and Olson, 1992). In contrast, MyoD is the first gene activated in avian somites, followed over a 12 hour period by the sequential activation of myf5 and myogenin (Pownall and Emerson, 1992). Species differences in myogenic gene expression therefore exist.

The fact that distinct temporal patterns of gene expression during embryogenesis exist suggests that each MyoD family member plays a unique role in the regulation of determination and differentiation of the myogenic lineage. BC3H1 cells (a murine muscle-derived cell line) are unable to fuse and commit to terminal differentiation. It was also found that these cells do not express MyoD, yet do express myogenin (Spizz et al., 1987; Taubman et al., 1989). When transfected with exogenous MyoD, BC3H1 cells regain the ability to fuse, suggesting that MyoD may be important for fusion and that myogenin cannot duplicate this function (Brennan et al., 1990; Miller, 1990). The temporal pattern of MyoD expression in muscle during limb development where it appears coordinately with myotube formation (Sassoon et al., 1989) is consistent with this potential function of MyoD. Myogenin appears to be required for differentiation. BC3H1 cells normally differentiate into mononucleated myocytes, yet when their myogenin expression is blocked by antisense oligonucleotides the cells are unable to express muscle specific genes (Brunetti and Goldfine, 1990). Myf5, also expressed by BC3H1 cells, appears unable to activate the myogenic program alone (Florini and Ewton, 1990).

In vivo studies have also been undertaken to elucidate the specific roles of the various MyoD family members. Inactivation of the MyoD gene through introduction of a null mutation of MyoD into the germline of mice (termed "knockout" mice) produces viable and fertile mice which present normal skeletal muscle morphology and normal levels of skeletal muscle-specific mRNA's (Rudnicki et al., 1992). This study also found that compared to normal control mice the postnatal mutant mice had significantly higher myf5 mRNA levels. These findings

indicate that MyoD is not essential for normal skeletal muscle development in mice which in turn suggests that some degree of functional redundancy exists in the control of the skeletal myogenic developmental program (Rudnicki et al., 1992). In contrast, it has been found that forced overexpression of MyoD1 during mouse embryogenesis is incompatible with normal development and leads to embryonic lethalities (Faerman et al., 1993).

Similar to the MyoD knockout studies, mouse *myf5* knockout reveals that *myf5* is not essential for skeletal muscle formation during embryogenesis. The homozygous mutant mice show no obvious defects in skeletal muscle development, however they suffer from severe rib abnormalities that cause perinatal death, (Braun et al., 1992). The *myf5* mutants express MyoD, myogenin and MRF4 which would suggest that myf5, the protein product of the first *MyoD*-related gene activated in mouse embryos, does not regulate activation of these genes. It appears, however, that *myf5* plays a crucial role in the initiation of myotomal muscle differentiation in somites of early embryos, and also in the formation of specific sclerotomal derivatives (Braun et al., 1992).

Unlike the apparent functional redundancy of MyoD and *myf5* (Rudnicki et al., 1992; Braun et al., 1992), myogenin appears to be essential for the development of functional skeletal muscle and other members of the MyoD gene family cannot compensate for its absence (Hasty et al., 1993; Nabeshima et al., 1993).

Differential expression of the MyoD family occurs between different muscle cell lines, and only subsets of the 4 genes are expressed. Every skeletal muscle line examined thus far expresses myogenin during differentiation, but MyoD, myf5 and MRF4 are expressed only in certain cell lines (Li and Olson, 1992). It has been suggested that the differential expression of these genes could be due to selective repression of certain family members by a specific inhibitor or the lack of a gene-specific activator (Peterson et al., 1990; Thayer and Weintraub, 1990). It is also possible that DNA methylation could cause the lack of MyoD expression in

certain muscle cell types and the lack of MyoD autoregulation in others (Jones et al., 1990b).

The multiplicity of myogenic determiation genes suggests that each might regulate a distinct subset of genes (Smith et al., 1993) and recent findings support the possibility that this differential myogenic determination gene expression might explain myogenic cell diversity (different fibre types, etc.) in developing and adult skeletal muscle tissue (Voytick et al., 1993). There are distinct patterns of expression of MyoD, myogenin, myf5 and MRF4 mRNA's in developing and adult rodent muscles (Smith et al., 1993; Bober et al., 1991; Hinterburger et al., 1991; Hannon et al, 1992). Changing the expression of myogenic determination genes alters myogenic cell line phenotypes (Brennan et al., 1990; Miller, 1990; Block and Miller, 1992). Finally, in transfection assays it has been found that the promotors of several muscle-specific genes are differentially activated by individual myogenic determination genes (Yutzey et al., 1990; Chakraborty et al., 1991b; Fujisawa-Sehara et al., 1992).

The literature reviewed thus far suggests that the myogenic determination genes are just that; they regulate the differentiation of uncommitted cells to cells expressing skeletal muscle-specific proteins. There is also evidence that the myogenic determination genes induce a complete block of muscle cell proliferation (Sorrentino et al, 1990; Crescenzi et al, 1990) prior to the differentiation process. Trouche and colleagues (1993) found that upon cell differentiation, concomitant with increased MyoD expression, there is a large decrease in c-*fos* expression (c-*fos* activates cell proliferation). They subsequently identified a MyoD binding site overlapping with the serum-responsive element in the c-*fos* promotor suggesting that MyoD may exert its negative effect on muscle cell proliferation by transcriptional inactivation of growth-responsive genes (Trouche et al., 1993).

In summary, it appears that myogenic determination genes are more complex than originally postulated. Rather that the existence of a simple single

"master gene" for myogenesis, there appears to be at least four genes, and many other putative factors, which form a complex network of myogenic determination genes.

2.3 The skeletal muscle satellite cell:

In mature adult muscle, damage, due to injury or disease, triggers proliferation of myogenic satellite cells during the process of muscle regeneration. Ever since Mauro (1961) described the satellite cells in frog skeletal muscle the satellite cell has been most frequently implicated in the reserve cell hypothesis of regeneration.

The satellite cell is a mononucleated cell which lies between the basal lamina and the plasma membrane of the skeletal muscle fibre (Muir, 1970; Campion, 1984).

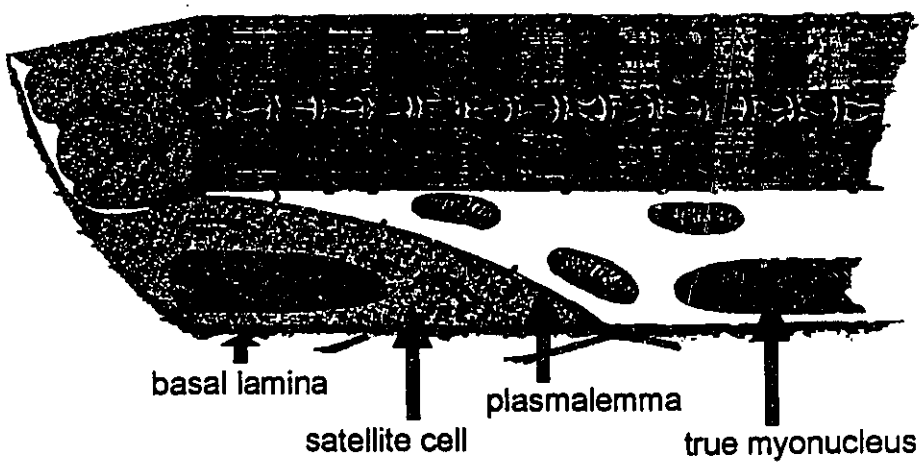


Figure 2.3.1: The skeletal muscle satellite cell (modified from Muir, 1970).

Satellite cells generally have a fusiform shape with their long axes directed along the associated muscle fibres. Small laterally-oriented processes or tails have been observed to emanate from the satellite cells of some species (refer to a review by Mazanet and Franzini-Armstrong, 1986, for more detail). Those, however, observed in the rat (Snow, 1977a; Schmalbruch and Hellhammer, 1977) and the mouse (Schultz, 1974) are generally the typical fusiform shape.

Satellite cells do not contain myofilaments in their cytoplasm, and they contain only a few mitochondria. Those mitochondria that are observed have less internal cristae and are smaller when compared to myofibre mitochondria. The satellite cells do contain some Golgi apparatus, a pair of centrioles and some occasional rough endoplasmic reticulum. The ribosomes, of which there are many, tend to be found free in the cytoplasmic matrix of the satellite cell. Some vesicles are also observed within the cytoplasm. These findings all demonstrate that the satellite cell does not have any specializations which would indicate major phagocytic or secretory activity; neither does it seem to demonstrate any other specialist activity characteristics. The existence of the centrioles, however, does indicate that the satellite cell has the capacity to divide mitotically. This is supported by the observation that the satellite cells on the myofibres are in the null phase of the cell cycle (G_0) when placed in culture (Bischoff 1986a, 1989). (See review on satellite cells by Campion, 1984; Muir, 1970).

Campion (1984) notes that evidence accumulated over the years has demonstrated that in young growing animals there is a high proportion of metabolically active satellite cells which are characterized by a lower nuclear-cytoplasmic volume ratio which is a result of the elaboration of cytoplasmic organelles. In contrast, the inactive satellite cells observed in older animals have a relatively greater nuclear-cytoplasmic volume ratio.

The gross structure of satellite cell nuclei is similar to myonuclei in the associated muscle fibre. However there are some key characteristics which distinguish a satellite cell nucleus from a myonucleus. Satellite cell nuclei are heterochromatic and slightly smaller (10-12 μm in length, 4.5 μm in width, 2.5 μm in depth) than the euchromatic myonuclei of the muscle fibres in which they reside (Muir, 1970; Campion, 1984). Venable (1966) noted the absence of a nucleolus, whereas there is a nucleolus present in myonuclei.

The location and distribution of satellite cells is neither constant between species, nor between skeletal muscle types within a species (Campion, 1984).

There appears to be a random distribution of satellite cells along the length of normal extrafusal fibres (approximately one satellite cell/125-250 μm length of skeletal muscle; Muir, 1970). The average percentage of satellite cell nuclei is less than 5% of the total nuclear population in adult extrafusal fibres (Moss and Leblond, 1971). However, this ratio does vary with muscle fibre type. Kelly (1978, 1979) has found that in adult rat red muscle (type I, slow oxidative and type IIa, fast oxidative; eg. soleus) there are more satellite cells present as a proportion of total nuclei in the muscle fibre (4.5%) than in rat white muscle (type IIb, fast glycolytic; eg. tibialis anterior superficialis, extensor digitorum longus) in which only 1% of the muscle fibre nuclei belong to satellite cells. Since it is known that the percentage of fibre types in certain muscles changes with age from fetal development to the adult state, it follows that the regenerative capacity of a particular muscle will change with age. Also the proliferation potential of satellite cells is inversely proportional to animal age (Schultz and Lipton, 1982).

Schmalbruch and Hellhammer (1977) observed that there was a positive relationship between the concentration of satellite cells per volume of muscle and the concentration of myonuclei per volume of muscle. Therefore, one may conclude from this finding that a muscle (or fibre type) that possesses a high concentration of myonuclei will have a correspondingly high concentration of satellite cells, and presumably a greater potential for regeneration.

Rumpelt and Schmalbruch (1969) and Snow (1977a) both reported that satellite cells are also present on intrafusal muscle fibres in the mouse. This relationship has also been found in numerous other species, such as the frog (Karlsson and Andersson-Cedergren, 1971), rat (Rumpelt and Schmalbruch, 1969), cat (Adal, 1969), dog (Banker and Girvin, 1971) and man (Rumpelt and Schmalbruch, 1969).

Fetal myoblasts (mouse e15-17) and satellite cells appear to be functionally equivalent both in vivo and in vitro, however much evidence exists suggesting that satellite cells and fetal myoblasts represent different subclasses of

myogenic cells (Cossu and Molinaro, 1987). In vitro, cells grown from adult satellite cells demonstrate a cell shape distinct from those originating from fetal myoblasts (Cossu and Molinaro, 1987; Lunt and Parry, personal observations). Fetal myoblasts typically exhibit a spindle-shape, while initially, satellite cells exhibit a round-shaped cell morphology. Most of the round-shaped satellite cells then elongate, contact each other, and begin to fuse into myotubes. Studies based on the ability of mouse satellite cells to incorporate [^{3}H]TdR (indicating proliferation) suggest that the round cell morphology is presented during the undifferentiated, proliferative phase of the adult myogenic precursor cell cycle (Cossu et al., 1980). A more recent study (Colley et al., 1990), however, demonstrates that fetal myoblasts from chick embryos (e12) present similar patterns of morphology to adult mouse satellite cells although it must be noted that the chick embryo fetal myoblasts only maintain the round cell morphology for approximately 24 hours, as compared to 2-3 days in the mouse satellite cells. In contrast to the findings of Cossu and coworkers, Colley et al. (1990) found many round cells, derived from fetal myoblasts, to be post-mitotic (no uptake of BUdR therefore indicating no proliferation), and in addition, some pre-mitotic, BUdR-positive cells. There were many BUdR-negative and BUdR-positive round cells which expressed muscle-specific proteins (titin, myofibrillar myosin heavy chain and zeugmatin) indicating that myogenic precursor cells exhibiting a round-shaped morphology are not always pre-mitotic or undifferentiated. Furthermore, muscle-specific proteins do not appear to be expressed exclusively in post-mitotic cells.

Differences between fetal myoblasts and adult muscle satellite cells are also seen in their biochemical responses in vitro. TPA (a tumour promotor) has no effect on mouse satellite cell differentiation, whereas it reversibly inhibits differentiation in mouse fetal myoblasts (Cossu et al., 1983). Accumulation and expression of muscle specific proteins also appears to differ between fetal myoblasts and satellite cells: rat satellite cells show some expression of desmin (Yablonka-Reuveni and Nameroff, 1985) and functional acetylcholine receptors (Cossu et al., 1987), whereas fetal myoblasts do not. The myotubes derived from fetal myoblasts,

however, demonstrate greater accumulation of α-actin per myonucleus than those derived from satellite cells (Allen et al., 1982).

2.4 Review of evidence for the satellite cell being the reserve myogenic cell:

Many lines of evidence indicate that the regeneration of skeletal muscle fibres following various injuries is directly linked to the ability of satellite cells to undergo mitosis. In other words, the satellite cells that are inactive under normal circumstances in adult muscle are triggered during injury to undergo mitosis and differentiate to form new myotubes, eventually forming new skeletal muscle fibres.

Early studies linking the satellite cells to the regenerative process were largely descriptive and the evidence was circumstantial. For example, regeneration was seen only in muscle that possessed satellite cells (skeletal muscle) and not in cardiac muscle which does not possess satellite cells. However, the conclusion drawn from this observation (that satellite cells are the source for regeneration) does not consider the fact that the trigger for regeneration may be missing in cardiac muscle, rather than the apparatus (presumed to be the satellite cells in this instance) for regeneration.

As introduced previously, there was controversy surrounding the origins of the myogenic cells in regenerating muscle. There were two possible explanations; a population of mononucleated cells with myogenic potential could be present in adult muscle prior to injury, or these cells could be formed as a response to injury.

Evidence mounted demonstrating that during normal post-natal growth a source of myogenic cells was contributing to muscle fibre nuclei. It was known that once myoblasts fuse to form myotubes their nuclei no longer synthesize DNA or divide, they are irreversibly post-mitotic (Stockdale and Holtzer, 1961). However, tritiated thymidine ([H^3]TdR)-labelled cells (indicating ongoing mitosis) were observed within the basal lamina of myofibres (Messier and Leblond, 1960), yet not of myofibre nuclei (Bintliff and Walker, 1960). This was consistent with Enesco and colleagues (1960, 1964) findings that the number of myonuclei increased with age while the number of myofibres remained constant. These conflicting observations could be reconciled by the possibility that the muscle satellite cells, and not the true muscle nuclei, were the nuclei which were undergoing mitosis.

Using [H^3]TdR autoradiography to label newly proliferating myonuclei and subsequent analysis with electron microscopy, Moss and Leblond (1970, 1971) presented compelling evidence that the source of new myonuclei which appear during post-natal muscle growth is invariably from muscle satellite cells, never from true muscle nuclei. The satellite cell nuclei undergo mitosis, following which half of the daughters of the satellite cells are incorporated into the muscle fibres to become myonuclei; the remaining half remain as satellite cell nuclei. Recently it has been shown that in adult rat (Rosenblatt and Parry, 1993; Rosenblatt, Yong and Parry, 1994) and adult mouse (Rosenblatt and Parry, 1992) muscle hypertrophy does not occur when the muscle contains satellite cells which are unable to undergo mitoses (made mitotically non-viable by a high dose of radiation), despite experiencing the same stresses which induce compensatory hypertrophy in control animals which contain healthy satellite cells. These studies indicate that satellite cells in young growing rodents have the capacity to divide repeatedly and, in normal adult muscle undergoing hypertrophy, they function as a source of new true myonuclei.

Subsequently, Bischoff (1974) demonstrated, through enzymatic liberation of myogenic cells from uninjured, non-regenerating mature muscle, that myogenic cells could be obtained only with enzymes that dissolved the basal lamina sheath surrounding the myofibres. This confirmed the earlier studies which suggested that a reserve population of myogenic cells resides beneath the basal lamina of normal, non-regenerating myofibres, the location of satellite cells.

It was suggested that satellite cells could be similarly capable of participating in myogenesis during regeneration following injury as they are during normal postnatal growth. The first true studies investigating the satellite cell - reserve myogenic cell theory during muscle regeneration indicated that following injury there is an increase in the number of satellite cells in the region of trauma (Church, 1970; Teräväinen, 1970) and that these satellite cells are the major source of regenerating myoblasts in rat skeletal muscle (Snow, 1977c).

2.5 Skeletal muscle regeneration:

It is well established that the essential process of skeletal muscle regeneration is similar regardless of the cause of injury or disease (Carlson, 1973; Allbrook, 1981). However, there are some obvious conditions that must be met in order for regeneration to occur successfully. They are:

i) necrosis and subsequent phagocytosis of the myofibre and myofibre debris, since regeneration is inhibited by persisting necrotic tissue (Grounds, 1987),

ii) satisfactory revascularization in order that the regenerating tissue receives the basic nutrients required for growth (Roberts and McGeachie, 1992),

iii) the presence of healthy myogenic cells (presumed to be satellite cells; Rosenblatt and Parry, 1992), and

iv) an intact basal lamina surrounding the myofibre to act as a framework around the regenerating fibres (if the basal lamina is torn, regeneration still occurs, yet at a lower level)

(Carlson and Faulkner, 1983; Marechal, 1986; Grounds, 1991). Furthermore, in order for complete regeneration to occur (ie. viable, functioning myofibres) regeneration of the neuromuscular junction and successful reinnervation must be achieved (Carlson et al., 1983; Cote and Faulkner, 1984; Bader, 1981). The myogenic cell requirement also implies that in order for regeneration to occur the injury inflicted upon the muscle cannot be so extensive as to destroy all of the myogenic cells present. For example, a completely frozen muscle cannot regenerate unless a new source of myogenic cells (namely minced muscle fragments) is introduced in that muscle (Schultz et al., 1986).

Research investigating skeletal muscle regeneration has used both in vivo and in vitro approaches. There are some important implications involved in each of these two methods, each with their advantages and disadvantages.

Many researchers (Carlson, 1970; Snow, 1979; Schultz et al., 1986; Carlson and Faulkner, 1989; Alameddine et al., 1989; Grounds and McGeachie,

1989) have studied regeneration of skeletal muscle in vivo, principally using the technique of minced tissue grafting by autotransplantation (grafting minced muscle back into its original location in the animal) or by cross-transplantation (grafting donor minced muscle into a space vacated in the host). The muscles are later studied through histological and autoradiographic analyses from cryostat sections.

The obvious problem with these methods is that the cells cannot be monitored dynamically as they grow, but can only be seen statically at progressively longer periods of time after injury and after sacrifice of the animal (Konigsberg, 1979), therefore one obtains a composite of results of many animals. Another problem with these in vivo experiments is the complicated and uncontrollable environment within and surrounding the experimental tissue. It is very hard to regulate, or even monitor, the environment and factors affecting the tissue in vivo.

One aspect of the regenerative response in vivo that an in vitro study cannot mimic is the spatial ordering of cell types within the embryo or adult. Bischoff (1974, 1975), Konigsberg (1979) and others have observed that in vivo cells fuse to form myotubes within a single plane and in one direction (that of the future muscle fibre). In vitro, however, the myotubes normally form in random directions over the surface of the petri dish. Evidence suggests that this is probably due to the lack of tension and mechanical forces, normally present in vivo, to act upon the regenerating fibres in vitro (Vandenburgh et al., 1991a,b).

There is also the problem of different selection pressures in the in vitro environment, compared to the in vivo environment. Cells less able to survive and proliferate in their new surroundings gradually disappear from the population. This aspect of cell culture can be minimized if one only studies primary cell cultures (cells recently transferred from a living organism), rather than studying secondary cell cultures. Established cell lines are a convenient and useful tool for exploring cell behaviour, however one must remember that strong selection pressures may have been exerted on the cell line. Those selection pressures may have caused deficiencies or alterations in the growth control pathways compared to those found in vivo. For

example, as mentioned earlier, some immortalized myogenic cell lines do not express MRF4 (Rhodes and Konieczny, 1989) while others do not express MyoD1 (Braun et al., 1989b). This therefore limits the knowledge transferrable from studies on these cell lines to the situation in vivo. Furthermore, cell lines are immortalized cells (in itself different from the true physiological condition) cloned from tumours, which are obviously not a normal or healthy physiological condition.

Primary cell cultures are more difficult to work with than cell lines. However, the cells are similar to those in vivo; they are not in culture long enough to be affected by selection pressures, and they can be derived from healthy tissue. Primary cell cultures still differ from the true situation in vivo since the surrounding environment is quite different. For initial studies on cell behaviour this fact offers an advantage; all of the extrinsic factors are controllable, unlike the situation in vivo (such as, nutrient delivery, oxygenation, presence of growth factors, etc.).

An ideal culture system should maintain a microenvironment similar to that seen in vivo in order that the cells might be subject to a similar physiological condition.

Studies of satellite cells in vitro generally utilize large populations of satellite cells released enzymatically from minced muscle tissue (Dusterhoft et al., 1990; Allen et al., 1984). For all future reference these will be referred to as mass satellite cell cultures. Mass satellite cell culture studies have resulted in a wealth of information regarding satellite cells and their behaviour in vitro. However, despite the use of various (time-consuming and complicating) techniques to purify the myogenic cultures (panning, pre-plating, Percoll density-gradient) there are always contaminating non-myogenic cells (generally fibroblasts) present in the final culture (Allen et al., 1984; Yablonka-Reuveni and Nameroff, 1987). These purifying techniques also increase the risk of selecting only a part of the population of myogenic cells.

It is generally accepted that fibroblasts and myogenic precursor cells cannot be distinguished morphologically in live cultures at the light microscopic level.

Distinction between the two is only possible after differentiation of the myogenic cells to multinucleated myotubes (can be observed in live cultures) or after staining fixed cultures for a muscle- or fibroblast-specific marker. There are conflicting reports as to the efficacy of the various markers; after extensive study most appear to be specific for only a portion of the total population of a given cell type. For example, desmin was reported to be present in proliferating rat satellite cells (Allen et al., 1991) as was neural cell adhesion molecule (NCAM, Covault and Sanes, 1986), therefore antibodies against desmin or NCAM could be used to immunolabel satellite cells. However, bovine satellite cells only express desmin post-mitotically, not when they are still proliferating. Therefore species differences in desmin expression exist (Allen et al.,1991). Further study of NCAM expression revealed that a subpopulation of fibroblasts also express NCAM (Gatchalian et al., 1989; Covault and Sanes, 1986; Grumet et al, 1982; Moore and Walsh, 1985).

Conversly, one may attempt to label any non-myogenic cells. This potentially presents a problem in that muscle fibroblasts appear to be a very heterogeneous population of cells (Yablonka-Reuveni et al., 1988). An antigen, Thy-1, is described to be present on mouse and rat fibroblasts (Stern, 1973) but absent from human skeletal muscle and human basement membranes (Reif, 1989). However, it has been shown that Thy-1 antigen is present on myoblasts but not myotubes of rat L6 muscle cells and rat muscle primary culture cells (Lesley and Lennon, 1977; Walsh and Ritter, 1981).

Cloning single cells from mass satellite cell cultures would result in a pure myogenic cell population. This technique would not employ primary cultures since at least one passaging of the original primary myogenic cell culture would have to be performed. Therefore, similar to the method of using cell lines, there would be an increased chance that selection pressures would be exerted on the cells in culture, as previously discussed. When picking out the individual cells to be cloned there is a chance that the cell may be a non-myogenic cell (wasted time for each non-myogenic cell plated) and, furthermore, the microenvironment surrounding the

cell would be very different from that normally experienced in vivo; ie. no adult myofibre, intact or degenerating would be present.

In summary, studies of the proliferative behaviour of satellite cells (for example, monitoring [H]^{3}TdR uptake by proliferating cells) are very difficult to do without including proliferating non-myogenic cells, as well as being inefficient and time-consuming.

In order to investigate the proliferative behaviour of satellite cells which results in skeletal muscle regeneration, a primary tissue culture method to isolate a pure population of satellite cells and their myogenic progeny, without the interference of contaminating non-myogenic cells, must be developed.

In 1974, Bischoff demonstrated that proteolytic enzymes, such as trypsin and pronase, which dissolve the myofibre basal lamina, are effective in releasing myogenic cells from adult rat muscle which subsequently proliferate and differentiate in culture. Other enzymes, such as collagenase, which leave the myofibre basal lamina intact, do not release myogenic cells and merely result in the release and growth of fibroblasts and macrophages. Further evidence demonstrating that satellite cells were the reserve myogenic cells was given by teasing single muscle fibres, with their basal lamina and satellite cells intact, away from a muscle fibre bundle and explanting them in culture (Bischoff, 1975; Konigsberg, 1979). The satellite cells could be induced to proliferate beneath the basal lamina sheath and form myotubes within it. This technique, however, was very labourious and did not lend itself easily to extensive testing protocols. Single muscle fibres (Bekoff and Betz, 1977a,b) were being used to examine single myofibre physiological properties, therefore Bischoff (1986a) modified their relatively simple technique by digesting muscle tissue in a collagenase solution. This resulted in the release of single muscle fibres which retained their satellite cells beneath the intact basal lamina sheath. These myofibre-satellite cell complexes remained viable for at least two weeks if the complex was not damaged during the dissociation process and if the fibre attached properly to the appropriate substratum. Furthermore, given the appropriate media

the satellite cells proliferated, forming myotubes within the myofibre basal lamina sheath (Bischoff, 1986a; 1989).

The evidence presented by previous research is a compelling statement as to the role of satellite cells in skeletal muscle regeneration: satellite cells appear to be the primary, if not the sole, source of myogenic precursor cells for vertebrate skeletal muscle regeneration. This leads one to ask another question: assuming satellite cells are the source of myogenic precursor cells, then what is the signal, or trigger, that normally causes the regenerative process in skeletal muscle? This has been the focus of more recent research into regeneration.

2.6 Satellite cell regulation:

Two processes are involved in satellite cell growth and development - cell proliferation and cell differentiation. With the onset of growth and development satellite cells, normally in the quiescent phase of the cell cycle (G_0), are triggered to enter the presynthetic period (G_1) of the cell cycle (first described by Howard and Pelc, 1951; see Fig. 2.6.1). A period of DNA synthesis (S) then occurs, followed by a postsynthetic phase (G_2) which leads up to cell mitosis (M). If conditions dictate cell proliferation then the daughter cells continue around this cell cycle.

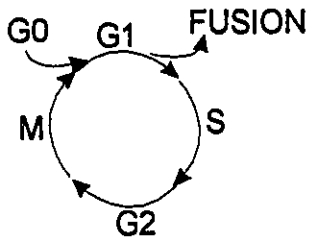


Figure 2.6.1: The cell cycle.

Studies on the MM14 cell line (derived from mouse satellite cells) suggest that once cells are in the S phase they are committed to complete the cell cycle through to the end of mitosis despite removal of the original proliferation stimulus (Clegg et al., 1987). Some daughter cells will withdraw from the cell cycle into G_0; however they may then be triggered to reenter the cell cycle given appropriate conditions for proliferation. At some point the cells become committed to differentiation, exiting from the G_1 phase of the cell cycle to eventually become post-mitotic myotubes with muscle gene expression.

Satellite cell growth control may be exerted at a variety of points through the cell cycle and effectors for this control may come from a variety of possible sources. Mounting evidence suggests that proliferation and differentiation are distinct processes. For example, fibroblast growth factor (FGF) is known to stimulate proliferation (Gospodarowicz et al., 1976; Linkhart et al., 1980) and inhibit

differentiation (Linkhart et al., 1980; Bischoff, 1986a; Allen et al., 1985) of muscle cells (discussed further in Section 2.7 - Fibroblast growth factor). However, using non-mitogenic conditions (no stimulation of cell proliferation; eg. low serum concentration) basic fibroblast growth factor still exerts its inhibitory effect on differentiation (Linkhart et al., 1982; Lathrop et al., 1985a,b; Spizz et al.,1986; Clegg et al., 1987) suggesting that differentiation is not simply a default condition when proliferation is not occurring. The possibility therefore exists that there are multiple effectors of satellite cell growth and development (see Figure 2.6.2). Bischoff (1990a,b,c) suggests that perhaps not only mitogens which act positively on growth and development exist, but also inhibitors of growth and development.

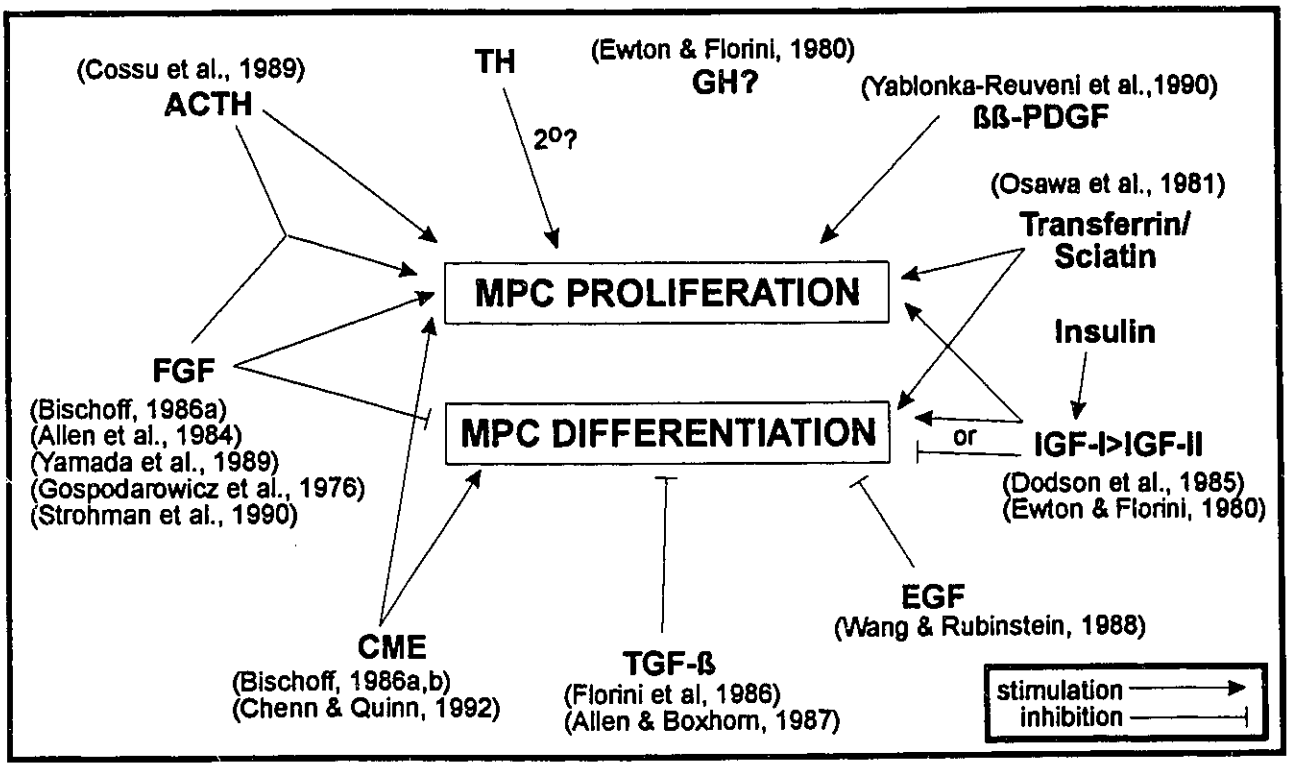


Figure 2.6.2: Putative effectors for the control of myogenic precursor cell (MPC) growth and development.

Recent evidence suggests that myogenic regulatory factors are expressed by activated satellite cells and they play a role in satellite cell growth and development during muscle regeneration. Fuchtbauer and Westphal (1992) found that during the regeneration process in vivo, mouse skeletal muscle expressed MyoD1 and myogenin. These proteins were also expressed in the nuclei of the newly regenerated myofibres for up to two weeks following the formation of myotubes. In contrast, neither regulatory factor protein was expressed in normal uninjured adult muscle. Yablonka-Reuveni and Rivera (1994) observed that satellite cells which were proliferating, but not those which were quiscent, coexpressed PCNA (Proliferating Cell Nuclear Antigen which labels proliferating cells; Bravo et al., 1987) and MyoD. There were also similar numbers of cells expressing MyoD and PCNA. Following a decline in PCNA and MyoD expression there was an increase in the number of cells expressing myogenin. The similarities in temporal appearance and disappearance of PCNA and MyoD would suggest that, during cell proliferation, some, if not all, satellite cells express MyoD only; following a decline in their proliferative activity the satellite cells appear to express myogenin, as well as the differentiation-specific, cytoskeletal proteins, developmental sarcomeric myosin and α-smooth muscle actin. Yablonka-Reuveni and Rivera (1994) also reported that inhibition of either satellite cell proliferation or differentiation resulted in a drastic decrease in the number of satellite cell nuclei expressing MyoD, and the complete elimination of myogenin expression. This offers further support that during skeletal muscle regeneration the two myogenic regulatory factor proteins, MyoD and myogenin, are expressed in satellite cells during, or subsequent to, their proliferation.

As mentioned above, FGF stimulates proliferation and inhibits differentiation of satellite cells and their progeny (Clegg et al., 1987; Allen and Boxhorn, 1989; reviewed by Grounds and Yablonka-Reuveni, 1993). bFGF, however, was not found to affect the time course of progression of activated satellite cells through the myogenic program; simply put, myogenic satellite cells appear to follow

a highly coordinated, multistep program of regulatory and structural protein expression (Yablonka-Reuveni and Rivera, 1994).

Evidence is accumulating which indicates antagonism between growth factor signalling pathways and pathways controlled by members of the MyoD family. This would suggest that the decision of a myoblast to proliferate or differentiate is determined by a balance between these antagonistic programs (Li and Olson, 1992). Growth factor signals (eg, FGF and TGF-ß) appear to inhibit myogenesis at multiple levels; at the level of MyoD and myogenin gene transcription (Davis et al., 1987; Vaidya et al, 1989), and, farther "downstream", by suppressing activity of the myogenic regulatory factors (Edmondson and Olson, 1989). It has been found that high concentrations of mitogenic growth factors or high levels of expression of activated oncogenes (eg. *ras, jun* or *fos*, which initiate a proliferation response) can overcome the actions of myogenic determination gene proteins, thereby preventing differentiation (Bengal et al., 1992; Li et al., 1992).

Despite much research, the exact mechanism(s) through which growth factor signals may inhibit myogenic gene activity remains unclear, although several possibilities are suggested (see Figure 2.6.3).

Growth factors may mediate the induction of inhibitory helix-loop-helix (HLH) proteins. One such inhibitory HLH protein is *Id* which lacks a basic domain and is able to form heterodimers with other non-DNA binding HLH proteins, such as, E12, E47 and weakly with MyoD (Jen et al., 1992). An E-box is a DNA consensus sequence motif, CANNTG, where N represents any nucleotide, present in the enhancer element of contractile protein genes (Olson, 1990; Wright, 1992; Brennan et al., 1991a; Davis and Weintraub, 1992). It is postulated that in order for MyoD protein to be able to activate muscle specific genes MyoD must form heterooligomeric complexes with another gene product, such as E12 or E47 (Murre et al., 1989b; Davis et al, 1990; Brennan and Olson, 1990; Chakraborty et al., 1991a; Murre et al., 1991). Proliferating myoblasts express relatively high levels of *Id*, but it is down-regulated with cell differentiation (Benezra et al., 1990). These three

findings suggest that *Id* expression, perhaps up-regulated by the presence of mitogenic growth factors, results in sequestration of E47 or E12 by its gene product, Id; thereby preventing MyoD from being able to exert its action on muscle-specific genes (Li and Olson, 1992; Emerson, 1993) (see Fig. 2.6.3, pathway A). This is further supported by the observation that in proliferating myoblasts that express high levels of *Id,* MyoD and myogenin are unable to bind the MCK (a muscle specific gene) enhancer (Buskin and Hauschka, 1989; Mueller and Wold, 1989; Brennan et al., 1991b). This however does not appear to be the case with differentiation

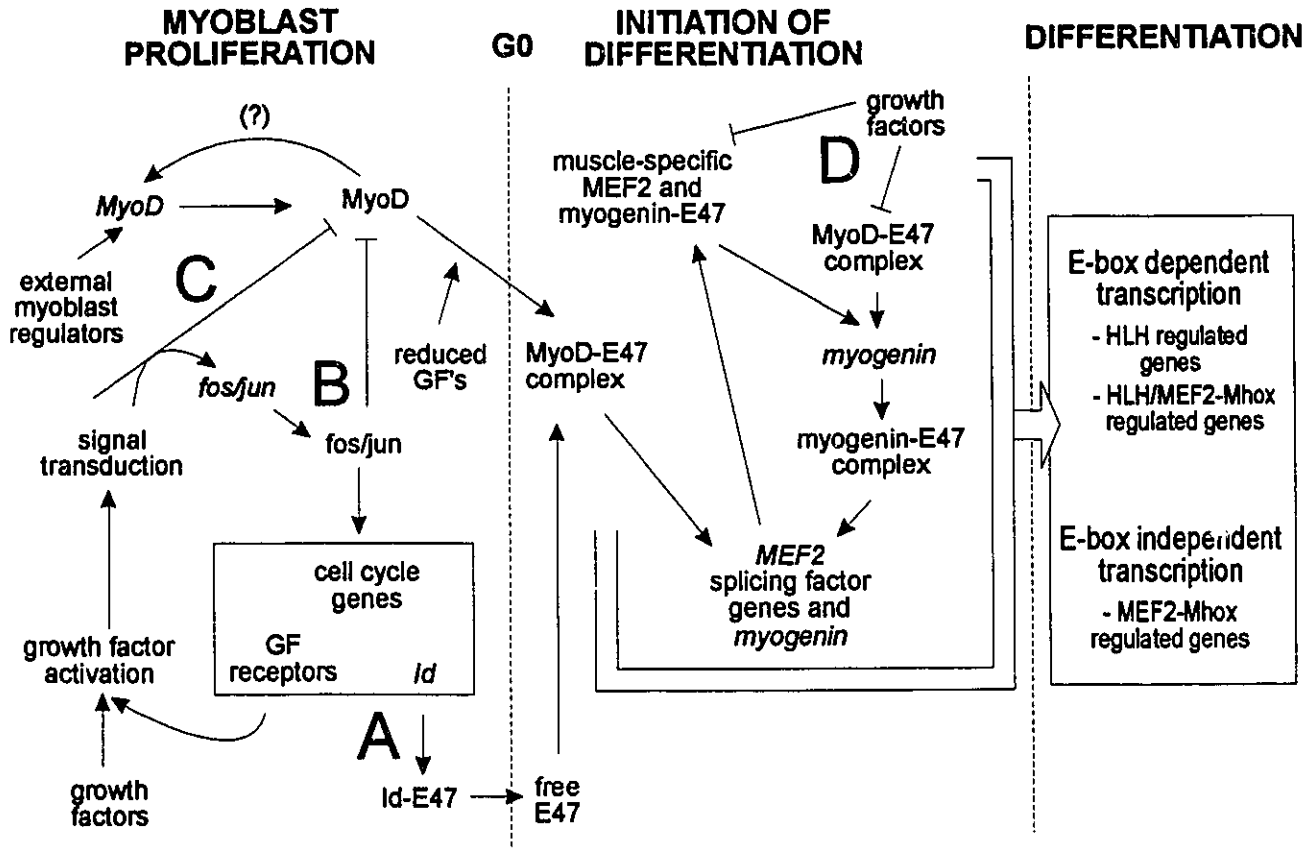


Figure 2.6.3: Possible genetic pathways for growth factor regulation of myoblast proliferation and differentiation (modified from Emerson, 1993).

suppression exerted by nonmitogenic peptide growth factors, such as TGF-ß. Myoblasts which have their differentiation capabilities blocked by TGF-ß express Id at low levels, similar to those seen in differentiated myotubes; however, myogenin and MyoD are still able to bind DNA (Brennan et al., 1991b). This suggests that nonmitogenic peptide growth factors inhibit the transcription-activating properties of myogenic HLH proteins through a mechanism independent of DNA binding.

Another posible pathway through which growth factors may exert negative control of myoblast differentiation is through inducing immediate early gene (eg. *fos, jun, myc*) products which activate cell cycling (see Fig. 2.6.3, pathway B) (Spizz et al., 1987; Li and Olson, 1992; Emerson, 1993). This hypothesis is supported by the fact that a variety of growth-related signaling pathways, which lead to the induction of *c-fos, junB, c-jun* and *c-myc*, are initiated at the cell membrane, the binding site of most growth factors in muscle (Lewin, 1991).

A third mechanism through which growth factors may suppress myogenic gene expression is through changes in myogenic HLH protein phosphorylation (see Fig. 2.6.3, pathway C). It is known that MyoD and myogenin are phosphorylated, and there are many examples in which transcription factors are regulated by phosphorylation; however, the sites in the genes of the MyoD family that are phosphorylated and the kinases for which they are substrates are presently unknown (Olson et al., 1991; Emerson, 1993).

The final hypothesis is that various proteins which collaborate with myogenic HLH, including coregulators (such as the previously discussed E12 and E47), may be targets for negative regulation of differentiation by growth factor signals (see Fig. 2.6.3, pathway D) (Li and Olson, 1992). Little is known of these putative myogenic HLH collaborators, however it has been shown that myogenin and MyoD activation of muscle-specific enhancers requires cooperative interactions with other enhancer-binding proteins that bind sites adjacent to the E-boxes (Sartorelli et al., 1990; Gossett et al., 1989; Lin et al., 1991; Weintraub et al., 1989).

Slater (1976) determined that the proliferation of myogenic cells in vitro is maximal with the addition of 5% chick embryo extract (CEE) to the culture medium. This suggests that chick embryo extract might contain a mitogenic factor(s) that directly trigger(s) muscle regeneration, or it presents an environment needed for an intrinsic signal in the satellite cells to act and trigger regeneration. Similarly, CEE may supress an inhibitory factor which normally maintains satellite cells in G_0 (inhibiting their growth and development) thereby allowing the satellite cells to become mitotically active.

Cossu et al. (1989) investigated the mitogenic effects of peptides derived from proopiomelanocortin (POMC) in murine satellite cell and fetal myoblast cultures. They found that adrenocorticotropin (ACTH) and α-, ß- and γ-melanocyte-stimulating hormones (MSH) caused a three- to fourfold increase in the proliferation of myogenic cells, reflected by an increased accumulation of skeletal myosin. No increase in proliferation of mouse embryo skin fibroblasts, muscle fibroblasts, or vertebral chondroblasts was seen. This mitogenic effect was greatly enhanced by the addition of basic fibroblast growth factor (bFGF). Therefore, it would appear that ACTH may be specific for myogenic cells. This would agree with the fact that any event which leads to muscle degeneration causes a stress response in the animal, resulting in a rise in the serum ACTH level; this may selectively stimulate the proliferation of satellite cells which would result in the observed regeneration rather than fibrosis. Cossu et al. (1989) also suggested that since bFGF potentiates the mitogenic effect of ACTH it might indicate that at any developmental stage, or in a given pathophysiological condition, the proliferation of a specific cell type may be regulated by the combined action of different growth factors.

These findings support those of Bischoff (1986a,b), who tested the mitogenicity of bFGF, epidermal growth factor, multiplication stimulating activity and platelet-derived growth factor (PDGF). It was found that only bFGF induced the proliferation of satellite cells to form myotubes, although it also stimulated the growth of fibroblasts (Bischoff, 1986a). Another study using crushed adult muscle

extract (CME) as the mitogen showed that this crushed muscle extract is specific to the myogenic satellite cells only, and not to the fibroblasts (Bischoff, 1986b).

Contrary to Bischoff's (1986a) findings on PDGF, Yablonka-Reuveni et al. (1990) have found that PDGF does exert a mitogenic effect on the myogenic cells, and, furthermore, that satellite cells from adult mouse skeletal muscle express receptors to PDGF. Moreover, they determined that, for the most part, only the ßß-PDGF isoform (PDGF with two ß chains) will bind to the myogenic cells, therefore indicating the presence of selective receptors with ßß subunits, rather than $\alpha\alpha$ or αß subunits. Bischoff (1986a) did not indicate the isoform of PDGF used in his experiments, therefore the discrepancy of results could be due to his use of $\alpha\alpha$-PDGF or αß-PDGF rather than ßß-PDGF.

Transforming growth factor-beta (TGF-ß), a widely and variably active protein, is present in relatively large quantities in the α granules of platelets, activated lymphocytes and macrophages, and it has been found (at least in small quantities) in every cell line examined (Roberts and Sporn, 1990). TGF-ß is a very potent reversible inhibitor of differentiation in all measured aspects (fusion, CK elevation, acetylcholine receptor appearance, transition from ß- and τ- to α-actin, the formation and expression of MHC and other muscle specific proteins and their mRNA's) in rat L6 and L8 myoblasts (embryonic myoblast cell lines; Massagué et al, 1986; Florini et al., 1986), mouse C2 (embryonic myoblast cell line) and BC_3H1 (non-fusing myogenic cell line) myoblasts (Olson et al., 1986) and rat satellite cells (Allen and Boxhorn, 1987). All of these studies, except for that on BC_3H1 cells, found that this inhibitory effect is seen only if TGF-ß is added early in the process, before the cells become irreversibly committed to myogenic differentiation. BC_3H1 cells only exhibit a partially irreversible commitment to differentiation; once BC_3H1 cells are in a differentiated state, TGF-ß exposure results in the myocytes losing their differentiated functions, however they still remain in the post-mitotic state (Olson et al., 1986).

TGF-ß involvement in regeneration appears feasible since during the degeneration process platelets, lymphocytes and macrophages invade the trauma site to clear debris during necrosis, therefore presenting a viable source for TGF-ß release. It could be argued that TGF-ß acts to inhibit differentiation of the satellite cells, thereby freeing them to continue cycling through mitosis fulfilling the proliferation phase of regeneration. Once necrosis ends and the source of TGF-ß withdraws, or deactivates, the newly generated population of myogenic cells could continue on to differentiate, forming new myotubes and eventually new muscle fibres. As previously discussed, TGF-ß does not appear to inhibit differentiation through an action on myogenic regulatory factors by affecting their DNA-binding abilities (Brennan et al., 1991b). Perhaps it inhibits the transcription-activating properties of myogenic HLH proteins through a mechanism independent of DNA binding (Li and Olson, 1992).

Although the normal role of fibroblast growth factor (FGF) has yet to be established, it is believed that bFGF (basic FGF) plays an important role in various regeneration processes; such as cartilage repair (Cuevas et al., 1988), brain wound repair (Finkelstein et al., 1988), mouse muscular dystrophy (DiMario et al., 1989) and normal wound repair (Broadley et al., 1989). Much evidence also exists showing FGF to be widely implicated in embryonic and adult myogenic cell growth and development. Strohman and coworkers (1990) found elevated levels of bFGF in hypertrophied rat muscle. Furthermore, bFGF is the only purified mitogen that has been shown to independently inhibit myogenic differentiation and stimulate myogenic proliferation, rather than simply stimulating one or both processes (reviewed by Florini et al., 1991). Also, as discussed in greater detail in the following section, bFGF is a heparin-bound growth factor stored in the extracellular matrix of the muscle fibre (Yamada et al.,1989). This is an ideal location for its release into action during injury to trigger the regenerative process in muscle.

The above evidence for bFGF implication in muscle growth presents a strong case for its involvement in the mitogenic trigger. This study, therefore, will

concentrate on the further investigation of bFGF as a component of the mitogenic trigger for muscle regeneration through the use of purified recombinant bFGF and its neutralizing antibody, anti-bFGF (αbFGF).

2.7 Fibroblast growth factor:

To date, six members of the FGF family have been discovered; basic FGF (bFGF; Gospodarowicz, 1974; Gospodarowicz and Moran, 1974), acidic FGF (aFGF; Gospodarowicz et al., 1975), *int-2* (Moore et al., 1986), *hst/K-fgf* (Sakamoto et al., 1986), FGF-5 (Zhan et al., 1987) and keratinocyte growth factor (KGF or FGF-6; Rubin et al., 1989). All are structurally related proteins (homologous within a "core" of 120 residues), with large divergence among family members' sequences (33-69% core homology). In contrast, each FGF type is highly conserved through mammalian evolution, with greater than 92% sequence identity within the core region and substantial homology in flanking regions between human and murine clones. This suggests that FGF's are not interchangeable in vivo and have evolved to possess distinct biological functions (Goldfarb, 1990). All FGF's are mitogenic for a broad range of mesoderm- and ectoderm-derived cells (reviewed by Burgess and Maciag, 1989), and are expressed in a broad range of developing and adult tissues. *Int-2* and *hst/K-fgf* are found in embryonic tissues only; FGF-5 and FGF-6 are found in restricted sets of non-muscle adult tissues, as well as in embryonic tissues (reviewed by Goldfarb, 1990). bFGF has been localized in embryonic and adult muscle (Anderson et al., 1991; Joseph-Silverstein et al., 1989; DiMario and Strohman, 1988; Yamada et al., 1989; DiMario et al., 1989; Vaca et al., 1989; Morrow et al., 1990), extracted from adult muscle (Kardami et al., 1985a) and its mRNA is expressed in primary rat satellite cell cultures (Alterio et al., 1990). Similarly, aFGF has been localized to, and extracted from rat satellite cells (Groux-Muscatelli et al., 1990), and its mRNA is expressed in primary rat satellite cell cultures (Alterio et al, 1990).

The export mechanisms of aFGF and bFGF are unclear since thay are not synthesized with signal sequences which direct the proteins into the secretory pathway. bFGF has been detected in the cytoplasm of producer cells growing in vitro (Schweigerer et al., 1987; Becker et al., 1989) and in vivo (Joseph-Silverstein et al., 1989). Many studies have verified that bFGF is deposited into the extracellular

matrix (ECM, also called the basal lamina) in both tissues and cultured cells (Baird and Ling, 1987; Vlodavsky et al., 1987; Folkman et al., 1988; Yamada et al., 1989; DiMario et al., 1989; Kardami et al., 1985a,b). The storage of aFGF is variable among producer cell types; newly synthesized aFGF is efficiently deposited into the ECM of cultured cardiac myocytes (Weiner and Swain, 1989), however a study by Groux-Muscatelli et al. (1990) suggests that aFGF is deposited in the cytoplasm, not the ECM, of rat satellite cells in vitro.

Not only are bFGF (Bischoff, 1986a; Allen et al., 1985) and aFGF (Clegg et al., 1987) mitogenic for satellite cells (ie. increasing cell proliferation) but they also delay myogenic differention of satellite cells (Allen et al., 1985; Bischoff, 1986a). As already discussed these two actions are independent of each other, most likely occurring through different mechanisms. The question therefore remains as to what stage of differentiation FGF acts upon to exert its inhibitory effect. Satellite cells replicate through the cell cycle. Each time they pass through G_1 phase they may either continue through the cell cycle (see Fig. 2.6.1) and continue to replicate, or they may exit the cell cycle and commit to differentiation. FGF must therefore act upon some point within the G_1 phase of the cell cycle. Much evidence has accumulated suggesting that FGF blocks an early event (Kelvin et al., 1989; Clegg et al., 1987). Lathrop and colleagues (1985b) suggest that FGF causes myoblasts to exit the G_0 phase to a point in G_1 from which commitment to differentiation is not possible. More recently, Glaser and Wice (1989) defined two restriction points in G_1 which determine the the cell cycle fate of the myoblasts. G_{1d} occurs in early G_1 and permits synthesis of muscle-specific proteins, while G_{1q} occurs approximately 4 hours later and does not permit synthesis of muscle-specific proteins. Addition of FGF causes movement of the cells from G_{1d} to G_{1q} and reversal of this pattern occurs with removal of the mitogen (Glaser and Wice, 1989).

Much circumstantial evidence exists to suggest that acidic and/or basic FGF is bound in the ECM ready for release during trauma to trigger skeletal muscle regeneration through activating the ECM apposed satellite cells. aFGF and bFGF

have very high affinity for heparin and heparan sulfate proteoglycans (Gospodarowicz et al., 1984; Burgess and Maciag, 1989) which are present in abundance in muscle fibre endomysium ECM (Fernandez et al., 1991; Bayne et al., 1984; Mayne and Sanderson, 1985). aFGF and bFGF have been localized to the ECM and shown to be mitogenic for satellite cells. bFGF was detected in the basal lamina of muscle fibres at a much higher concentration in muscle from mdx mouse mutants (muscles are continuously regenerating) than in normal non-dystrophic muscle (DiMario et al., 1989). Furthermore, Strohman and coworkers have shown that heparin inhibits the myogenic activity of bFGF in chick myoblasts (Kardami et al., 1988) and of adult mouse skeletal muscle derived satellite cells (DiMario and Strohman, 1988). Therefore, it could be postulated that modulation of FGF binding to heparin components of the ECM may determine availability of bound FGF to satellite cells. Disruption of the normal FGF-heparin interaction may range from mechanical distortion of the muscle fibres (eg. physical activity; Irintchev and Wernig, 1987), to an inflammatory response which accompanies focal or more extensive damage to muscle fibres (Naparstek et al., 1984).

Despite much research into FGF effects on growth and differentiation little is known about cell receptors for FGF (Olwin and Hauschka, 1989). Both aFGF and bFGF bind to the same receptor (Olwin and Hauschka, 1986; 1989) which could be expected due to the degree of structural homology between them. The FGF receptor is highly specific for FGF only, and neither bFGF or aFGF bind to other growth factor receptors (Gospodarowicz et al., 1986a,b).

During myogenic differentiation of rat L6 and mouse Sol 8 myoblasts the mRNA encoding aFGF, bFGF and FGF receptor are coordinately downregulated while the expression of myogenin, a myogenic determination gene, increases (Moore et al., 1991). It is suggested that a coordinate decrease in endogenously produced aFGF, bFGF and their receptor may participate in the regulation of myogenic differentiation through the regulation of expression of myogenic determination genes (Moore et al, 1991; Vaidya et al, 1989; Brunetti and Goldfine, 1990).

Similarly, proliferating skeletal muscle-derived MM14 and C2C12 cells express FGF receptor; following terminal differentiation FGF receptor expression is lost (Olwin and Hauschka, 1988). In contrast to the findings of Moore et al. (1991), Olwin and Hauschka (1988; 1989) reported that rat L6 cells did not express FGF receptor. It must be noted, however, that Olwin and Hauschka's studies identified receptors through a ^{125}I-aFGF binding assay, working on the assumption that aFGF and bFGF mediate their actions through a common receptor. Moore's group used the more sensitive technique of a FGF receptor mRNA assay.

There appear to be similar patterns of aFGF mRNA expression in both developing muscles and cultured satellite cells. Alterio and colleagues (1990) found that during myogenic growth (bovine fetal muscle and proliferating bovine adult satellite cells) aFGF mRNA was expressed at high levels, while there was no, or very low levels of expression in the differentiated state (neonatal bovine muscle and myotubes derived from bovine adult satellite cells). This, along with the finding that FGF receptor expression is lost following terminal differentiation (Olwin and Hauschka, 1988), and the, previously discussed, fact that exogenous FGF keeps cells in a proliferative state, suggests a causal relationship between proliferation ability and the presence of FGF mRNA. Considering the previously discussed findings that bFGF is more ubiquitous and usually detected in higher concentrations in muscle ECM than aFGF, it is surprising that Alterio and colleagues (1990) found that bFGF mRNA was only weakly expressed, as compared to aFGF mRNA, in bovine proliferating satellite cell cultures. All of these findings together, however, suggest that aFGF, as well as bFGF, may play a role in myogenesis, possibly through autocrine action.

2.8 Crushed muscle extract studies:

The preceding review of various mitogenic substances for myogenic precursor cells demonstrates the vast spectrum of contenders for the trigger of skeletal muscle growth, development and regeneration. All of these factors fulfill at least one of the criteria for a role as the trigger for regeneration (ie. stimulates proliferation of myogenic precursor cells, stored in a structure within the muscle environment, etc.). For all of these substances, however, there is at least one contradictory aspect of its involvement in muscle. For example, TGF-B does not promote satellite cell proliferation (Allen and Boxhorn, 1987), therefore it could not be the sole factor involved in the regeneration triggering process. Another example is bFGF which not only triggers satellite cell proliferation, but also fibroblast proliferation (Gospodarowicz, 1974; Gospodarowicz and Moran, 1974). During normal skeletal muscle regeneration only satellite cells appear to be affected.

Numerous studies have demonstrated that extracts from damaged muscle {for example, muscle undergoing stretch-induced (chicken, Summers et al., 1985) or exercise-induced hypertrophy (rat, Chromiak et al., 1991), minced muscle (mouse, Vandenburgh et al., 1984; rat, Chromiak et al., 1991) or crushed muscle (rat, Bischoff, 1986b; mouse, Chen and Quinn, 1992; rat, Johnson and Allen, 1993)} induce proliferation of myogenic but not non-myogenic cells (including fibroblasts) in culture. Cultures grown in control conditions (ie. extracts from undamaged muscle) did not induce myogenic cell proliferation beyond basal levels.

Some intriguing work by Bischoff (1986a,b; 1990a) investigated the properties of a crude saline extract of crushed rat muscle. The crushed muscle extract was able to induce proliferation of all the satellite cells on a single intact myofibre, yet muscle extract from intact muscle (not crushed) only yielded basal levels of proliferation. This response to crushed muscle extract was greatly enhanced if the myofibres were killed by brief exposure to the myotoxic local anaesthetic Marcaine (which kills the myofibres, but does not affect the apposing satellite cells (Hall-Craggs, 1974b), therefore indicating that some aspect of the undamaged

myofibres suppressed satellite cell proliferation. In fact, Bischoff's experiments (1990a) demonstrated that contact of the satellite cell with the differentiated end cell (viable adult myofibre) was able to reverse or even suspend the cell cycle commitment of its precursor stem cell (satellite cell) produced by exposure to muscle extract. Bischoff (1989) demonstrated that in both live and dead myofibres the satellite cells are in contact with the basal lamina and they appear to be the same shape. The basal lamina, therefore, does not appear to be involved in this effect seen with live fibre contact. Instead, Bischoff (1990b) concluded that the growth suppression seen during contact with live fibres appears to be mediated by contact with the plasma membrane of the myofibre, since following myofibre death the plasmalemma disappeared and satellite cell proliferation subsequently occured.

The satellite cells remained in the cell cycle for a period of two to three days and then cell fusion occured at 84 hours in vitro (Bischoff, 1990a). The crushed muscle extract appears to be specific for satellite cell proliferation, since very little fibroblast growth was seen (Bischoff, 1986b). Also the crushed muscle extract appeared to act simply as the *trigger* for satellite cell proliferation, and was not required to remain present for the proliferation cycle to continue. The crushed muscle extract alone, however, could not support proliferation. The serum factors present in the growth medium were needed for satellite cell proliferation and for subsequent fusion of satellite cells to continue once they were triggered by the crushed muscle extract. Consequently, Bischoff (1990a) proposed that the mitogens present in the crushed muscle extract act transiently as a "competence factor," while serum factors appear to be a "maintenance factor."

Attempts to characterize the mitogen responsible for these specific myogenic effects have been largely unsuccessful. This may very well be because the mitogenic effects are exerted by more than one factor which, acting together, constitute the mitogenic trigger. It has been determined that the activity in the extract has a molecular weight greater than 30kD (Bischoff, 1986b; 1990c) and is heat and trypsin senstive (Bischoff, 1986b). Chen and Quin (1992) tested the additive

activity of crushed muscle extract from adult mouse with saturating doses of other growth factors. When the optimal concentration of crushed muscle extract was added with optimal concentrations of bFGF alone or bFGF and IGF-I there was an increase in myogenic cell proliferation over that produced by these factors without crushed muscle extract. Similar results were found with the addition of crushed muscle extract to saturating doses of transferrin, IGF-I, bFGF, EGF, PDGF, macrophage colony-stimulating factor (M-CSF) and ACTH alone or combined together. Western blot analysis found transferrin present in the crushed muscle extract, estimated at a concentration of 30 μg/mg crushed muscle extract protein. All these results (Chen and Quinn, 1992) indicate that crushed muscle extract contains transferrin as well as one or more uncharacterized mitogens for myoblasts which are distinct from transferrin, the IGF's, bFGF, EGF, PDGF, M-CSF and ACTH.

2.9 The dy, dy^{2J} and mdx mouse models of muscular dystrophy:

The hypotheses and questions raised thus far concerning skeletal muscle regeneration have been related to regeneration of damaged normal muscle. Degeneration and subsequent regeneration can occur, not only as a result of mechanical trauma, but also as a result of disease, such as in Duchenne muscular dystrophy. The trigger for regeneration seems to be from the degenerating fibres. This is supported by the finding that during externally induced trauma the fibres begin to degenerate before the satellite cells appear to enter the regeneration process (Komorowski et al., 1990; Carpenter and Karpati, 1989).

Human Duchenne muscular dystrophy (X-linked recessive inheritance) is characterized histologically by greatly increased intrafascicular connective tissue (collagen and fat) with consequent fibre rounding, atrophy of some fibres and hypertrophy of others (resulting in a wide range of muscle fibre sizes), macrophage invasion and splitting of hypertrophic fibres (Mrak, 1985). Ultimately, the degeneration of muscle overrides the regenerative capacity of the muscle resulting in severe atrophy and fibrosis of the skeletal muscles.

The first recognition of a hereditary muscular dystrophy in an animal model was reported by Michelson et al. (1955). The murine dystrophy (designated as dy/dy) was found to display simple autosomal recessive inheritance. By two weeks of age the affected animals start to show clinical symptoms of a waddling gait with splaying and stiffening of the hindlimbs and toes. With progression of the disease there is overt dragging of the hindlegs, while there is much less functional impairment of the forelimbs. The histological features (Mrak, 1985) of the dystrophic mouse (dy/dy) muscle are quite similar to those seen in human muscular dystrophies; including variation in myofibre size, increase in endomysial connective tissue, segmental necrosis of myofibres, internal nuclei and regenerative activity. Also similar to the human condition is an elevation in serum level of muscle-specific enzymes. There is some fatty replacement of fibres, however this is not as extensive as that seen in human Duchenne muscular dystrophy (DMD).

Due to the fact that the life-span of dy/dy mice is considerably shorter than normal, healthy mice (less then 6 months, as compared to greater than 2 years) and the mice are so severely incapacitated by the time they reach sexual maturity, another hereditary myopathy in mouse (dy^{2J}/dy^{2J}) is more commonly used as a model. The dy^{2J}/dy^{2J} myopathy gives rise to a slower developing, more mild disease as compared to dy/dy. However, when the two alleles (dy and dy^{2J}) are expressed in the same strain of mice, the two myopathies (dy/dy and dy^{2J}/dy^{2J}) are clinically and histologically similar (Macpike and Meier, 1976). Another advantage of the dy^{2J}/dy^{2J} form is that it appears to be capable of successful regeneration in vitro (Hamburgh et al., 1975; Parsons, 1974) and in vivo (Dribin and Simpson, 1977), at least in the early stages of the disease, whereas most of the studies on dy/dy muscle have suggested that it is virtually incapable of regenerating either in vitro (Moore, 1975; Parsons, 1974; Paul and Powell, 1974) or in vivo (Banker, 1960, 1967; Bray and Banker, 1970; Denny-Brown, 1960; West and Murphy, 1960). Successful in vivo regeneration in dy^{2J}/dy^{2J} mice is clearly age-dependent. For example, Schiaffino et al. (1993) have shown that in dy^{2J}/dy^{2J} mice older than six months there is much less regeneration following cold injury, than in age-matched healthy C57BL mice, or younger dy^{2J}/dy^{2J} mice.

Human DMD is characterized by persistent skeletal muscle necrosis and is believed to be the consequence of a deficiency of dystrophin (Hoffman et al., 1988), a component of the sarcolemma. This is not the case in dy^{2J}/dy^{2J} murine dystrophy; like normal mice, the dy^{2J}/dy^{2J} animals still have dystrophin. Therefore, the cause of the dystrophy in dy^{2J}/dy^{2J} mouse muscle is not a lack of dystrophin.

Another mouse dystrophy model, mdx, which lacks dystrophin is better suited to study the effects of missing dystrophin. The mdx genotype, like human DMD, is inherited as an X-linked recessive and, similarly, necrosis of myofibres occurs as a result of the dystrophin deficiency. In fact, recent research has demonstrated that the genes for both the human and the murine defects can be mapped to the same region of the X-chromosome (Brockdorff et al., 1987). The two

diseases also share some histopathological features. The degeneration/regeneration characteristic of human DMD muscle fibres appears very similar to the pathology initially seen in the mdx mouse (Hoffman et al, 1987). However, unlike the human condition and the dy/dy and dy^{2J}/dy^{2J} models, the extensive connective tissue proliferation (fibrosis) appears to be largely absent in mdx muscle (Bridges, 1986).

Mdx mice display a pronounced, but transient phase of debilitating muscle degeneration that starts at about ten days of age and lasts until approximately the third week after birth. Thereafter necrosis progresses at a much slower pace (Woo et al., 1987). Most of the degenerated fibres from the initial massive degeneration phase are replaced by newly regenerated myofibres and adult mdx mice display few, if any, clinical symptoms (Anderson et al., 1987; Coulton et al., 1988). This is unlike the human and dy^{2J} mouse conditions where the degeneration process proceeds at a constant pace throughout life.

As a result of this initial phase of massive muscle degeneration and regeneration mdx mice present a high proportion of centrally nucleated muscle fibres and a large variation in muscle fibre diameter (Morgan et al., 1989). Therefore, one may assume that the high proportion of centrally nucleated myofibres in adult mdx mice is a reflection of the complete replacement of degenerated fibres with newly regenerated myofibres derived from, or the repair of only partially damaged myofibres by, satellite cells. These new myofibres are still missing dystrophin, as a result of a mutation in the gene encoding this protein. Yet the new myofibres remain healthy for the life of the animal. This may indicate that the function of dystrophin is taken over by another protein or dystrophin analogue (Campbell and Kahn, 1989; Turner et al., 1988; Franco and Lansman, 1990); perhaps utrophin, previously known as dystrophin-related protein (DRP; Matsumara et al., 1992; Takemitsu et al., 1991).

Dystrophin is the protein product of the Duchenne muscular dystrophy (DMD) gene (Hoffman et al., 1987). It is associated with a large oligomeric complex of sarcolemmal glycoproteins (reviewed by Matsumara and Campbell, 1994), including dystroglycan which provides a linkage with laminin, an extracellular matrix

component (Ibraghimov-Beskrovnaya et al., 1992). Recent evidence suggests that dystrophin is also linked to the subsarcolemmal cytoskeleton (Klietsch et al., 1993). Dystrophin therefore appears to be an important component of the structural dystrophin-glycoprotein complex and its absence may disrupt the linkage of the dystrophin-associated proteins to the subsarcolemmal actin-cytoskeleton in DMD skeletal muscle. Under such conditions the sarcolemma may be vulnerable to physical stress.

Evidence (reviewed by Hoffman et al., 1987) indicates that, like the human condition, the deficiency of dystrophin in mdx mice can be presumed not to be a secondary consequence of a non-homologous genetic disorder. More simply, the major mdx lesion, the persistent sporadic necrosis of muscle fibres, is innate to the muscle of mdx genotype and is not secondary to any other neuropathic, or extramuscular primary lesion.

The fact that the adult mdx mouse shows very little of the clinical phenotype seen in the later stages of the human DMD could be due to the fact that the mdx mouse has successfully regenerated its muscle fibres, whereas in human DMD regeneration is not as successful. The inverse relationship between myogenesis and fibrosis suggests that perhaps these two phenomena are linked; ability to regenerate seen in mdx mice and loss of it in DMD may be a function of the difference in endomysial fibrosis occurrence between the two disease states. Hoffman et al. (1987) state that the prominent fibrosis seen in human DMD muscle is probably an indirect or secondary consequence of the primary lesion causing myofibre degeneration, the absence of dystrophin. The endomysial fibrosis may interfere with the ability of individual muscle fibres to regenerate: as the muscle ages its fibre number would progressively decrease as the connective tissue content would increase. This process would ultimately result in insufficient muscle fibre numbers for mobility and respiration. This hypothesis agrees with the clinical manifestation of human DMD. In comparison, the muscles of mdx mice do not exhibit significant fibrosis (Bridges, 1986; DiMario et al., 1991 and there is some evidence suggesting

that they retain the ability to regenerate throughout the life of the mouse (DiMario et al., 1991), suffering no threat to either mobility or normal life span of the mouse. Furthermore, and perhaps the most significant difference between the two disease states is the relative absence of continued degeneration in mdx mice.

Following the initial massive muscle degeneration phase that occurs in the early life of the mdx mouse the rate of degeneration decreases, thereby allowing the muscles of the mdx mouse to regenerate almost completely. On the other hand, dy/dy and dy^{2J}/dy^{2J} mice exhibit a similar clinical manifestation of the disease to the human condition (continuing myofibre degeneration, eventual loss of regeneration capabilities, and fibrosis), yet they do not lack the protein dystrophin. It has been hypothesized that mdx mouse muscle is able to achieve normal muscle function, following the initial phase of degeneration and regeneration, perhaps by producing a dystrophin analogue or other molecule(s) which compensates for the lost function of dystrophin (DiMario et al., 1991; Campbell and Kahn, 1989; Turner et al., 1988; Franco and Lansman, 1990) and thereby escape from further myofibre degeneration.

Table 2.9.1: Summary of the murine models of Duchenne muscular dystrophy.

	Human DMD	dy^{2J}/dy^{2J} mouse	mdx mouse
inheritance	X-linked recessive	autosomal recessive	X-linked recessive
dystrophin status	absent	present	absent
muscle necrosis	persistent	persistent	transient
regenerative ability	eventually disappears	eventually disappears	yes (persistent?)
endomysial fibrosis	yes	yes	no

Study of the *loss* of skeletal muscle regeneration ability in human DMD is best achieved through the most clinically similar murine model, dy^{2J}/dy^{2J}.

To summarize, in both the human DMD and murine dy^{2J} disease state skeletal muscle regeneration eventually fails. It is not clear from the literature whether this is true for mdx mice since degeneration does not continue at the same rate. It is not known whether the lack of dystrophin plays a role in the decreased regenerative capacity of DMD. If it does, however, then this may appear to present a paradox in the mdx mouse if their muscles retain the ability to regenerate.

2.10 Thesis objectives:

1. To investigate the effect of various "regeneration factors" on growth and differentiation of satellite cells in culture in order to explore the mechanism which results in normal activation of muscle regeneration.

2. To assess the effects of these same factors on satellite cells from dystrophic muscle in an attempt to determine the nature of the fault which results in the eventual absence of the regeneration process in the dy^{2J}/dy^{2J} murine model of muscular dystrophy.

3. To investigate the regenerative capacity of old mdx mice to gain further insight into the ongoing ability of mdx mice to regenerate in contrast to dy^{2J}/dy^{2J} mice.

In order to carry out these objectives it is essential to;

A. establish a method of obtaining a pure population of myogenic precursor cells from adult mouse muscle, and

B. characterize the growth and development of this pure population of satellite cells.

CHAPTER III

MATERIALS AND METHODS:

3.1 Animals and muscles

Mice were originally obtained from Jackson Laboratories (Bar Harbour, Maine, USA) and have since been bred in the animal care facility in Roger Guindon Hall, University of Ottawa. The mice were quartered in standard cages with food (Prolab RMH 4020 pellets, Agway, Syracuse, NY) and water supplied *ad libitum*. Constant temperature and relative humidity were maintained for optimal health conditions.

Normal mice were divided into two groups as a function of age. Young mice were between 3 and 5 months of age. Old mice were 12-26 months of age.

No significant difference was found among satellite cell cultures derived from single myofibres obtained from +/+ or +/? C57BL mice, therefore, skeletal muscle from phenotypically normal (+/?) or normal (+/+) mice of the C57BL strain was used as the control.

Genetically dystrophic mice of the strains C57BL dy^{2J}/dy^{2J} and C57BL mdx were used.

All muscles were removed while the animals were anaesthetized with an intraperitoneal injection of sodium pentobarbital (0.07 mg/g body weight; Somnotol, M.T.C. Pharmaceuticals, Cambridge, Ontario, Canada). The animals were sacrificed by an overdose of Somnotol.

The flexor digitorum brevis (FDB) muscle of the mouse was used as the source for single fibres since it has relatively short fibres compared to other muscles. FDB is a multi-pennate muscle, and is the most superficially placed muscle in the plantar surface of the foot.

3.2 Tissue Cultures

Explants of intact muscle fibres with their associated satellite cells and basal lamina sheath were isolated from mouse flexor digitorum brevis muscle (FDB) using a method similar to that described by Bischoff (1986a, 1989). Initially, many fibres were plated per dish (as per Bischoff's method), however much fibroblast-like cell growth was observed. This complicated the quantification of satellite cell growth and development, since satellite cells and fibroblasts are morphologically very similar. To increase the purity of the culture (ie. decrease the number of fibroblast cells present) the isolated fibres were plated one at a time on multiwell plates, so that there was one fibre per well. Once the myogenic purity of cell growth, which resulted following plating of single fibres, was confirmed (see Results) it was assumed that any growth observed on the culture plate must be the progeny of the satellite cells associated with that individual myofibre.

The donor animal was sterilized by spraying with 70% ethanol following anaesthesia and sterile procedures were employed during removal of the muscle from the animal and during the subsequent plating and culturing procedure to minimize the risk of contamination to the cultures.

Following exposure of the FDB muscle, the muscle was gently removed so as to avoid damage to muscle fibres and stretching of the muscle. First, the proximal FDB tendon was severed close to the calcaneous bone. The muscle was then gripped by the proximal tendon and peeled distally. Rather than pulling the muscle against the force of the fascia retaining the FDB, fine scissors (Fine Science Tools Inc., Vancouver, B.C.) were used to cut the fascia. The muscle was peeled down to the point where the toes joined, so that the three distal FDB tendons were well exposed (FDB is a multipennate muscle) and could be cut easily. As a result, any adjacent excess tendons, connective tissue, fat, blood vessels, nerves and extraneous muscle were also taken to minimize damage to the FDB. The muscle was carefully pinned by its four tendons to a 60 mm petri-dish containing cured silicone elastomer (SYLGARD 184 silicone elastomer, Dow Corning) bathed by sterile Tris-buffered saline (TBS, pH 7.4). Under a dissecting microscope, fine dissecting instruments (8.5cm Vanna-Tubingenspring scissors with 5mm cutting edge, Dumont #5 Biologie fine forceps, Fine Science Tools Inc., Vancouver, B.C.) were used to remove any non-FDB muscle, visible connective tissue, blood vessels and nerves. Following removal of excess tendon, the cleaned muscle was transferred by its tendon stumps to another sterile 60 mm petri dish containing a sterile filtered, freshly prepared solution of 0.2% crude collagenase (Sigma Type I, lot no. 12H 0539) in Minimal Essential Media (MEM, GIBCO). The muscle was then digested in a 37°C gentle shaker water bath. Following the first hour of digestion gentle trituration (about 10 cycles) using a pasteur pipette with a broken and fire-polished 2 mm wide tip helped loosen the outer layers of digested connective tissue from the underlying muscle tissue. It was generally found that muscles from old normal and old dystrophic mice of both strains required up to three hours of collagenase digestion, while those from young normal mice needed no more than a two hour digestion. Following the collagenase treatment the muscle was transferred, by grasping the remaining wisps of collagenous tendons, to a new petri dish containing 10% Horse Serum Medium. Very gentle trituration was performed using a wide-mouth pipette

(2 mm wide mouth). Progressively smaller-mouthed (1.5 mm and 1 mm wide mouths) pasteur pipettes were used for subsequent trituration. The loss of fibres through adhesion to the pipette walls was lessened considerably by pre-flushing the pasteur pipettes with 10% horse serum medium, therefore all pipettes were treated this way just prior to their use. Trituration was continued until there were mainly single fibres covering the bottom of the plate, and a few remaining on the tendons. Any large muscle/tendon chunks, remaining collagen wisps and fibre clumps (2 or more fibres) were removed using fine forceps. The remaining single fibres were transferred, using a normal size pasteur pipette (pre-flushed with 10% horse serum), to a 10 cm column of MEM in 15 ml conical tube (Falcon polystyrene, Becton Dickinson, Mississauga, Ont.) for sedimentation at 1 g for 15-20 minutes, until the fibres could be seen at the bottom of the column and the debris was still in suspension. Following sedimentation the upper layer of basal medium and non-myofibre tissue was gently removed so as to leave about 5 mm of medium over the muscle fibres at the bottom of the tube. This sedimentation cycle was repeated twice more. Due to the fact that fewer fibres were recovered from the old, dy^{2J} and mdx muscles often only one sedimentation was done under these conditions. This allowed for a higher single fibre yield (some fibres are inadvertantly lost with each sedimentation), but did result in a slightly larger proportion of the wells being contaminated with non-myogenic cells. The tubes used for the 3 sedimentation columns were pre-treated with 10% horse serum medium at room temperature for 2 hours and air dried prior to use. This prevented attachment of the myofibres to the walls of the tube. Following the final sedimentation the concentrated myofibre suspension was diluted into 10 ml MEM in 60 mm petri dishes pretreated with 10% horse serum (to prevent attachment of the fibres to the plate). These dishes were incubated at 37°C with 5% CO_2 for approximately 15 minutes. This allowed any damaged cells to hypercontract so they were visibly non-viable and lowered the chances of plating a non-viable cell, damaged by the dissociation process. Only single intact viable myofibres were plated since it was observed that non-viable fibres do not attach to the substratum but tend to float

which decreases the likelihood that their associated satellite cells would proliferate (their growth is attachment-dependent). A pasteur pipette was then used to carefully pick out single viable myofibres which were subsequently plated onto a thin, dilute extracellular matrix substrate (Matrigel, Collaborative Biomedical Products, 1:10 in MEM) in tissue culture wells (1 fibre per well, Falcon Primaria surface-modified 24 multiwell plate). A substratum such as Matrigel was required as satellite cells and myofibres are attachment-dependent cells. It was found that the myofibres attached to the culture surface much more readily under serum-free conditions. Before adding any media to the culture, the multiwell plate and its plated fibres were incubated at 37°C for 60 minutes to allow some time for the fibres to settle onto the substratum. 0.5 ml of the specified medium was then added to each well. The cultures were incubated at 37°C with 5% CO_2 and humidified with milli-Q water. Unless noted otherwise, the specified medium was added at the time of fibre plating and not changed throughout the 7 day culture period. The amount of proliferation resulting from the satellite cells derived from one myofibre was small enough that a regular change of media was not required. It was desirable to maintain the myofibre in the satellite cell environment so it could exert any of its potential effects on the satellite cells. It was decided that this was more similar to the true physiological condition. Furthermore, the myofibre may have contained some satellite cells which remained within the basal lamina sheath.

3.3 Solutions:

All powdered media and other solutions were made using Milli-Q water and filter-sterilized using Millipore 0.22μm sterilizing filters (Millipore Corporation, Bedford, MA). The Collagenase Solution and any media used to feed the cultures were filter-sterilized, after addition of all the media components, with Millipore 0.45μm (to catch large debris often present in horse serum and/or CEE) and 0.22μm (to sterilize) low protein-binding filters.

Collagenase Solution (muscle digestion)

98.7% Minimal Essential Medium (MEM)
1% penicillin/streptomycin (100 U/ml)
0.1% Fungizone
0.2% Collagenase type I (Sigma, lot no. 12H 0539)

10% Horse Serum Medium

88.9% Minimal Essential Medium (MEM)
10% horse serum (HS)
1% penicillin/streptomycin (100 U/ml)
0.1% Fungizone

Tris-buffered Saline (TBS)

50 mM Tris (Bio-Rad Laboratories, Richmond, CA)
0.9% NaCl (BDH Inc., Toronto, Ont.)
- brought to pH 7.4 with HCl

The Media (to feed the cultures) were as follows :

<u>Basal Medium</u> (BM)
78.9% Minimal Essential Medium (MEM)
20% horse serum (HS)
1% penicillin/streptomycin (100 U/ml)
0.1% Fungizone

<u>CEE Growth Medium</u> (CM)
78.4% Minimum Essential Medium (MEM)
20% horse serum (HS)
0.5% Chick Embryo Extract (CEE)
1% penicillin/streptomycin (100 U/ml)
0.1% Fungizone

<u>CPSR-2 Medium</u>
77.9% Minimal Essential Medium (MEM)
20% Controlled Process Serum Replacement Type-2 (CPSR-2)
1% horse serum (HS)
1% penicillin/streptomycin (100U/ml)
0.1% Fungizone

bFGF Media
- the required amount of bFGF (recombinant human bFGF, R&D Systems) was added to Basal Medium, which had its volume of MEM adjusted to account for the extra volume of bFGF

All of these reagents were from GIBCO (Grand Island, New York), unless otherwise specified.

3.4 Crushed Muscle Extract:

Similar to the tissue culture methods, sterile procedures and instruments were used. The mice were anaesthetized with sodium pentobarbitol (80 mg/kg body weight, intra-peritoneally; Somnotol, M.T.C. Pharmaceuticals, Cambridge, Canada), the hindlimbs were shaved and sterilized by spraying with 70% ethanol. The muscles of both hindlimbs were exposed so that the anterior thigh and leg, and posterior leg muscles could be excised by cutting proximal and distal tendons with as little pulling as possible. The muscles were weighed and then crushed gently at 7-10 sites along each muscle length with a pair of broad pattern forceps (2.5 mm wide, Fine Science Tools Inc., Vancouver, B.C.) which had had their serrations filed smooth. The tissue from one animal was placed in 1 ml cold Tris-buffered saline (TBS, pH 7.4) and incubated at 4°C with very gentle shaking for 90 minutes. Subsequently, the entire muscle plus TBS was centrifuged for 10 minutes at 1500g and the supernatant (CME) transferred to a sterile eppendorff tube. The CME could be stored at 4°C until needed (however, it was never stored more than 24 hours). The protein content of each sample isolated in this manner was determined by the Bradford microassay technique (Bio-Rad Protein Assay Dye Reagent Concentrate, Bio-Rad Chemical Division, Richmond, CA) using bovine serum albumin (BSA) in TBS to generate a standard curve.

3.5 Determination of Myogenicity:

Two criteria were tested to prove the myogenicity of cultures in the preliminary experiments. The first was the formation of myotubes, identified visually in live cultures. The second was the expression of sarcomeric myosin heavy chain, identified immunohistochemically using a mouse monoclonal antibody, MF-20, to all sarcomeric myosin heavy chain (Bader et al, 1982; generously donated by Dr. M. McBurney, University of Ottawa).

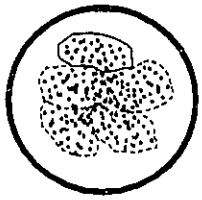
Cultures were fixed in AFA Fixative (20:2:1 - ethanol:10% buffered formalin:glacial acetic acid) for 15 minutes at room temperature (RT), rehydrated in 50% ethanol, and washed in phosphate buffered saline (PBS) for 30 minutes, changing the PBS every 10 minutes. Fixed cultures were incubated at RT for 16 hours with MF-20 diluted 1:5 in 5.0% bovine serum albumin (BSA) in 25 mM phosphate buffered saline (PBS). This high BSA concentration was found to markedly reduce non-specific staining. Each well was incubated with 200 µl diluted MF-20. The PBS was made by mixing 25 mM K_2HPO_4 with 25 mM KH_2PO_4 to pH 7.4, adding NaCl to a final concentration of 0.9% w/v. At the end of the incubation the MF-20 was removed by suction and each well was washed in PBS (3x10 minutes). Subsequently, each well was incubated for 2 hours at RT with a horseradish peroxidase (HRP)-conjugated (Cedarlane Laboratories, Hornby, Ont.) goat-antimouse IgG secondary antibody. The amount of non-specific staining found with the Cedarlane HRP-conjugated anti-IgG was very much less than that observed with comparable antibodies from other companies. The secondary antibody was removed by suction and each well was again washed in PBS (3x10 minutes). The immunohistochemistry was developed following the final PBS wash using 500ml of 0.1% (w/v) 3'3 diaminobenzidine (DAB; Sigma Chemical, St. Louis, MO) and 0.03% (v/v) H_2O_2 in PBS for 30 seconds only. Development was stopped by removal of the DAB solution and washing in tap water. A maximum of 6 wells were developed at a time due to the short development time. Each well was coverslipped with glycerol and a 12mm coverslip circles (Fisher Scientific Inc., Nepean, Ont.)

Once it was established that the culture method resulted in pure populations of satellite cells and I was satisfied that I could visually identify those cultures that were or were not myogenic these criteria (myotube formation and sarcomeric myosin expression) were not necessarily tested. All of the culture conditions tested did not necessarily result in differentiation of the myogenic precursor cells, therefore it was not always possible to test these criteria. For example, as shown in the results section (Figure 4.2.1.1), the presence of bFGF in concentrations greater than 1.0 ng/ml inhibits satellite cell differentiation. Therefore, most of the myogenic cells resulting from satellite cell proliferation under these conditions do not express sarcomeric myosin, nor do the cells fuse to form multinucleated myotubes, since the cells remain in the cell cycle and do not differentiate. As shown in the results section (Figures 4.1.1.2 and 4.1.2.2) fibroblast-like cell growth was clearly identifiable following 7 days in culture.

3.6 Assessment of culture growth and development

Quantitative assessment of cultures was initially carried out by means of cell counts of live cultures taken at 24 hour intervals over a 7 day culture period. Live cultures were observed through an inverted microscope (Leitz Wetzler, Leica, Nepean, Ont.) on the highest power possible, such that the entire colony could be seen in a single field of view. Visual counts were made of single cells and recorded using a hand counter. This was relatively simple to do with fewer than 100 cells in the field of view, however following 4 days in culture the colonies usually consisted of more than 100 cells and had a high density, especially when grown in CEE Medium. For large colonies a representative area of 100 cells was counted and then the total area population was estimated by:

(total area/100 cell area) x 100 cells



eg. $\frac{\text{total area}}{\text{100 cell area}} = 5$

⇒ approx. 500 cells

Any myotubes or multinucleated structures were not included in these cell counts; only single cells were counted. This method allowed for proliferation rates of the satellite cells to be determined, as shown in Results, Section 4.1.3.

The number of single cells approximated for a colony is obviously very inaccurate for those colonies with more than 100 cells, therefore those counts taken following 4 days in culture would be expected to have a large error factor.

Success was achieved through a method whereby counts of the nuclei of fixed cells were taken. The cultures were grown undisturbed for 7 days, after which they were fixed in AFA fixative, rehydrated in 50% ethanol, and washed in PBS for 30 minutes, changing the PBS every 10 minutes. MF-20 immunohistochemistry was then done as described (Section 3.5), followed by hematoxylin staining at RT to identify all nuclei. Each plate was stained in 1000 ml beaker containing Harris-modified Hematoxylin (BDH Inc., Toronto, Ont.) for 7 minutes. Following rinsing in tap water to remove all excess hematoxylin the plates were dipped for 1-2 seconds in 1% acid-alcohol (v/v HCl in 70% ethanol) to differentiate the stained nuclei, and again rinsed thoroughly in tap water. The stain was blued in Lithium Carbonate (50% of a saturated, filtered Lithium Carbonate solution) for 1 minute and then rinsed under running tap water for 10 minutes. Those wells containing cellular growth were coverslipped as described previously.

Following MF-20 immunohistochemistry and hematoxylin histochemistry all nuclei contained within differentiated (MF-20 positive) or undifferentiated (MF-20 negative) cells could be visualized. Nuclei were then scored, as being in either MF-20 positive cells or in MF-20 negative cells, using a digitizer tablet (Hipad Plus, Houston Instrument, Austin, TX) connected to an IBM 486 computer with SigmaScan software (Jandel Scientific, San Rafael, CA). The satellite cell colonies were visualized using a microscope (Leitz Laborlux 11, Leica, Nepean, Ont.) with a drawing tube attachment. The colony was centred in the field of view through the 4x objective. While maintaining the colony at the same coordinates the 10x objective was flipped into place. Nuclei were then scored using the digitizer and

tally function of SigmaScan, and stored in the computer. Later, the nuclei counts were easily retrieved into SigmaPlot for graphing purposes and SigmaStat for statistical analysis. It must be noted that the entire colony was not necessarily counted; only the central 1.70 mm^2 area was counted (all nuclei within the field of view seen through the 10x objective). This area, the same for all colonies analyzed, is referred to as "standard area" in the text.

3.7 Histochemistry of whole muscle cross-sections

The FDB muscle was removed from the animal, bathed in a solution of 0.9% saline and blotted dry. The muscle was placed in a drop of O.C.T. Compound (Tissue Tek, Miles Canada, Inc., Etobicoke, Ont.) such that the O.C.T. Compound surrounded the muscle. The muscle, together with its coat of O.C.T. Compound, was frozen in melting isopentane that had been frozen in liquid nitrogen. The frozen muscle was placed in a small plastic vial and stored at -80°C until further processing. The muscle was mounted on the specimen holder in an upright position such that the long axis of the muscle was perpendicular to the flat surface of the specimen holder. Transverse sections, 10 μm thick, were cut from the mid-belly region of the muscle using a Microm MedGV Class 4 cryostat microtome (Heidelberg, Germany) which was set at -22°C. The cut sections were placed on slides (Superfrost Plus, Fisher Scientific, Nepean, Ont.) and then stored at -20°C, or stained immediately with either Hematoxylin and Eosin or with monoclonal antibodies (mAb) against myosin heavy chains (MHC).

Hematoxylin and Eosin Stain

The slides with the attached muscle cross-sections were brought to room temperature (RT) and then stained with Harris-modified Hematoxylin for 7 minutes. Following rinsing in tap water to remove all excess Hematoxylin the sections were dipped for 1-2 seconds in 1% acid-alcohol (v/v) to differentiate the stained

nuclei, and again rinsed thoroughly in tap water. The stain was blued in Lithium Carbonate solution for 1 minute and then rinsed under running tap water for 10 minutes. The cytoplasm was counter-stained with Eosin Y (EM Diagnostic Systems, Inc., Gibbstown, NJ) for 1 minute. The sections were briefly rinsed in water, dehydrated in successive alcohol baths of increasing concentration, cleared in Xylene substitute (BDH Inc., Toronto, Ont.) and mounted in Permount (Fisher Scientific, Nepean , Ont.).

Myosin Immunohistochemistry

Monoclonal antibodies (mAb) against type I MHC (BA-D5), type IIA MHC (SC-71), all MHC isoforms excluding type IIX (BF-35) and type IIB MHC (BF-35) were used (Schiaffino et al., 1986, 1989). They were generously provided by Dr. S. Schiaffino, Padua, Italy. Sections were incubated in a moist chamber with the primary mAb at RT for approximately 16 hours. All antibodies were optimally diluted (see Table 3.7.1) in 0.5% BSA in PBS. The sections were then rinsed in PBS (3x10 mins.). Excess PBS was removed from the spaces between the sections with a Kimwipe tissue and then incubated for 2 hours at RT with a HRP-conjugated rabbit-antimouse IgG secondary antibody (Cedarlane Laboratories, Hornby, Ont.) or, in the case of BF-F3, anti-IgM (Kirkegaard & Perry Laboratories, Gaithersburg, MD). The sections were rinsed again in PBS (3x10 mins), developed by submerging in DAB

Table 3.7.1: Myosin heavy chain monoclonal and secondary antibodies and relative concentrations used for immunohistochemistry.

MHC	Primary mAb	Dilution	Secondary mAb	Dilution
I	BA-D5	1:100	IgG HRP	1:50
IIA	SC-71	undiluted	IgG HRP	1:50
IIB	BF-F3	1:250	IgM HRP	1:50
I,IIA,IIB	BF-35	undiluted	IgG HRP	1:50

solution and processed in the dark for 5-10 minutes. The sections were washed in tap water, dehydrated through successive alcohol baths of increasing concentration, cleared 2x5 minutes in Xylene substitute and mounted in Permount.

3.8 Experimental protocols and definitions

This report uses the term "experiment" to describe all those single fibres, and their subsequent progeny, which were isolated and plated at the same time. All experiments using young mouse fibres utilized one mouse per experiment. As discussed briefly in Section 3.2 (Tissue cultures) the FDB muscle from old mice gave a much lower yield of single myofibres. The yield was even lower with the FDB muscle from dy^{2J} and mdx mice. This would be expected since old age and dy^{2J} dystrophy are conditions in which there is more connective tissue or fibrosis. In order that there were enough viable single fibres to be able to plate similar numbers of fibres for each condition I usually used two mice per experiment for old mouse experiments (13/16) and always used two mice per experiment when isolating dy^{2J} fibres. If possible, the two donor mice were littermates. The fibres were combined together from both animals during the fibre isolation procedure. All mdx mouse experiments used one mouse per experiment due to a shortage of old mdx mice.

In each experiment for testing optimal growth conditions and bFGF effects in young and old mice one plate (24 wells) of single fibres was plated per condition. In experiments using old dy^{2J}, old mdx and age-matched normal mice two plates (48 wells) of single fibres for Basal Medium and CEE Medium, and 4 plates for each bFGF concentration were tested. The total number of fibres plated per experiment was similar (8-10 plates; 192-240 fibres), thus explaining the need for similar fibre yields for each experiment during the single fibre isolation process.

Inadvertently, an occasional well would contain no fibre or more than one single fibre. Therefore, immediately following plating, each well was inspected and marked if it contained no, or more than one, fibre. These wells were not

included in the total number of fibres plated, nor assessed for myogenic cell growth and development in the reported analyses. Those wells containing more than one fibre and which exhibited myogenic growth were usually used during immunohistochemistry as secondary antibody controls.

Observations showed that, following 7 days in culture, myogenic cells are morphologically distinct from non-myogenic cells (see Results, Figure 4.1.2.2). Any colonies exhibiting stellate-shaped cells were assumed to be contaminated with fibroblasts and/or macrophages. These colonies, despite the presence of myogenic cell growth, were not assessed.

Unless indicated otherwise, those single myofibres which were replaced by a single myotube within the original basal lamina sheath (see Results, Section 4.1.3) were also not assessed. Quite frequently, single fibre replacement by a myotube occurred along with a satellite cell colony growing external to the parent myofibre. These cultures were assessed; nuclei in the colony and nuclei in the single fibre myotube were combined together as one colony.

Adoption of the above criteria resulted in the rejection of approximately 25% of the single fibres initially plated.

In addition to the plates of single fibres being tested for various conditions, each experiment had one plate of single fibres cultured in Basal Medium and another cultured in CEE Medium. This served as a control to check that the viability, morphology and proliferation of the satellite cells and fibres from that particular experiment were consistent with that of similar experiments. The viability, morphology or proliferation could have potentially been affected by slight differences in the isolation procedure and plating technique, batch number of the various media components, or bacterial or fungal contamination.

All single fibres and their progeny were cultured for 7 days (the day of plating being considered as day 0) prior to fixation, unless indicated otherwise.

For the remainder of this report the term "satellite cell colony (culture)" refers to the colony of myogenic precursor cells which were derived from a single myofibre.

3.9 Statistical analyses

All statistical analyses were done on SigmaStat (Jandel Scientific, San Rafael, CA), except for split plot ANOVA's (analysis of variance) and Duncan's multiple comparison tests which used SAS (SAS Institute Inc., Cary, NC).

All data tested for significant difference were first tested for normal distribution (Kolmogorov-Smirnov test) and equal variance (Levene Median). If the data passed both tests parametric tests were performed, otherwise nonparametric tests (for single factor analysis) or transformation of the data (for two factor analysis) were performed. All nonparametric data, analyzed for the effects of two factors, (Figures 4.3.2 and 4.3.3) were transformed using a log function. The transformed data passed the normality and equal variance tests, yet there were no alterations to the statistical conclusions obtained using parametic (transformed data) as compared to using the original, nonparametric data.

The unpaired student t-test (parametric data) or Mann-Whitney rank sum test (nonparametric data) were used for comparing two treatment groups. To compare the effect of a single factor on more than two treatment groups one way ANOVA (parametric data) or the Kruskal-Wallis ANOVA on ranks (nonparametric data) were used. The effects of two different factors, and their possible interaction, on two or more treatment groups was tested using a general linear model two way ANOVA (parametric data) or a general linear model split plot two way ANOVA (transformed nonparametric data). The split plot two way ANOVA is designed to accept data from test groups of different sizes, as was the case for all results pertaining to dy^{2J} and mdx genotypes of mouse.

If a statistically significant difference was indicated by the test (ANOVA's) then a suitable multiple comparison test (M.C.T.) was used to determine exactly which groups were different, and the size of the difference. If the one way ANOVA (parametric data) or two way ANOVA (parametric data) indicated a significant difference then the Student-Newman-Keuls test (all pairwise comparisons) or Dunnett's test (multiple comparisons against a single control group) were used to locate these significant differences. If a significant difference was indicated by the Kruskal-Wallis ANOVA on ranks (nonparametric data) then the nonparametric Student-Newman-Keuls test, nonparametric Dunnett's test or Dunn's test (used for nonparametric data when the sample sizes in the different treatment groups are different) were used. If the split plot ANOVA indicated a significant difference then Duncan's multiple range test was used.,

The level of significance for all differences was defined as being $p<0.05$.

Normalization for variation among experiments was performed for all data obtained from fixed cultures (counts of nuclei). Data from fibres obtained from the same treatment group within the same experiment were first averaged. The results reported here represent the means of the averages obtained from similar treatment groups from all experiments. For additional clarity, I have included in the results the total number of fibres scored in all experiments, which resulted in satellite cell proliferation, combined together, indicated as *f*; or, when appropriate, the total number of fibres scored in all experiments, which did or did not result in satellite cell proliferation, combined together, indicated as t.

CHAPTER IV

RESULTS

4.1 Establishing single fibre-derived satellite cell cultures

4.1.1 Development of single fibre isolation method

Much time was spent developing and optimizing the method of isolated single fibre cultures. Therefore, a brief description of the evolution of the method from that originally described by Bischoff (1986a, 1989) to the final method used is included below.

All data and observations presented in this section were derived from satellite cell cultures from young, normal mice, unless indicated otherwise. Multiple single fibre cultures (as per Bischoff's method) or isolated single fibre cultures were used as specified.

The flexor digitorum brevis (FDB) muscle was used as the source of single myofibres. Immunohistochemical fibre typing revealed that FDB has very few, if any, fibres which express IIB myosin isoform. Approximately 60% of the fibres

express type IIX, 30% express type IIA, and the remaining 10% express type I myosin (see Figure 4.1.1.1).

As mentioned briefly in Methods Section 3.2 (Tissue cultures), the method for obtaining single-fibres with their associated satellite cells and basal lamina, as described by Bischoff (1986a, 1989), was initially used. At first, very few intact myofibres were obtained. Most fibres were enucleated fibre pieces as shown in Figure 4.1.1.2 (Panel A). Neither hematoxylin nor ethidium bromide (nucleus-specific stains) identified nuclei in these fibre pieces. Further proof of the fibres being non-intact was that the fibre ends were square and ragged, rather than tapered as described by Bischoff. The collagenase digestion of the muscle was found to be the crucial step for obtaining single intact myofibres. Various solutions (Dulbecco's PBS, Gibco, NY; Tyrode's Solution) were tried as the solution used for collagenase dilution. MEM was found to give the best results. It was also found that the lot of Collagenase greatly affected the digestion results. Optimal muscle digestion, to obtain single intact fibres (see Figure 4.1.1.1, Panel B) was found with Collagenase type I, lot number 12H 0539 (Sigma, St. Louis, MO). This lot of Collagenase had the following specific activities:

Collagenase (FALGPA hydrolysis)	0.80 units/mg
Collagenase (Collagen digestion)	320 units/mg
Neutral Protease (Caseinase)	53 units/mg
Clostripain	0.60 units/mg

Other lot numbers tried were either too harsh, leaving the outer fibres as a sticky mass with the inner fibres undigested, or too gentle and unable to digest the muscle beyond large fibre clumps.

The sedimentation procedure suggested by Bischoff (1986a; 3x7 minutes) resulted in a large loss of single fibres which remained suspended in the supernatant. The sedimentation time was therefore lengthened to approximately 3x15 minutes, or until few fibres were seen remaining in the supernatant. It was possible to see any single fibres remaining in suspension by holding the sedimentation tube up to the light.

Further changes were made to the sedimentation procedure when it was found that the fibres were not sticking to the culture plate. It was realized that horse serum prevents adherence of cells to surfaces. Until this point sedimentation was done through columns of 10% Horse Serum Medium, thereby exposing the fibres to horse serum up until just prior to plating. Also the fibres were plated as a suspension in a drop of 10% Horse Serum Medium. This problem was resolved by sedimenting the fibres through a column of plain MEM. However, as described in the methods section, the tubes for these columns were pre-treated with 10% horse serum to prevent sticking of the fibres to the tube walls.

Problems remained concerning inadequate attachment of the fibres to the culture surface. Various substrata, and combinations thereof, were tried. Collagen (Vitrogen 100, Collagen Corporation, Palo Alto, CA) allowed relatively good fibre attachment, however the Collagen coat frequently detached from the surface of the culture dish. Gelatin and poly-L-lysine permitted essentially no fibre attachment. Better results were achieved with a poly-L-lysine treatment of the culture surface followed by a coat of collagen. The best fibre attachment was obtained with a thin coat of Matrigel (diluted 1:10 in MEM).

As discussed previously, the presence of fibroblasts was a constant problem. Following 3-4 days in culture the surface of the culture dish was frequently covered with a mat of fibroblast-like growth. The single fibres were often completely enmeshed, and obscured from observation, in a clump of fibroblasts (see Figure 4.1.1.3). During the dissociation procedure many of the single fibres stuck to each other and were inadvertently plated along with the single fibres. These fibre clumps were thought to be fibroblast "traps" which retained fibroblasts during the dissociation and plating procedure.

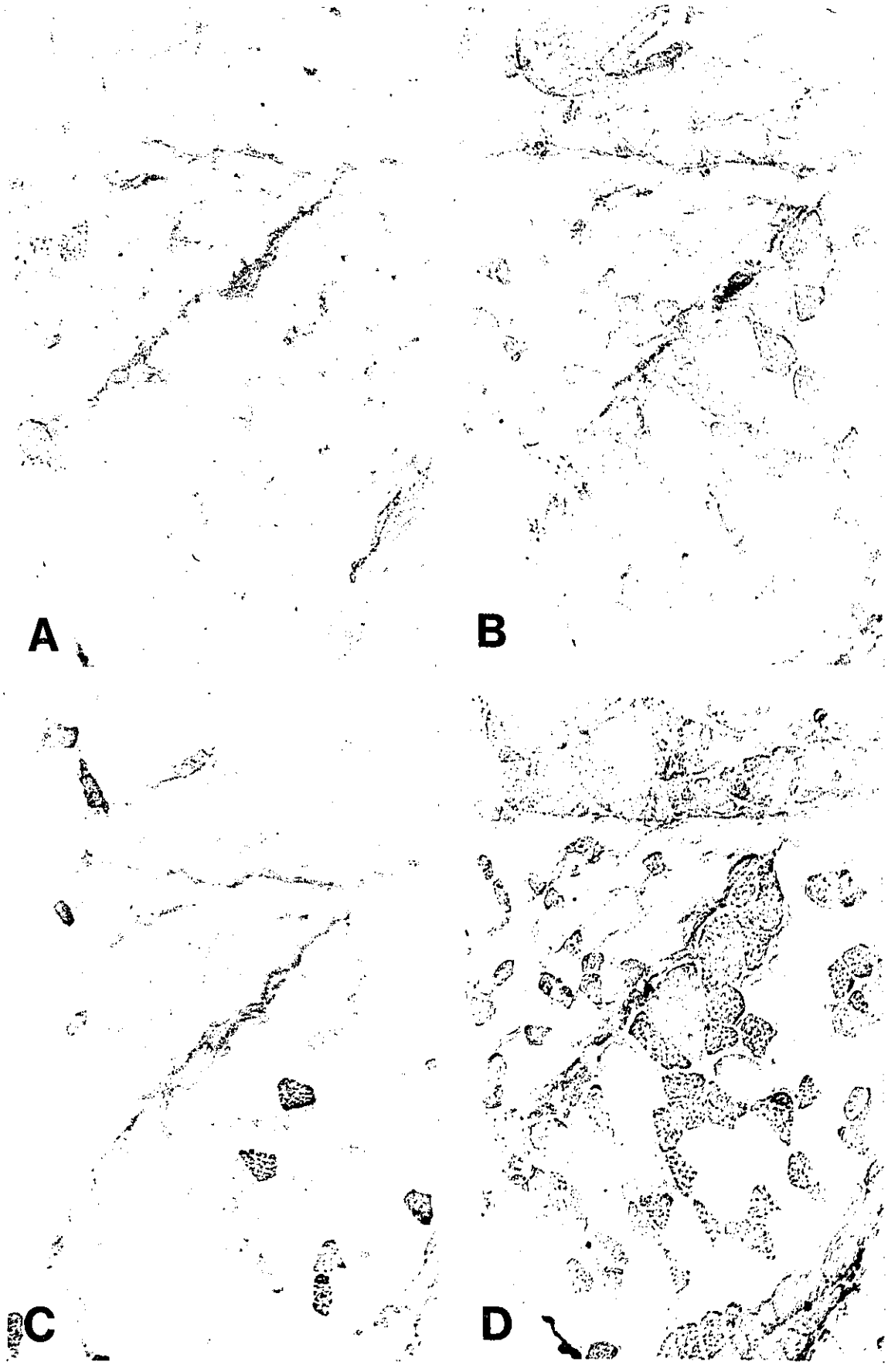
In order to decrease the chances of plating fibroblasts, single fibres were plated on Matrigel-coated multiwell plates so that there was one single myofibre per well. Furthermore, Primaria plates, rather than regular tissue culture grade dishes, were used since Primaria plates have a special modified surface which

inhibits fibroblast growth. It was found that the fibres attached quickly and securely. Only the occasional well was observed to have fibroblast-like cell growth. Cellular growth on the plate was easily observed, mainly in the form of round-shaped and spindle-shaped cells, which finally gave rise to myotubes. These cell forms were seen in the previous multiple single fibre cultures, however their growth and development was hard to observe due to overgrowth by fibroblast-like cells.

Viability of the plated single fibres was confirmed by trypan-blue (BDH Inc., Toronto, Ont.) exclusion. As shown in Figure 4.1.1.4, all those fibres in a hypercontracted state were non-viable (allowed trypan blue entry), while those appearing as slender striated fibres with tapered ends were viable (excluded trypan blue). Most single fibres plated hypercontracted at times ranging from a few hours after plating to the third day in culture; however, on occasion the myofibre remained viable for longer and was replaced by myotubes growing within the basal lamina sheath (shown later in Results Section 4.1.3, Figures 4.1.3.1 and 4.1.3.2)

For each set of single fibres plated there were a few wells which had no fibres plated. These wells conveniently served as a control to ensure that the cells, assumed to be satellite cells and their progeny, were not simply derived from connective tissue cells (for example, fibroblasts, macrophages, etc.) released during the dissociation procedure and floating free in the medium containing the single fibres ready for plating. No satellite cell-like growth was observed on these fibre-free wells, however on rare occasions small colonies of stellate-shaped cells were observed. These were assumed to be fibroblasts originating from some debris or collagen from the digestion procedure that were inadvertantly plated. As mentioned previously any wells containing cells with this stellate-shaped morphology were not used in the analysis.

Figure 4.1.1.1 Immunohistochemical fibre typing of the serial cross-sections of the flexor digitorum brevis muscle from a young mouse. Mouse monoclonal antibodies to (**A**) type IIB myosin heavy chain (MHC) (BF-F3), (**B**) type IIA MHC (SC-71), (**C**) type I MHC (BA-D5), and (**D**) all MHC's, except type IIX (BF-35), followed by HRP-conjugated goat anti-mouse IgG were used. BAR = 50 μm.



74.A

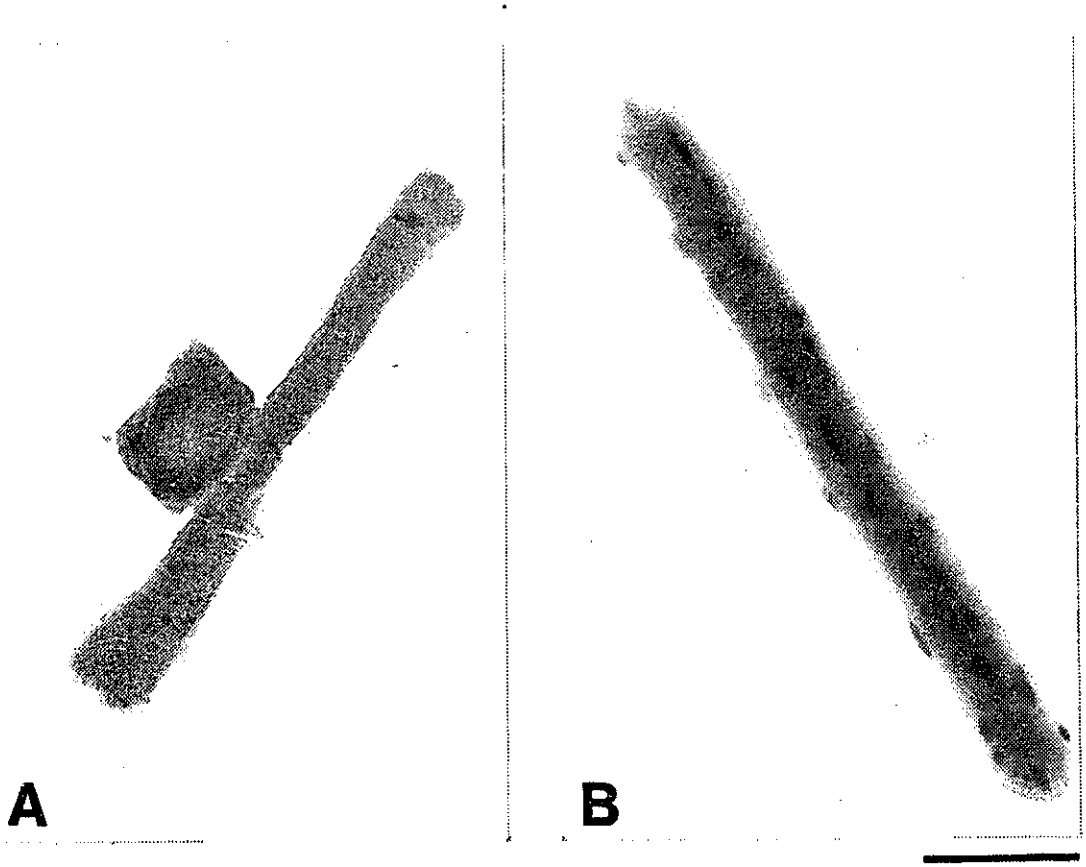


Figure 4.1.1.2

(A) A typical enucleate fibre piece isolated from mouse flexor digitorum brevis (FDB) muscle fixed and stained with hematoxylin (specific for nuclei) and counterstained with eosin (cytoplasm). The fibre ends are square and ragged.

(B) A typical intact myofibre isolated from mouse FDB muscle, fixed and stained with hematoxylin and eosin immediately following the fibre isolation and sedimentation method. The nuclei are hematoxylin-positive and the fibre ends are tapered.

BAR = 50 μm.

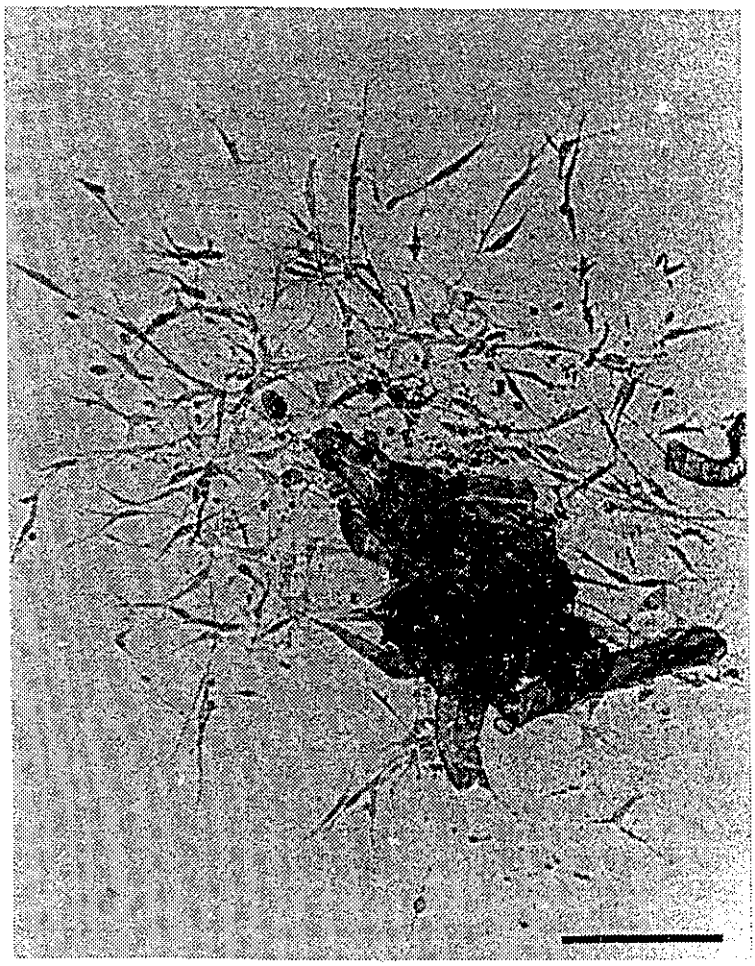


Figure 4.1.1.3 A live culture of single fibres isolated from mouse flexor digitorum brevis and plated many fibres per plate. Fibroblasts have surrounded the myofibres by the 7th day in culture, obscuring them from view. BAR = 200 μm.

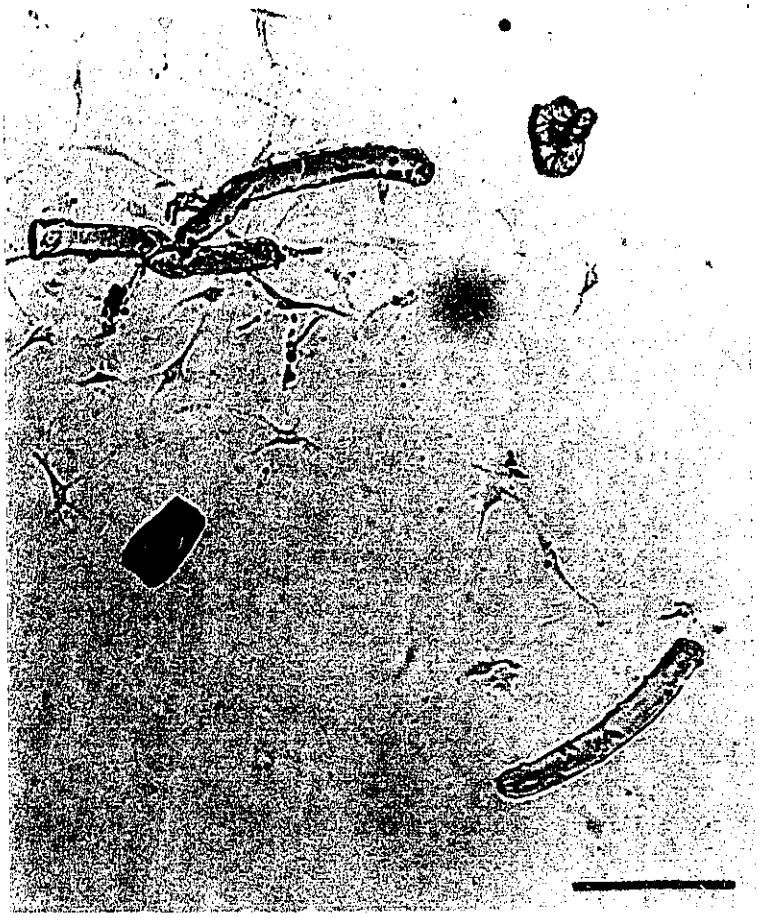


Figure 4.1.1.4 A live culture of single myofibres exposed to trypan blue on the fourth day in culture. Viable cells are trypan blue-negative and non-viable cells are trypan blue-positive. BAR = 200 μm.

4.1.2 Purity of satellite cell culture

All satellite cell colony data presented in this section were derived from single fibres from young, normal mice.

Figure 4.1.2.1 shows a representative area of the progeny derived from a single intact myofibre stained with MF-20, a monoclonal antibody to sarcomeric myosin, following a seven day (7d) culture period in a growth medium containing 20% horse serum and 0.5% CEE. This clearly shows multinucleated myotubes staining positively for sarcomeric myosin. Most of the "round cells" are also positive for myosin.

Figure 4.1.2.2 illustrates cells with fibroblast-like, flattened, stellate-shaped morphology that were considered non-myogenic. None of these cells were ever observed to be positive for sarcomeric myosin. Those colonies containing cells with this stellate-shaped morphology were not included in cell counts.

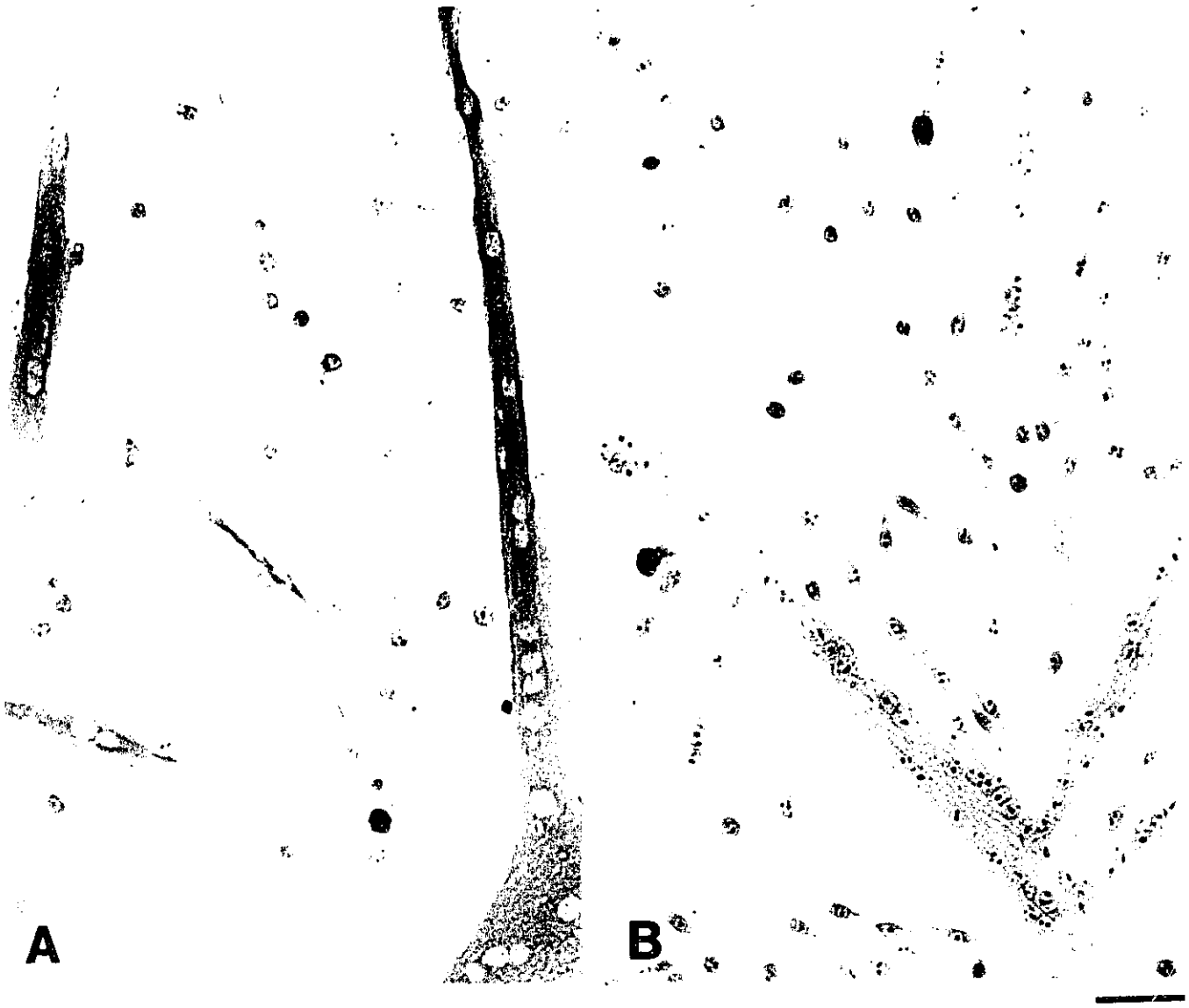


Figure 4.1.2.1 (A) Immunohistochemistry of a satellite cell culture fixed following 7d in culture (CEE Medium) and labelled for sarcomeric myosin heavy chain using MF-20 followed by HRP-conjugated goat anti-mouse IgG. (B) A parallel culture in which the primary antibody, MF-20, was not applied. All of the multinucleated myotubes, and occasionally round-shaped cells, were labelled for sarcomeric myosin. BAR = 50 μm.

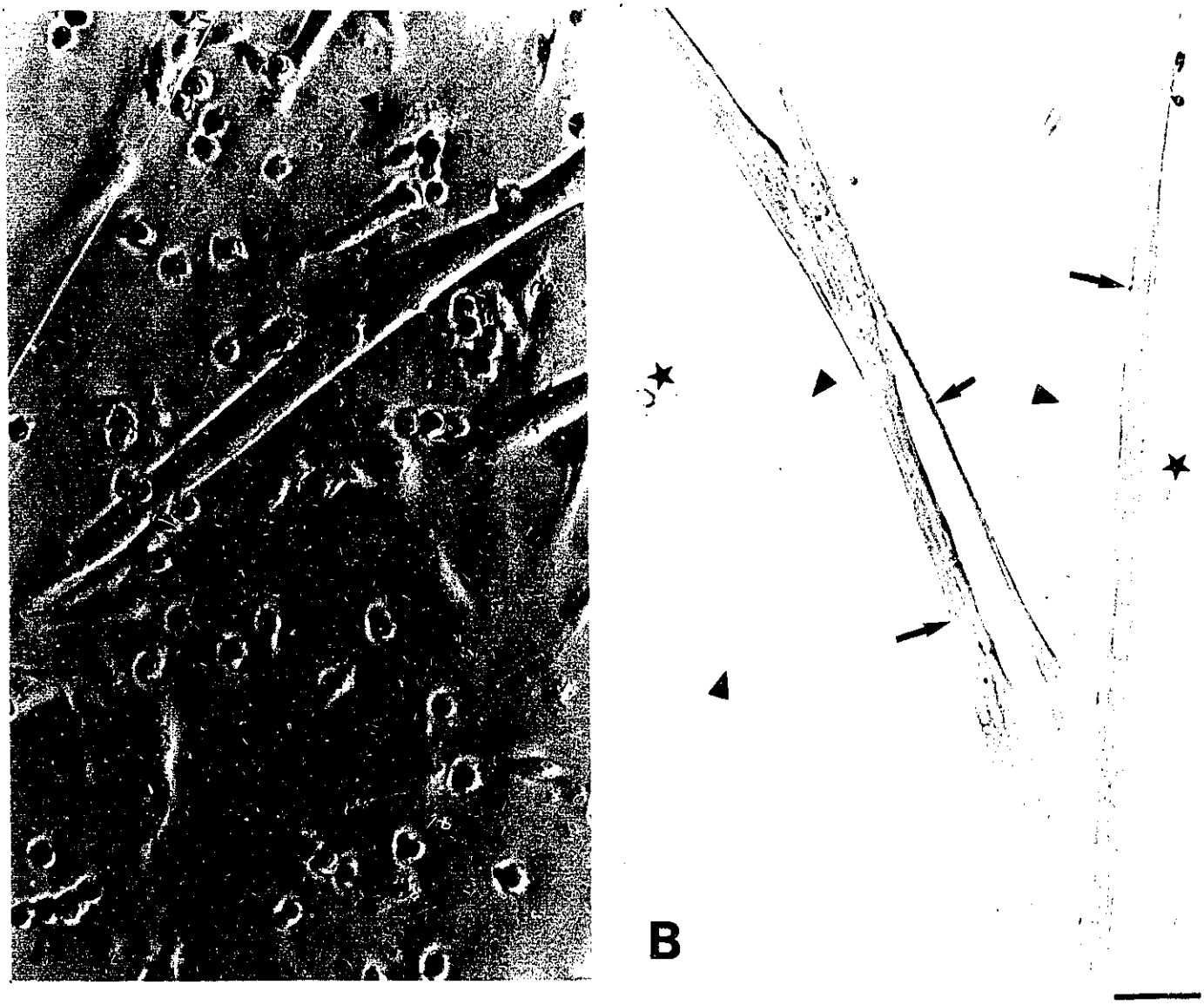


Figure 4.1.2.2 Photomicrographs of cells demonstrating stellate-shape morphology:
(**A**) A live 5d culture (phase contrast optics), demonstrating flattened stellate-shaped cells (▸) and round-shaped cells (★).
(**B**) A culture fixed and stained (immunohistochemistry against sarcomeric myosin and hematoxylin counterstain for all cell nuclei) following 7d in culture, demonstrating myosin-positive myotubes (→), stellate-shaped cells (▸) and round-shaped cells (★).
BAR = 50 μm.

4.1.3 Assessment of myogenicity

Unless noted otherwise, all satellite cell colony data presented in this section were derived from single fibres of young, normal mice. Initial analysis was by single cell counts of live cultures (Figures 4.1.3.5/.6/.7), while later analysis was by counting the number of nuclei/standard area from the centre of a colony that was fixed following 7 days in culture and stained with hematoxylin (Figure 4.1.3.2/.8).

Figure 4.1.3.1 shows photomicrographs of the same live culture over time. This shows the typical proliferation of myogenic precursor cells derived from an isolated single myofibre. Fusion of the myogenic precursor cells started by the sixth day (6d) and was almost complete by the tenth day (10d). Note that by 2 days in culture (2d) the myofibre hypercontracted and died. This myofibre hypercontraction happened in most cultures, usually occurring between a few hours after plating and the third day in culture. On occasion, however, the myofibre remained viable for longer and was replaced by myotubes growing within the basal lamina sheath as seen in Figure 4.1.3.2 (Panel A). This situation was seen most frequently with fibres isolated from old dy^{2J} and old mdx mice, and very infrequently in young normal mouse myofibres (shown later in Results Section 4.3, Figure 4.3.4, Panel A).

Figure 4.1.3.3 demonstrates that given the correct media conditions virtually all of the satellite cell progeny fused to form sarcomeric myosin-positive multinucleated myotubes.

Figure 4.1.3.4 demonstrates that medium containing 0.5% or 1% CEE resulted in high cell proliferation as compared to BM which contained no CEE. However, those cultures grown with 1% CEE Medium stopped proliferating and started to fuse quite early, as seen by the sharp drop in single cells observed after 4 days in culture.

Cells which are triggered by a growth factor to exit the quiescent (G_0) phase of the cell cycle may exhibit three patterns of growth as compared to control conditions (no growth factor present). One may see (1) earlier proliferation, (2) a

greater probability of any given cell to enter mitosis, or (3) faster progression through the cell cycle. Evidence suggests that the time to go through one cell cycle remains constant among cells (Bischoff and Holtzer, 1969; Yablonka-Reuveni and Rivera, 1994; reviewed by Johnston, 1990). Therefore, the effects of growth factors on this culture system using simple proliferation curves would be a result of two distinct variables (1 and 2 above). It would follow that the proliferation curve would shift to the left for the first pattern of growth and/or would have a greater slope for the second. Given these assumptions the proliferation curve in Figure 4.1.3.4 demonstrates clearly that with increasing CEE content (up to 0.5 % CEE) there was an increase in the number of cells which entered the cell cycle, illustrated by a steeper slope. Since there is no shift of the curve the start time for proliferation appears similar under all conditions.

The results of Figure 4.1.3.4 therefore indicate that the use of at least 0.5% CEE Medium induced optimal cell proliferation in this single myofibre-derived satellite cell culture system.

Determination of the optimal culture conditions are summarized in Figure 4.1.3.5 and 4.1.3.6. Figure 4.1.3.5 shows that addition of 20% horse serum to Basal Medium (BM) or CEE Medium (CM) did not lead to enhanced proliferation over that induced in the presence of 10% horse serum. CM, however, clearly enhanced cell proliferation as compared to BM. Assessment of CEE Medium (CM) effects compared to Basal Medium (CM) effects was better evaluated by comparing counts taken from cultures fixed and stained with hematoxylin (stains all nuclei) following 7 days in culture (Figure 4.1.3.6). The counts were obtained as number of nuclei/standard area from the centre of a colony. Cultures grown in BM maintained a relatively low level of cell proliferation, indicated by fewer nuclei. Data was normalized for each experiment. As discussed in Methods Section 3.8 (Statistical analyses), within each experiment the data obtained from the same treatment group was averaged. The results that are reported here represent the mean of the averages from each experiment. Therefore, the reported mean value for a given treatment

group represents the mean from different experiments (which comprise many colonies) and not of each satellite cell colony observed.

Figure 4.1.3.1 Photomicrographs of the same live fibre over time to show typical proliferation of satellite cells derived from an isolated single myofibre. Round-shaped cells predominated prior to the sixth day in culture (6d) when fusion started to occur (→). Fusion of the myoblasts to form large multinucleated myotubes was almost complete by the tenth day in culture (10d). Note that by 2 days in culture the myofibre had hypercontracted. BAR = 200 μm.

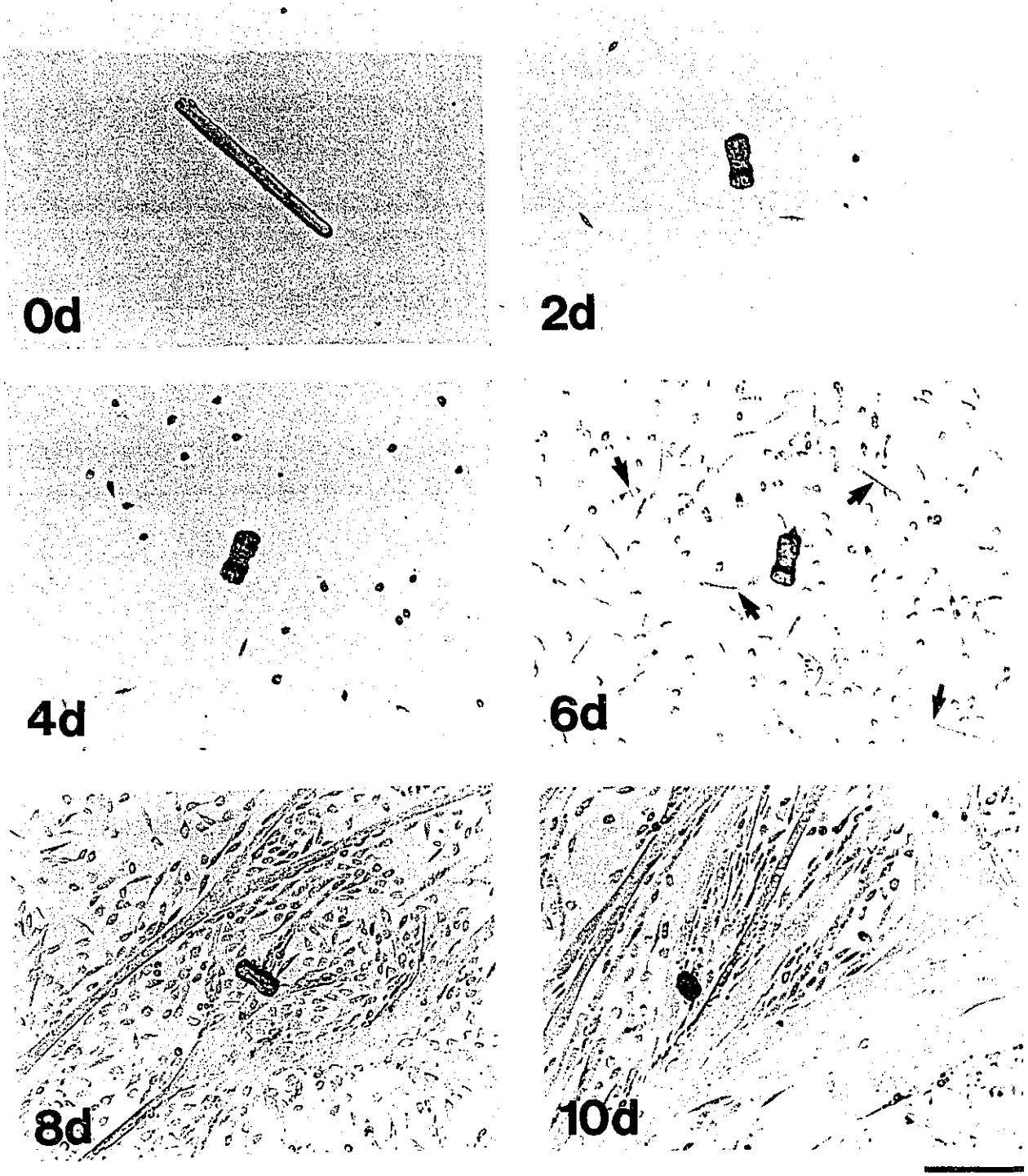
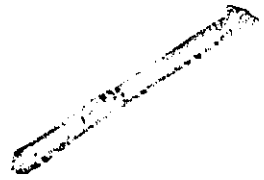

0d
2d
4d
6d
8d
10d

Figure 4.1.3.2 (A) Photomicrographs of the same myofibre in culture over time to show the replacement of the fibre with a new myotube. The appearance of myotubes out of both ends of the viable myofibre occurred on the fourth day in culture (4d). By the sixth day in culture (6d) the original myofibre had hypercontracted and myotubes were clearly visible out of both ends of the myofibre. The basal lamina sheath of the original myofibre was clearly visible surrounding the new myotube on the eighth and tenth days in culture (→, 8d and 10d). BAR = 200 μm.

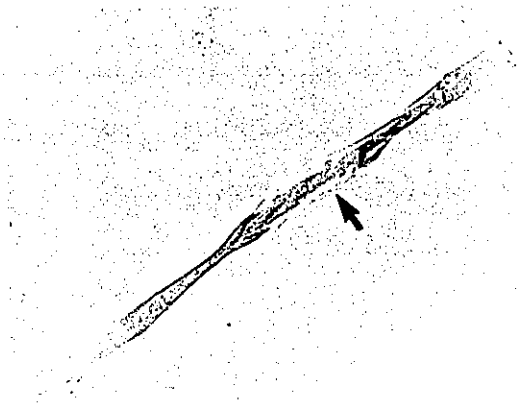


0d

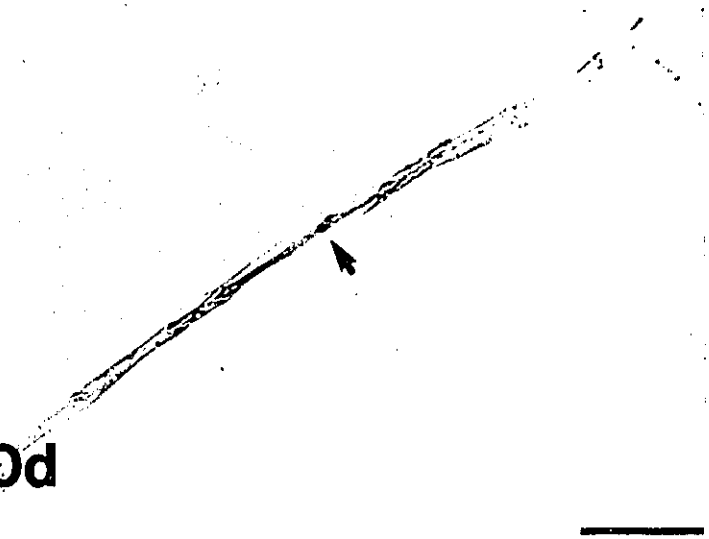
2d

4d

6d



8d



10d

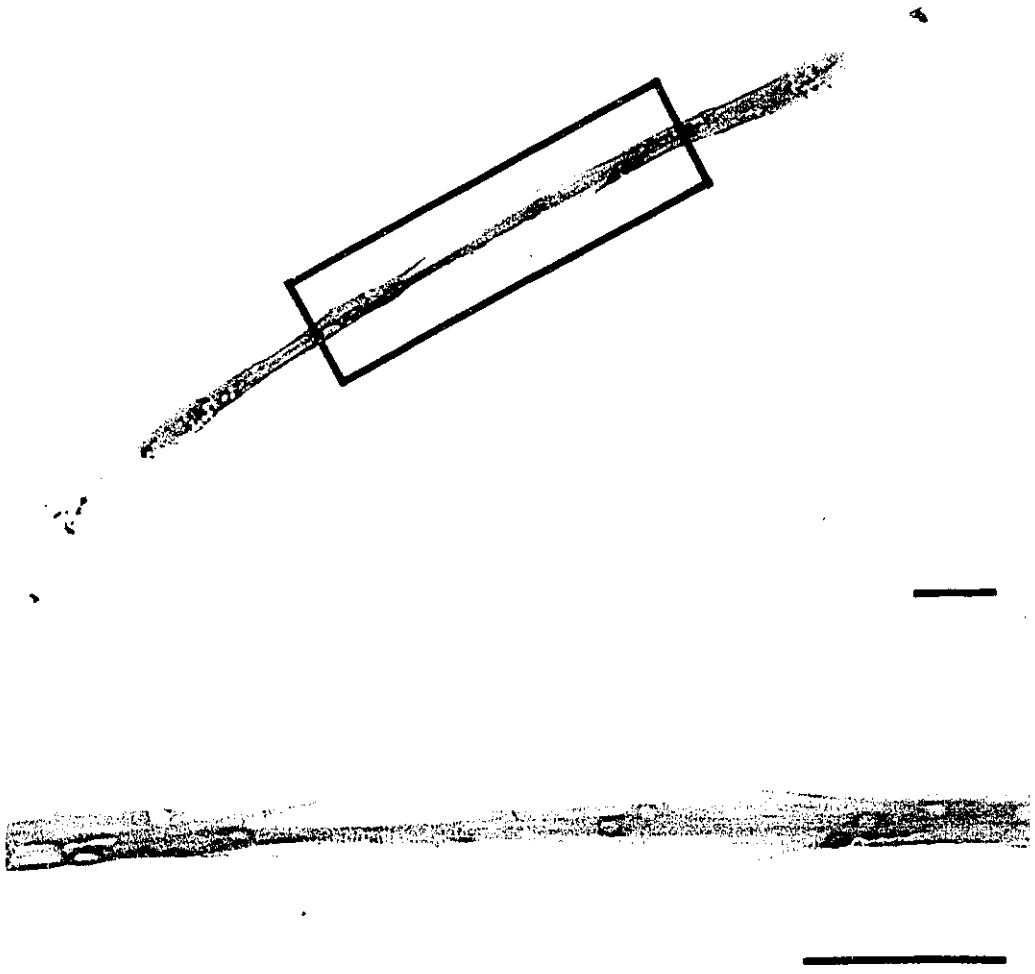


Figure 4.1.3.2 (B) The same culture as in (A) fixed following 10 days in culture. Immunohistochemical labelling for sarcomeric myosin (using the monoclonal antibody, MF-20; nuclei counterstained with hematoxylin) reveals the new myotube within this basal lamina sheath. BAR = 100 μm.

Figure 4.1.3.3 Photomicrograph of the progeny derived from satellite cells contained within a single intact myofibre. The culture was grown in Basal Medium for 10 days, fixed, stained with MF-20/DAB immunohistochemistry, and the nuclei counterstained with hematoxylin. This culture demonstrated the typical morphology throughout the culture period as described in Figure 4.1.3.1. Virtually all of the cells have fused by seven days and stain positively for sarcomeric myosin. There remain only a few round- and spindle-shaped cells, some of which are myosin-positive. BAR = 200 μm.

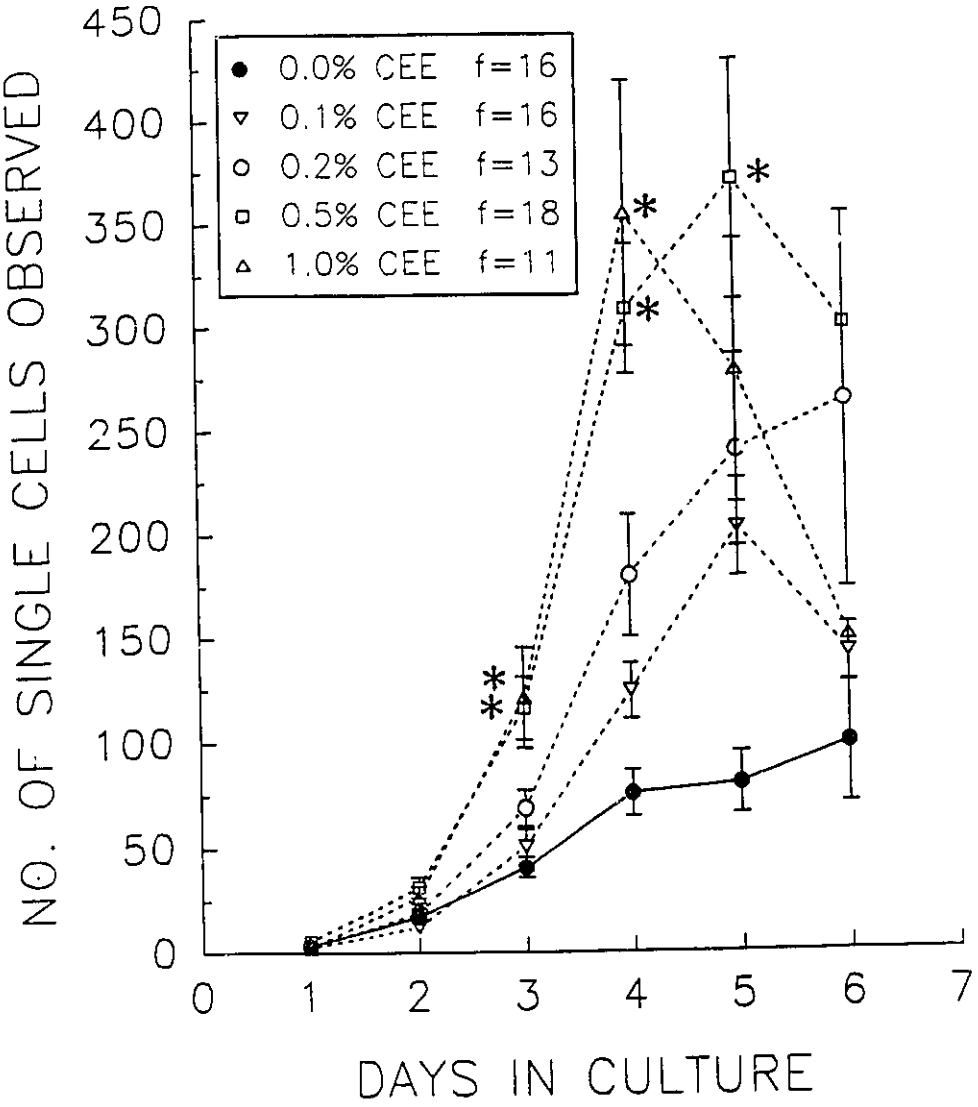


Figure 4.1.3.4 Proliferation curves of myogenic precursor cells as a function of chick embryo extract (CEE) concentration. All cultures were grown in the presence of 10% horse serum. Bars represent standard error of the mean number of single cells scored from *f* satellite cell colonies. Data were combined from 5 experiments (5 mice).

* : significantly different from value for Basal Medium (BM) at the same day in culture (Kruskall-Wallis one way ANOVA on ranks; $p<0.05$).

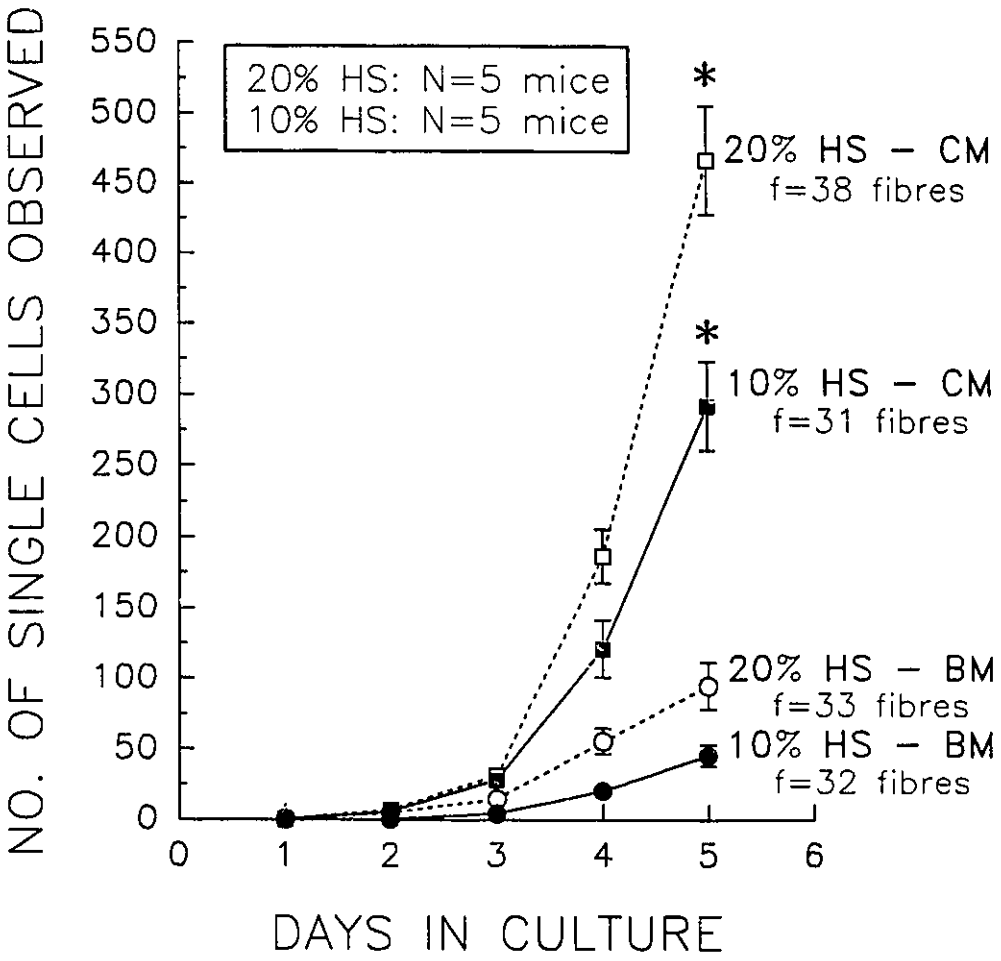


Figure 4.1.3.5 Daily single cell counts of satellite cell colonies derived from single myofibres grown in various medium conditions. Bars represent standard error of the mean of *f* colonies, combined from N mice. Statistical analysis was done only on those counts taken on the 5th day in culture. 10% horse serum (HS) is not significantly different from 20% HS (Kruskal-Wallis one way ANOVA on ranks).

* : significantly different from value for Basal Medium (BM) at the same day in culture (Kruskal-Wallis one way ANOVA on ranks and Dunn's method of pairwise M.C.T.; $p<0.05$).

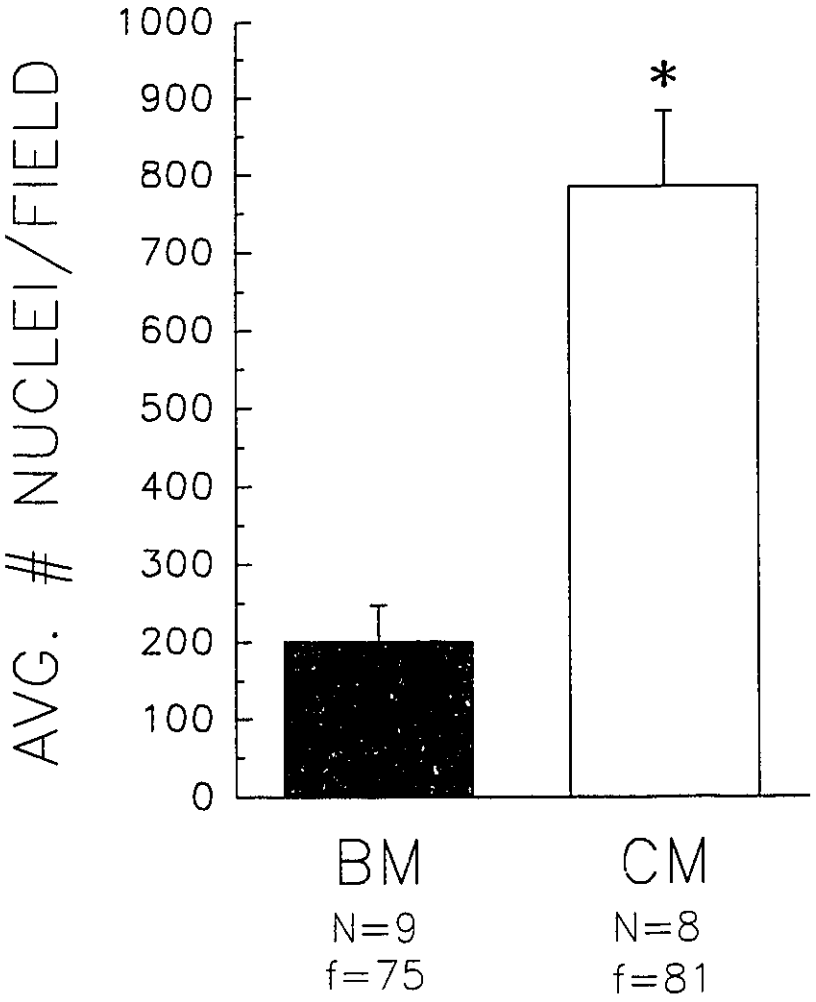


Figure 4.1.3.6 Assessment of myogenicity of satellite cell colonies derived from single myofibres at 7 days. Counts were obtained as number of nuclei/standard area from the centre of a fixed and hematoxylin stained colony. Cultures were grown in Basal Medium (BM) containing 20% horse serum or in CEE Medium (CM) containing 20% horse serum and 0.5% CEE. Bars represent mean ± standard error of the mean of N experiments. In total, *f* satellite cell colonies derived from single myofibres were scored.

* : significantly different from value for BM (paired student t-test; $p<0.05$)

4.2 Effects of bFGF on satellite cell growth and development

4.2.1 Effects of bFGF on satellite cells from young normal mice

All satellite cell colony data presented in this section were derived from single fibres from young normal mice. All cultures were grown in medium containing 20% horse serum, and any additional components as indicated. For all results in this section counts were obtained as number of nuclei/standard area from the centre of a colony that was fixed and stained with MF-20 and hematoxylin following 7 days in culture.

Satellite cells derived from young mice proliferated in a dose dependent manner to bFGF as shown in Figure 4.2.1.1, panel A.

Effects of bFGF on the differentiation of satellite cells derived from single myofibres was assessed by counting those nuclei contained in cells which stained positively for MF-20 as a percent of the total nuclei. Those cells which expressed sarcomeric myosin (MF-20 positive) were assumed to be differentiated, while MF-20 negative cells were assumed to still be undifferentiated. Figure 4.2.1.1, panel B demonstrates that with increasing bFGF concentration there were proportionally fewer nuclei contained within cells which expressed sarcomeric myosin.

In order to demonstrate that we were able to inhibit bFGF activity we tested for the effective dose of a neutralizing antibody for bFGF, αbFGF (R&D Systems, Minneapolis, MN). The effects of crushed muscle extract (CME) on the proliferation of satellite cells from normal and dystrophic mice would be clearer if any potential bFGF effects were inhibited. As discussed in Section 2.8 (Crushed muscle extract studies), it has been suggested that CME contains some bFGF. αbFGF elicited a dose-dependent inhibitory response in satellite cell cultures derived from single adult myofibres grown in the presence of 1.0 ng/ml bFGF (see Figure 4.2.1.2). It appears that αbFGF concentrations of 5 μg/ml or greater reduced the mitogenic effects of 1.0 ng/ml bFGF by more than 50%. Statistical analysis was not

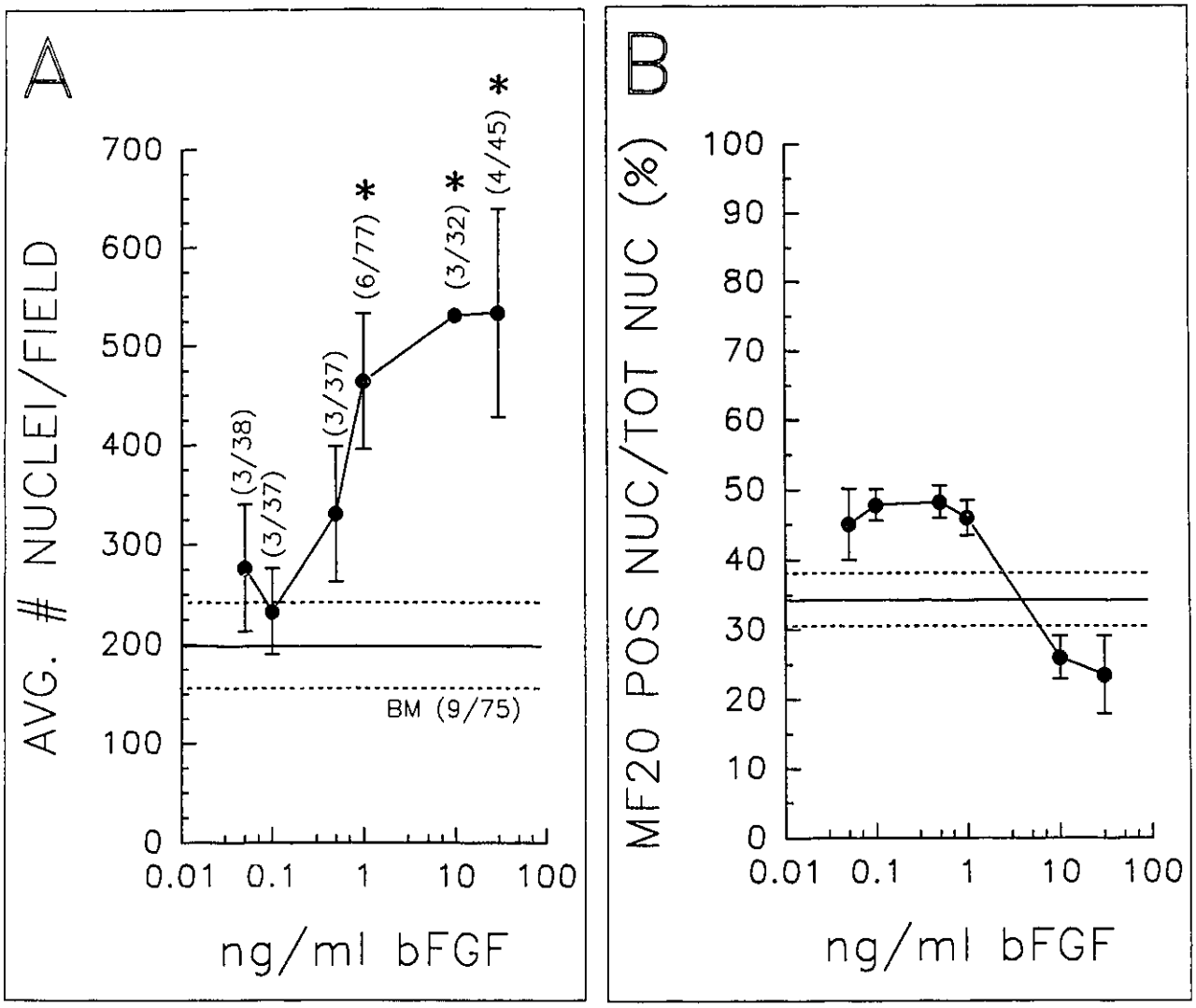
performed since the N values were too low, however an obvious pattern of bFGF inhibition, dependent on αbFGF concentration, could be seen.

Figure 4.2.1.1

(A) bFGF dose response curve of satellite cells derived from young mice. Bars represent mean ± standard error of the mean of N experiments. In total, *f* colonies were scored. Numbers in parentheses represent (N/*f*). Basal Medium (BM) -induced level of proliferation is represented by the solid (mean of N experiments) and broken (standard error of the mean) lines.

* : Significantly different from Basal Medium (BM; one way ANOVA and Dunnett's method of M.C.T.; $p<0.05$).

(B) bFGF effect on the differentiation of satellite cells derived from young mice. Bars represent mean ± standard error of the mean percent of nuclei within MF-20 positive cells per total number of nuclei scored from N experiments. The Basal Medium (BM) -induced value is represented by the solid (mean of N experiments) and broken (standard error of the mean) lines. With increasing bFGF concentration there were proportionally fewer nuclei contained within cells which were MF-20 positive.


A
AVG. # NUCLEI/FIELD
700
600
500
400
300
200
100
0
(3/38)
(3/37)
(3/37)
(6/77) *
(3/32) *
(4/45) *
BM (9/75)
0.01
0.1
1
10
100
ng/ml bFGF
B
MF20 POS NUC/TOT NUC (%)
100
90
80
70
60
50
40
30
20
10
0
0.01
0.1
1
10
100
ng/ml bFGF

93. A

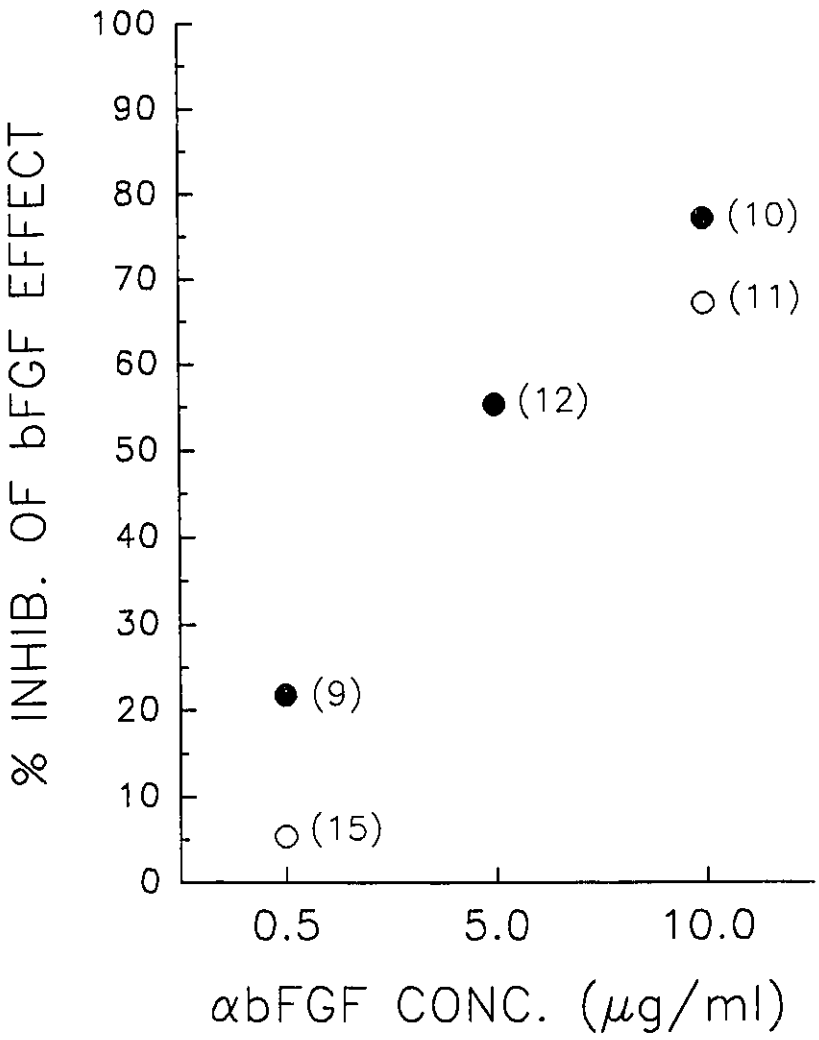


Figure 4.2.1.2 Percent inhibition of the effect of bFGF in basal medium by the neutralizing antibody, *α*bFGF. All cultures were grown in the presence of 1.0 ng/ml bFGF dissolved in basal medium (containing 20% horse serum). *α*bFGF was added to the media prior to feeding the plated fibres. Similar symbols represent cultures from the same experiment. Statistical analysis was not performed since the N value was low, however the *f* value, representing the number of fibres scored for each experiment, is shown.

4.2.2 Influence of age of mice on the effect of bFGF

All satellite cell colony data presented in this section were from single myofibres from young normal or old normal mice, as indicated. All cultures were grown in medium containing 20% horse serum, and any additional components as indicated. For all results in this section counts were obtained as number of nuclei/standard area from the centre of a colony that was fixed and stained with MF-20 and hematoxylin following 7 days in culture.

Two variables may be assessed when studying the regeneration of muscle by growing satellite cells in culture which originate from single myofibres. Firstly, it may be possible that the ability to regenerate may depend on the proportion of myofibres which contain viable satellite cells. Furthermore, the ability to regenerate may also depend on the capacity of those viable satellite cells to proliferate. Therefore, when appropriate, these two variables were assessed; the former by counting the percentage of single fibres plated from which satellite cell proliferation occurred, and the latter by assessing the proliferation potential (number of nuclei) of those satellite cell colonies which were observed. These two variables were assessed separately to simplify interpretation of the results (see Discussion for more detail).

Figure 4.2.2.1 shows the proportion of single myofibres plated which contained satellite cell colonies after 7 days in culture. The proliferation potential of those colonies is shown in Figure 4.2.2.2. Both graphs revealed statistically significant differences between young and old mice (two way ANOVA's; $p<0.05$). Therefore, in this culture system, young and old mice differed with respect to both the number of myofibres which gave rise to satellite cell proliferation and the proliferative capacity of those satellite cells and their progeny.

Further analysis of both figures reveals the conditions under which these differences existed. Figure 4.2.2.1 demonstrates that fibres derived from young mice were more likely to exhibit satellite cell proliferation in CEE Medium (CM)

than when cultured in Basal Medium (BM). Fibres derived from old mice showed satellite cell growth with a similar frequency regardless of culture medium used. It is also interesting to note that fibres derived from old mice showed growth with a frequency similar to that seen in cultures of fibres derived from young mice grown in BM. In CM, however, colonies were seen more frequently from myofibres from young mice than from myofibres from old mice. This suggests that there are fewer satellite cells which are available, or which have the ability, to proliferate in myofibres from old mice.

Figure 4.2.2.2 shows that within the same age group CM enhanced satellite cell proliferation. However, when comparing young to old mouse satellite cells, those derived from old mice exhibited a significantly lower proliferation potential in the presence of CM than those from young mice. In the presence of BM, no significant differences in the proliferation potential were observed between satellite cells derived from young as compared to old mice. This suggests that, in this culture system, satellite cells derived from old mice have a lower capacity for proliferation than those derived from young mice.

Further evidence to support this finding may be revealed through using other known myogenic growth factors, such as bFGF, to induce proliferation of, and compare the effects on, satellite cells derived from single myofibres from young and old mice. Figure 4.2.2.3 reveals differences between bFGF dose response curves generated for young and old normal mice. The most striking difference was the inability of satellite cells derived from old mice to proliferate beyond a certain number of cells (313 ± 36), despite high concentrations of bFGF (≥ 1.0 ng/ml bFGF). This again suggests that satellite cells from old mice have a lower capacity for proliferation than those from young mice. With increased bFGF concentrations the proliferation of satellite cells derived from young mice increased to levels significantly higher than that induced by BM. In satellite cell cultures derived from old mice similar numbers of nuclei were seen with bFGF concentrations less than 1.0 ng/ml when compared to those derived from young mice. However, with bFGF

concentrations of 1.0 ng/ml, or greater, there were significantly fewer nuclei present as compared to satellite cell cultures derived from young mice. Furthermore, except for those cultures grown in 10.0 ng/ml bFGF, bFGF failed to induce satellite cell proliferation to levels significantly higher than that seen in the presence of BM.

It is important to note that the proliferation potential of satellite cells from old mice, in the presence of Basal Medium, though not significantly different, is half that of satellite cells from young mice. If old mice have fewer satellite cells per fibre, perhaps due to a progressive decrease in the number of viable satellite cells with ageing which would eventually result in some fibres having no viable satellite cells, then we might expect the result seen in Figure 4.2.2.1. This could also explain the decrease in the number of cells derived per myofibre since, for example, if there was only one satellite cell in the old myofibre and two in the young then one would expect to see half as many cells possible even if the proliferative capacity of all satellite cells is identical.

Similar to the analysis on satellite cell cultures derived from young mice (see Section 4.2.1), the bFGF effect on differentiation of satellite cells derived from old mice was assessed (see Figure 4.2.2.4). Similar to satellite cells derived from young mice, there was a decrease in the proportion of nuclei contained within MF-20 positive cells as bFGF concentration was increased.

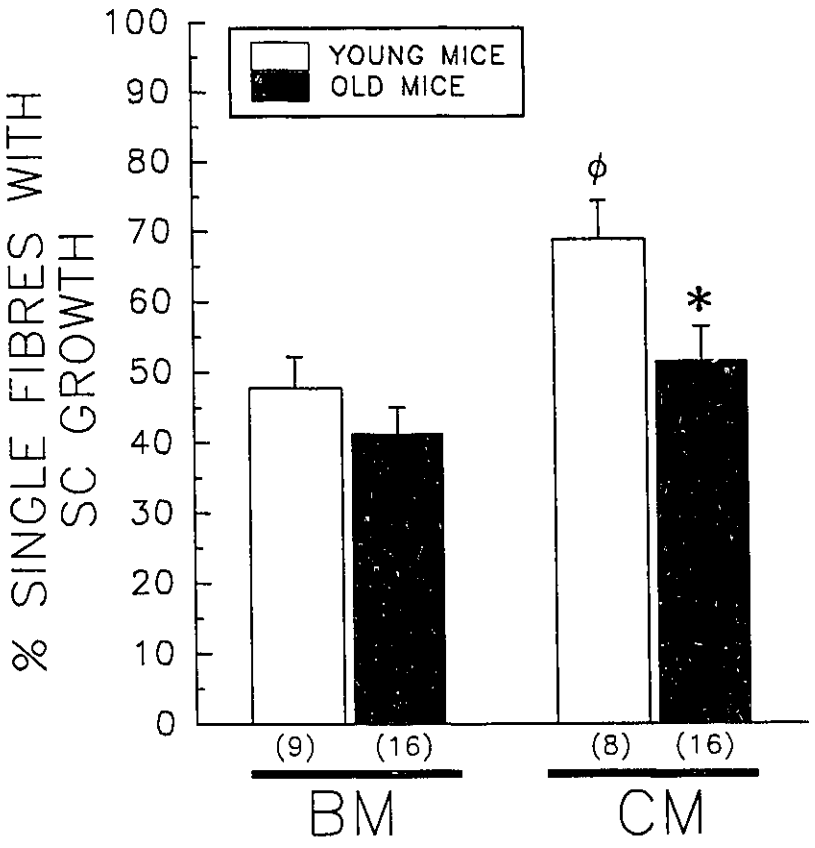


Figure 4.2.2.1 The percentage of single fibres from which satellite cell (SC) proliferation was observed as a function of media type and mouse age. Bars represent mean ± standard error of the mean of N experiments for each age group and media condition. There was no significant interaction between media type and mouse age (two way ANOVA).

ϕ : Significantly different from Basal Medium (BM) value within the same age group (two way ANOVA and Student-Newman-Keuls M.C.T.; $p<0.05$).

* : Significantly different from young mouse value within the same media treatment group (two way ANOVA and Student-Newman-Keuls M.C.T.; $p<0.05$).

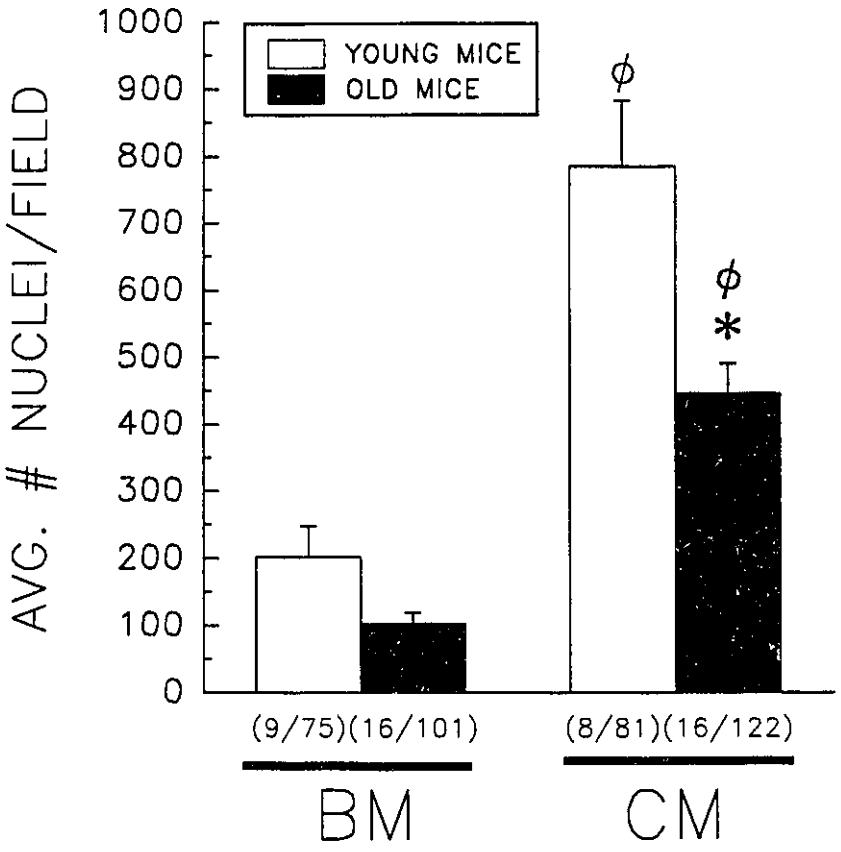


Figure 4.2.2.2 The effect of media type on the proliferation potential of satellite cells derived from young and old normal mice. Bars represent mean ± standard error of the mean from N experiments. A total of *f* single fibre colonies were scored. Numbers in parentheses represent (N/*f*). There was a significant interaction between mouse age and media condition (2 way ANOVA; $p<0.05$).

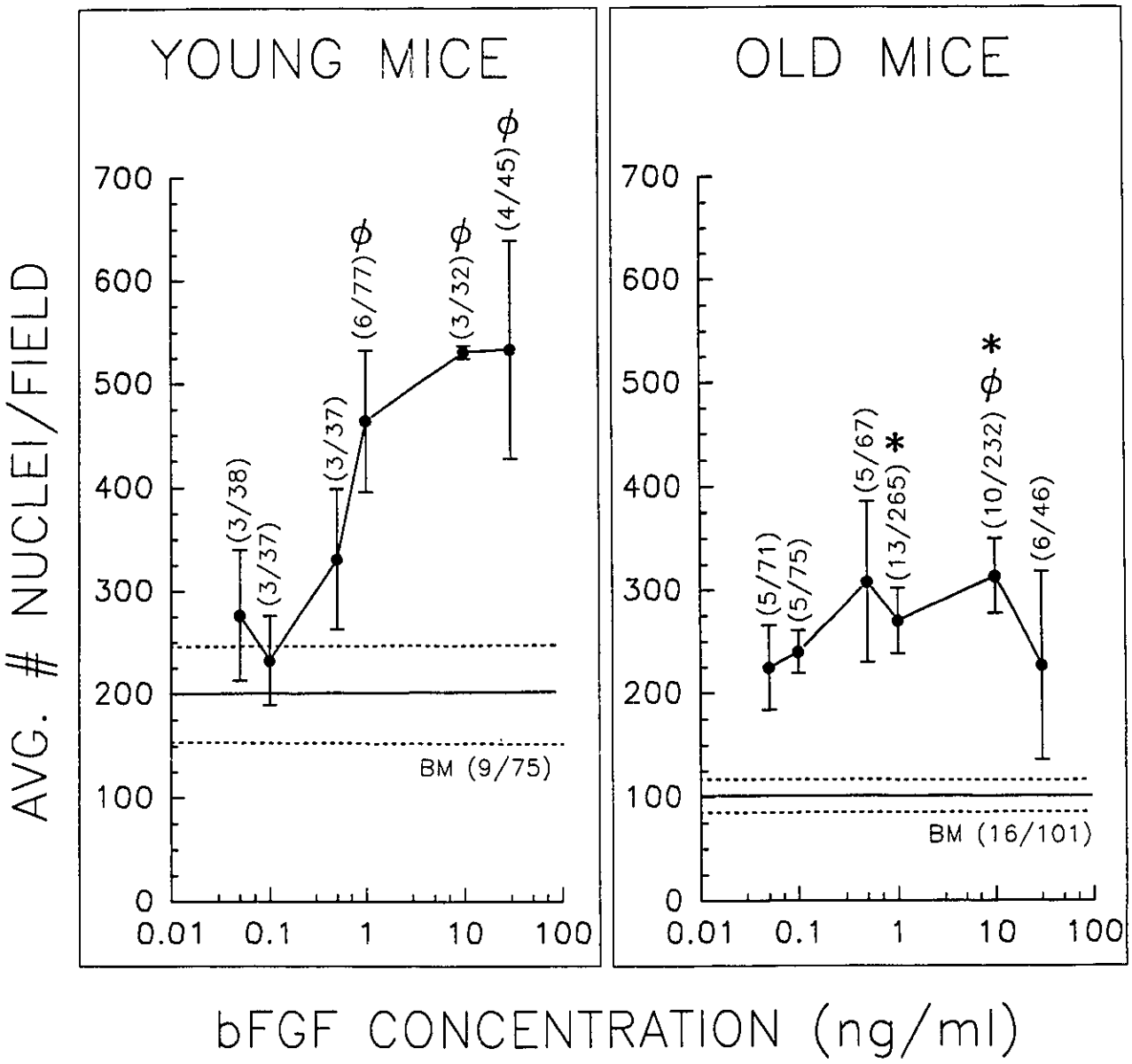
ϕ : Significantly different from Basal Medium (BM) value within the same age group (two way ANOVA and Student-Newman-Keuls M.C.T.; $p<0.05$).

* : Significantly different from young mouse value within the same media treatment group (two way ANOVA and Student-Newman-Keuls M.C.T.; $p<0.05$).

Figure 4.2.2.3 Comparison of bFGF dose response curves of satellite cells derived from young and old normal mouse single myofibres. Bars represent mean ± standard error of the mean of N experiments. A total of *f* colonies were scores. Numbers in parentheses represent (N/*f*). Basal Medium (BM) -induced level of proliferation is represented by the solid (mean of N experiments) and broken (standard error of the mean) lines. Age of the donor mouse had a significant effect on the growth response of the satellite cells derived from those mice (two way ANOVA; $p<0.05$). No significant interaction between donor mouse age and bFGF concentration was found (two way ANOVA).

ϕ : Significantly different from Basal Medium (BM) value within the same age group (one way ANOVA and Dunnett's method of M.C.T.; $p<0.05$).

* : Significantly different from young mouse value within the same media treatment group (paired student t-test; $p<0.05$).


YOUNG MICE
OLD MICE
AVG. # NUCLEI/FIELD
700
600
500
400
300
200
100
0
0.01
0.1
1
10
100
(3/38)
(3/37)
(3/37)
(6/77)
(3/32)
(4/45)
BM (9/75)
(5/71)
(5/75)
(5/67)
(13/265)
(10/232)
(6/46)
BM (16/101)
bFGF CONCENTRATION (ng/ml)

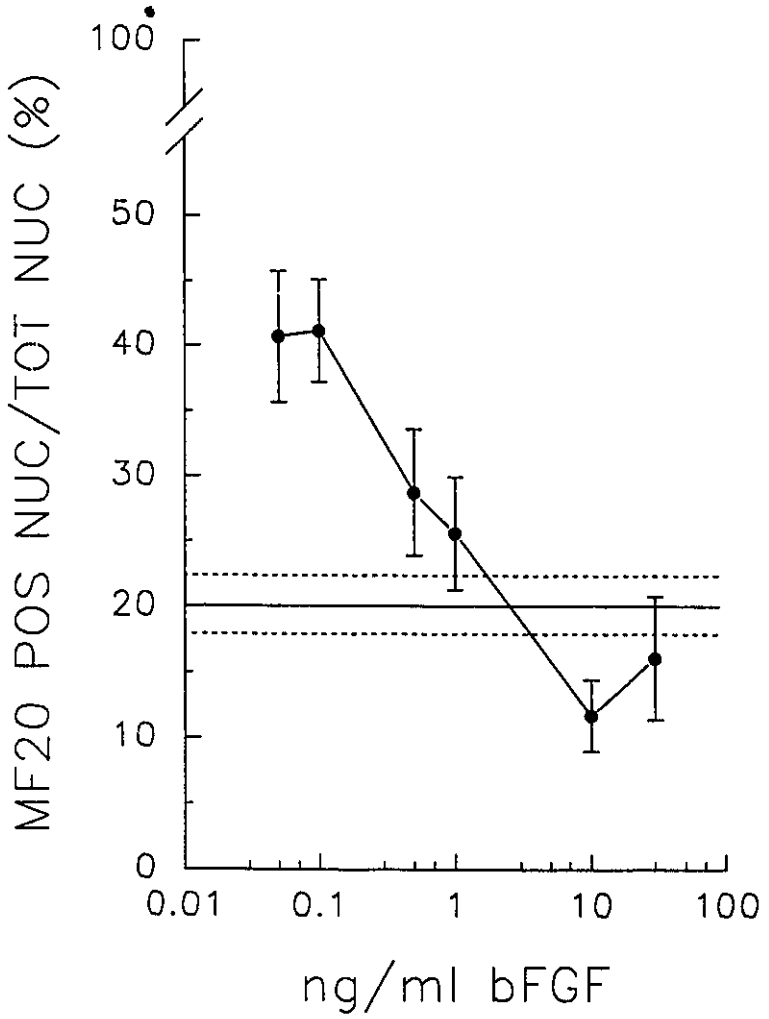


Figure 4.2.2.4 bFGF effects on the differentiation of satellite cells derived from old mice. Bars represent mean ± standard error of the mean percent of nuclei within MF-20 positive cells per total number of nuclei scored from N experiments. With increasing bFGF concentration there were proportionally fewer nuclei contained within cells which were MF-20 positive.

4.3 Proliferation and differentiation of satellite cells derived from dystrophic, dy^{2J} and mdx, mice

As discussed in Section 2.9 (The dy, dy^{2J} and mdx mouse models of muscular dystrophy), there appears to be much less regeneration in dy^{2J} mice older than six months than in age-matched controls. Since we were interested in the possible causes of the loss of the regenerative capacity of muscle in old dy^{2J} mice, old mice were used as the source for muscle in all experiments which addressed this question. Therefore, all satellite cell colony data presented in this section were from single myofibres from old normal (control group), old dy^{2J}, or old mdx mice as indicated. All cultures were grown in medium containing 20% horse serum, and any additional components as indicated. For all results in this section counts were obtained as number of nuclei/standard area from the centre of a colony that was fixed and stained with MF-20 and hematoxylin following 7 days in culture.

Histological examination of flexor digitorum brevis muscle from old normal, old dy^{2J} and old mdx mice revealed extensive fibre atrophy and fibrosis in both dystrophic genotypes (see Figure 4.3.1).

Morphologically, those satellite cell cultures derived from single fibres from dy^{2J} and mdx mice appear similar to age-matched normal mice (see Figure 4.3.2). No obvious abnormalities in shape or size of either the satellite cell progeny or the myotubes are apparent.

As in Section 4.2.2, the two variables which may affect the ability of muscle to regenerate were assessed; the proportion of myofibres which contained viable satellite cells, and the proliferation potential of those viable satellite cells.

Figure 4.3.3 clearly demonstrates that the percentage of fibres which contained mitotically viable satellite cells was significantly lower in old mdx and old dy^{2J} mouse fibres than those from old normal mice. Except for fibres derived from mdx mouse which were cultured in the presence of bFGF, there were no significant differences among the various media conditions as compared to Basal Medium values. Despite the differences in media for mdx mice a clear pattern was evident for

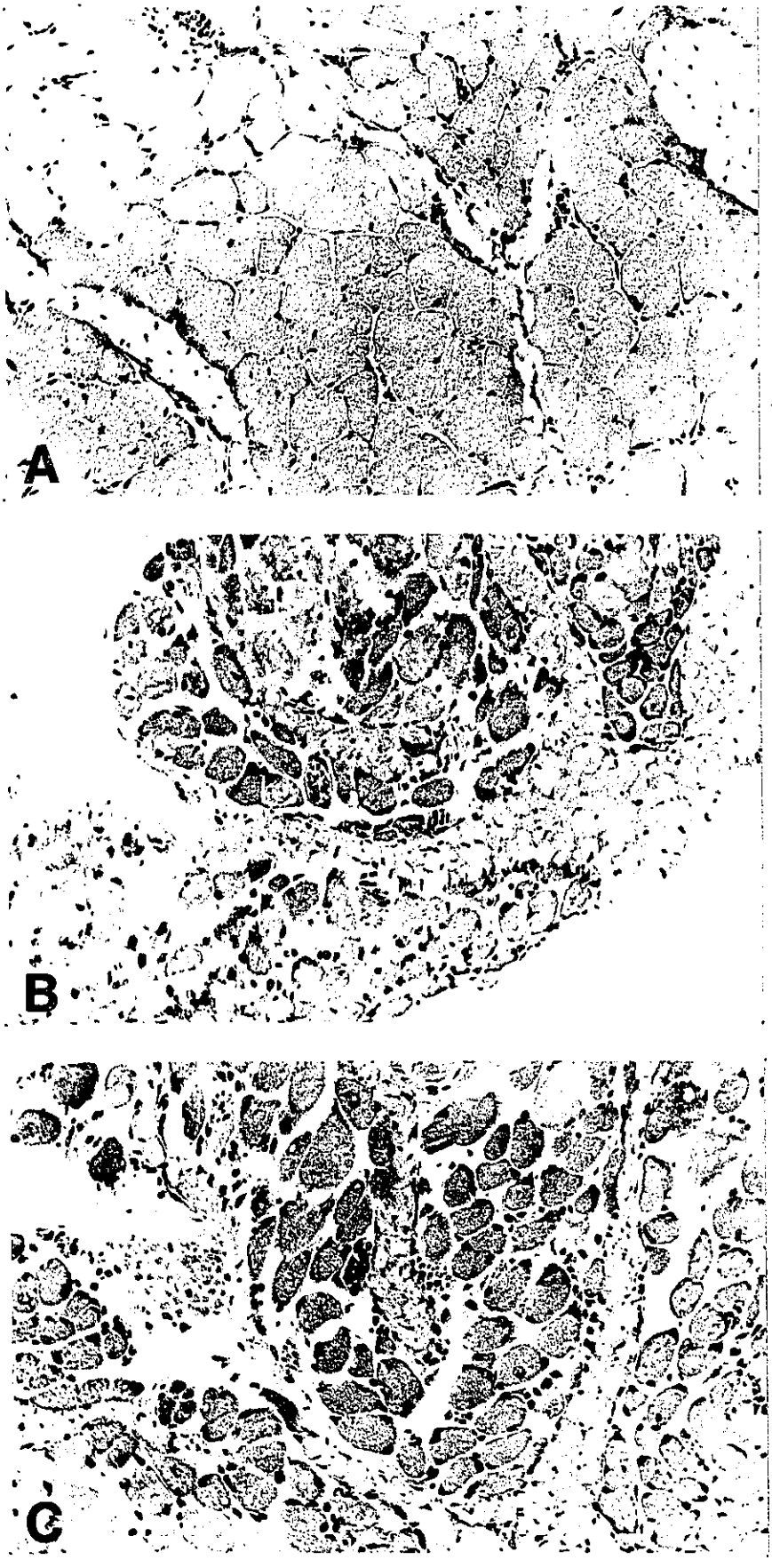
the different mouse genotypes: the proportion of fibres which contained viable satellite cells was greatest when the fibres were derived from old normal mice and least when they were derived from old dy^{2J} mice.

Those cultures which exhibited satellite cell proliferation were analysed for their proliferation potential as a function of media type and mouse genotype (see Figure 4.3.4). Statistical analysis was difficult to perform since so few fibres from mdx and dy^{2J} genotypes showed satellite cell proliferation. It would appear that those satellite cells from old dy^{2J} mice, which remain capable of proliferating do so with a similar (in the presence of CEE Medium, Basal Medium or 10.0 ng/ml bFGF) or greater (in the presence of 1.0 ng/ml bFGF) proliferation potential to those from old normal mice. Satellite cells derived from mdx mice, which remain capable of proliferating, show similar proliferation potentials to old mouse satellite cells in the presence of Basal Medium and 1.0 ng/ml bFGF. However, in the presence of CEE Medium or 10.0 ng/ml bFGF their proliferation potential is lower than that of old mouse satellite cells under similar conditions. Another difference between the proliferation potential of satellite cells derived from mdx mouse and the two other genotypes, normal and dy^{2J}, is that it does not vary with media type present in the mdx mouse, however the proliferation potential of normal and dy^{2J} mouse satellite cells does depend on the media type present.

As discussed briefly in Results Section 4.1.3, the plated myofibres occasionally remained viable for longer and were replaced by myotubes growing within the basal lamina sheath as shown in Figure 4.1.3.2 (Panel A). The frequency of this phenomenon was not affected by the type of medium present in the culture (split plot two way ANOVA; $p<0.05$), therefore for each mouse genotype data from all medium conditions tested were combined (see Figure 4.3.5). Single myofibres were replaced by a single myotube most frequently with fibres isolated from old mdx mice, and very infrequently in young normal mouse myofibres. However, the replacement myotubes had similar numbers of nuclei despite originating from different mouse genotypes or being grown in different media (except for satellite

cells derived from old dy^{2J} mice grown in the presence of CEE Medium, see Figure 4.3.6).

Figure 4.3.1 Histology of a cross-section of the flexor digitorum brevis muscle of (**A**) old normal mouse, (**B**) old dy^{2J} mouse, and (**C**) old mdx mouse, as revealed by hematoxylin (nuclei) and eosin (cytoplasm) staining. BAR = 50 μm.



IO5. A

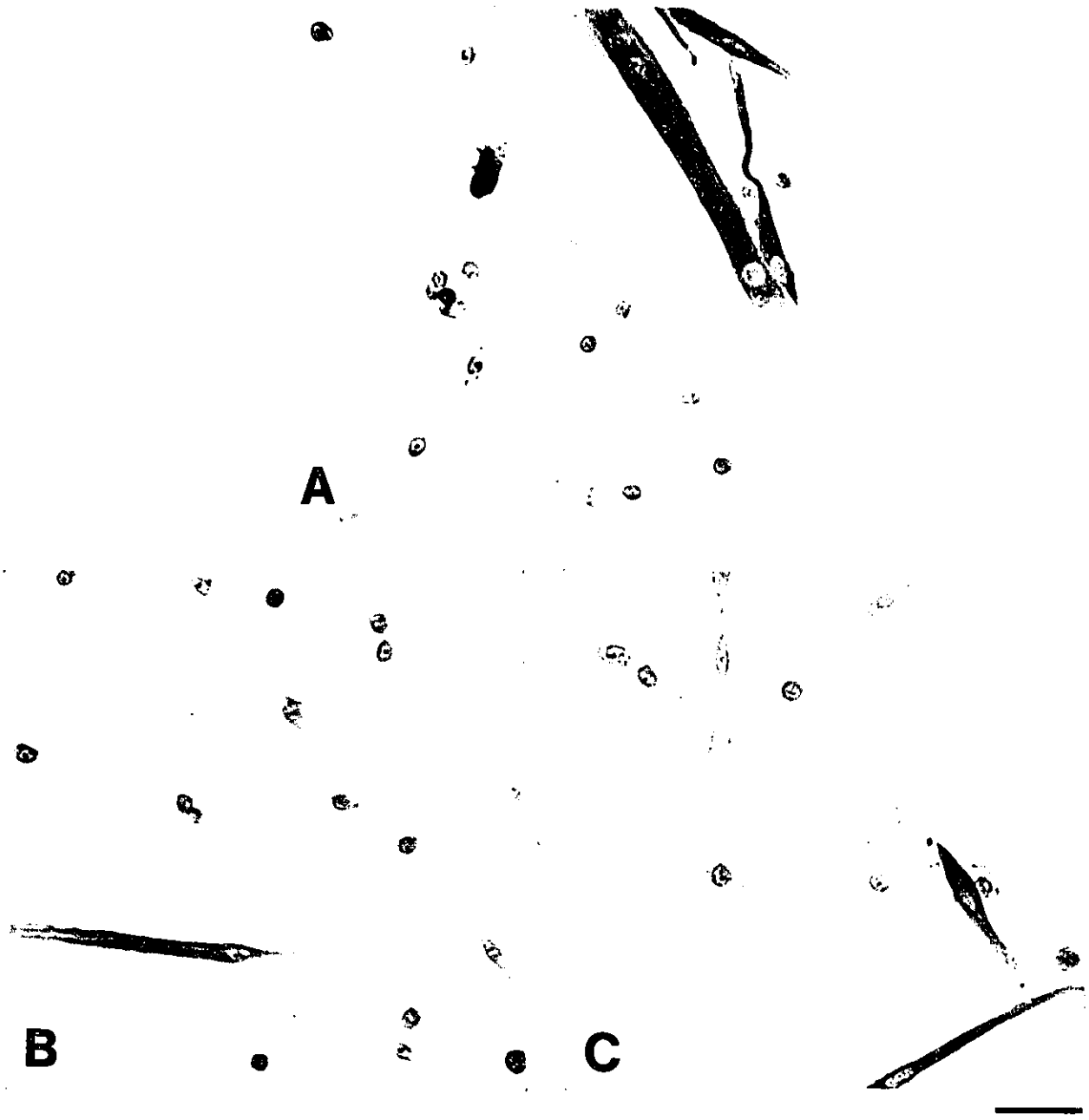


Figure 4.3.2 Satellite cell cultures, derived from single fibres, fixed and stained following 7d in culture from (A) old normal, (B) old dy^{2J}, and (C) old mdx mice. All cultures were grown in Basal Medium. All cultures show immunohistochemical labelling against sarcomeric myosin and hematoxylin stain for all nuclei.
BAR = 50 μm.

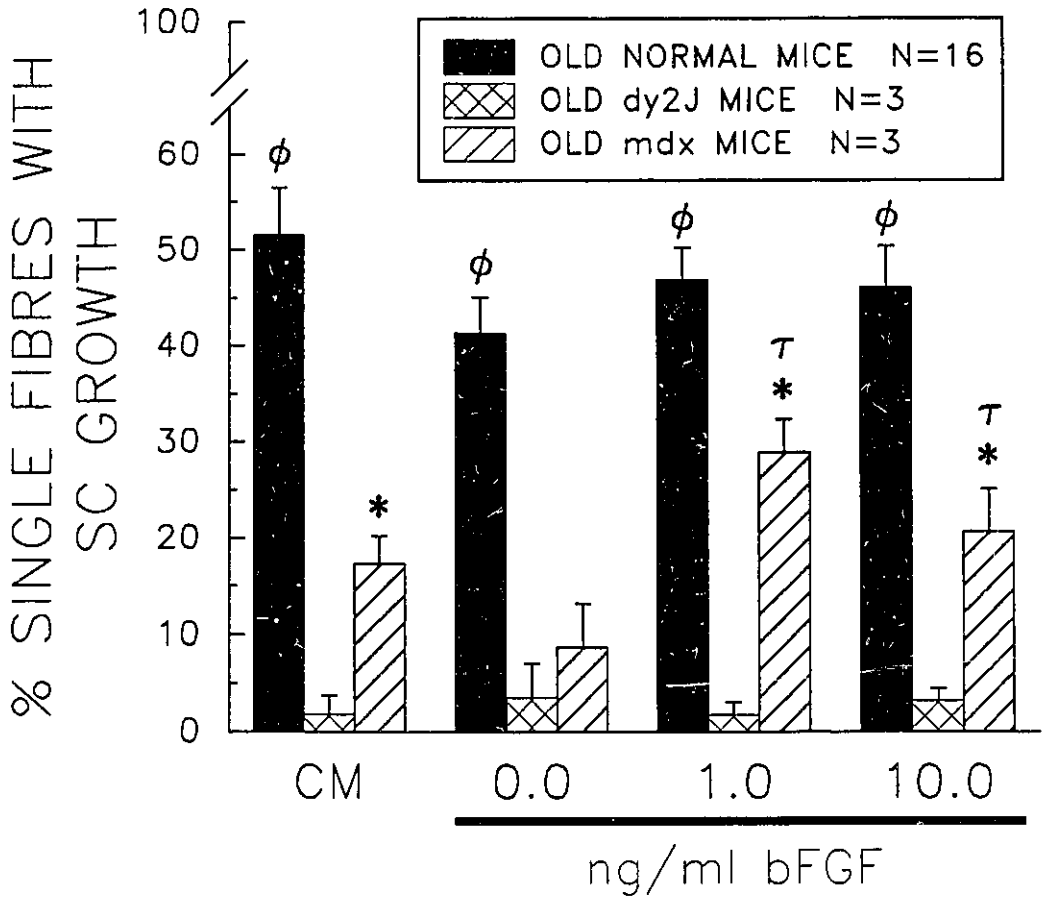


Figure 4.3.3 The percentage of single fibres from which satellite cell (SC) proliferation occurred as a function of media type and mouse genotype. Bars represent mean ± standard error of the mean of N experiments for each age group and media type. The data did not fit a normal distribution, therefore a log transformation of the data was performed.

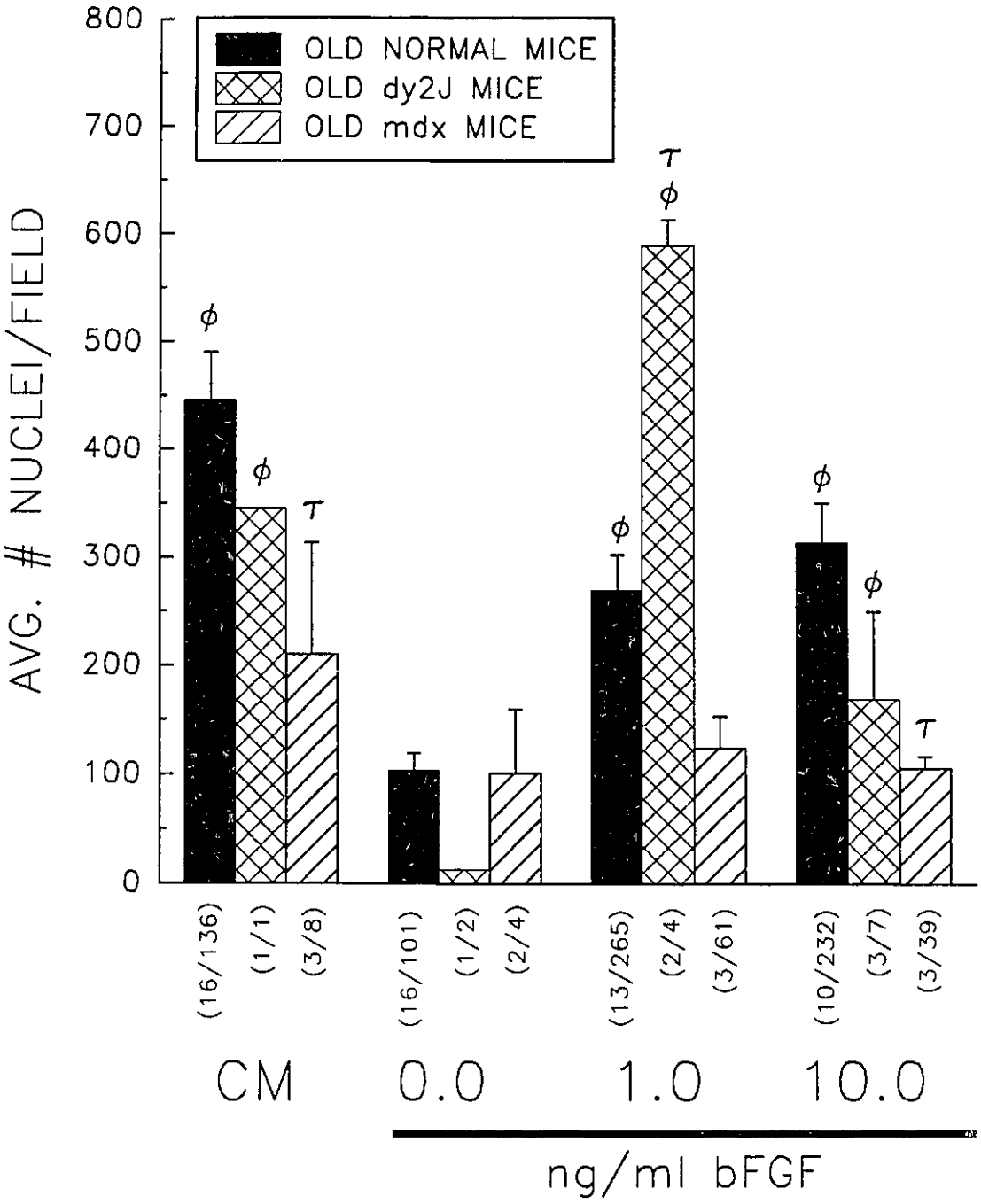
ϕ : Significantly different from dy^{2J} and mdx values within the same media type (split plot two way ANOVA and M.C.T.; $p<0.05$).

* : Significantly different from dy^{2J} values within the same media type (split plot two way ANOVA and M.C.T.; $p<0.05$).

τ : Significantly different from Basal Medium (0.0 ng/ml bFGF) values within the same mouse genotype (split plot two way ANOVA and M.C.T.; $p<0.05$).

Figure 4.3.4 The proliferation potential of satellite cells as a function of media type and mouse genotype. Bars represent mean ± standard error of the mean of N experiments for each age group and media condition. A total of *f* single fibre-derived satellite cell colonies were scored. Numbers in parentheses represent (N/*f*). The data did not fit a normal distribution, therefore a log transformation of the data was performed. There was a significant interaction between mouse genotype and media type (split plot two way ANOVA; $p<0.05$).

ϕ : Significantly different from Basal Medium (0.0 ng/ml bFGF) within the same mouse genotype (split plot two way ANOVA and M.C.T.; $p<0.05$).
τ : Significantly different from old normal mouse values within the same media type (split plot two way ANOVA and M.C.T.; $p<0.05$).


OLD NORMAL MICE
OLD dy2J MICE
OLD mdx MICE
AVG. # NUCLEI/FIELD
800
700
600
500
400
300
200
100
0
(16/136)
(1/1)
(3/8)
(16/101)
(1/2)
(2/4)
(13/265)
(2/4)
(3/61)
(10/232)
(3/7)
(3/39)
CM
0.0
1.0
10.0
ng/ml bFGF

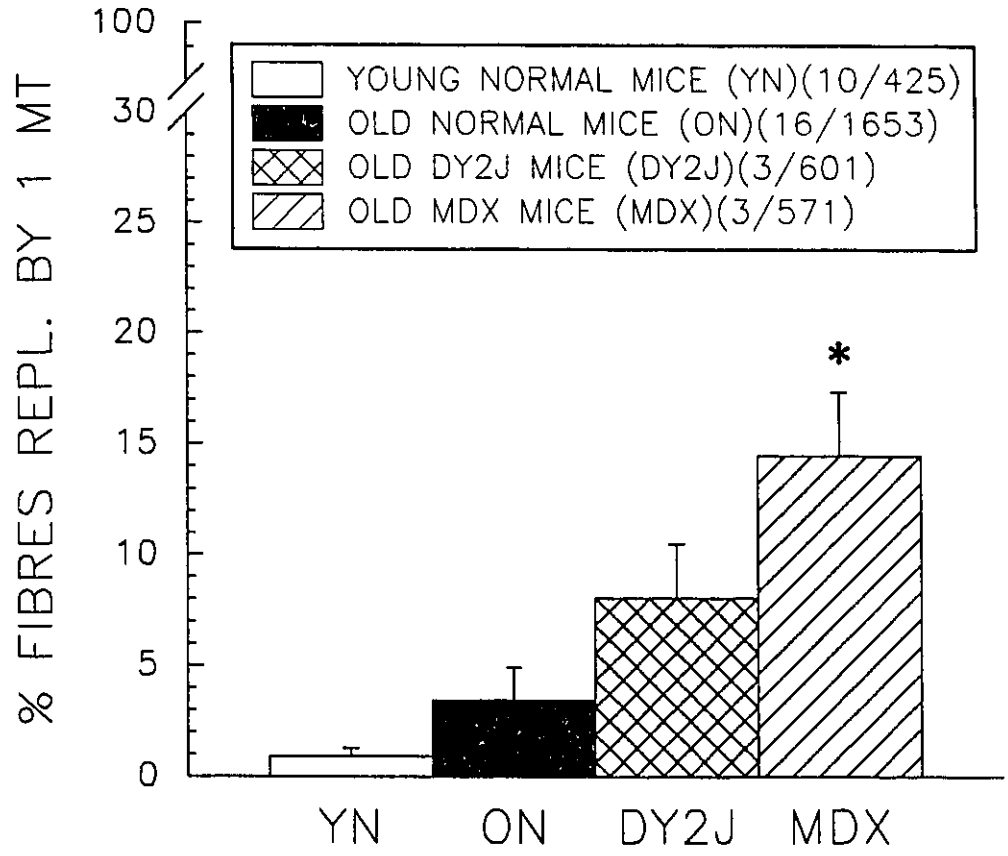


Figure 4.3.5 The proportion of plated fibres replaced by a single myotube growing within the original fibre basal lamina sheath. For each mouse genotype the data from all media conditions tested (CM, BM, 1.0 ng/ml bFGF and 10.0 ng/ml bFGF) were combined. Bars represent mean ± standard error of the mean of N experiments. Numbers in parentheses represent (N/t), where t is the total number of single fibres scored (with or without single myotube replacement of the myofibre) for the analysis.

* : Significantly different from young normal mouse genotype (Kruskall-Wallis one way ANOVA on ranks and Dunn's method of M.C.T.; $p<0.05$).

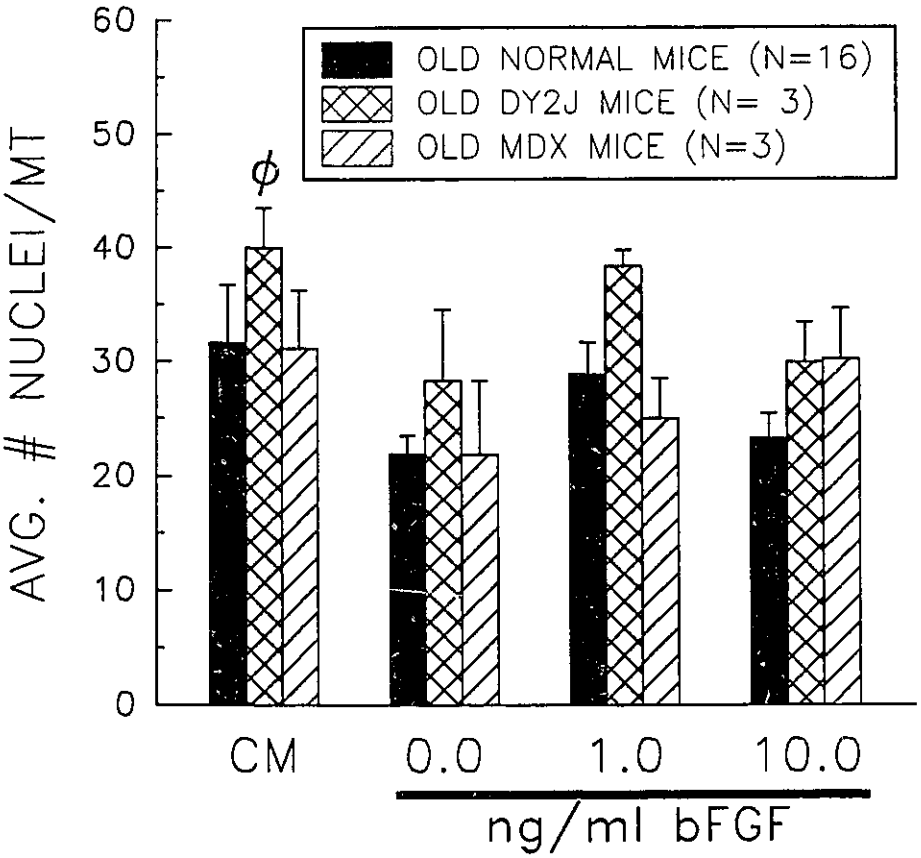


Figure 4.3.6 Average number of nuclei per single myotube which replaced the parent fibre. All cultures were grown in the indicated medium for 7 days. Bars represent mean ± standard error of the mean of N experiments. No significant interaction was found between media type and mouse genotype (split plot two way ANOVA). The data did not fit a normal distribution, therefore a log transformation of the data was performed. No significant differences were detected among values from different genotypes within the same media type (split plot two way ANOVA and Duncan's M.C.T.; $p<0.05$).

ϕ : Significantly different from Basal Medium (0.0 ng/ml bFGF) values within the same mouse genotype (split plot two way ANOVA and M.C.T.; $p<0.05$).

4.4 Effects of crushed muscle extract on single fibre-derived satellite cell growth and development

By using the method described in the Methods (Section 3.5), we were unable to obtain crushed muscle extract that could be used on this satellite cell culture system. This method and variations of it were originally described by Bischoff (1986b), Chen and Quinn (1992) and Johnson and Allen (1993). Slight modifications of the method following personal communication with L. Quinn improved the CME somewhat, however major problems remained such that it was not usable for testing the thesis questions as originally intended. Therefore, this section of the results is dedicated to a description of the CME we were able to obtain, the problems that it presented and our attempts at solving these problems.

Bischoff (1986b) extracted soluble proteins from crushed rat muscle in Dulbecco's Phosphate Buffered Saline (D-PBS) and concentrated the supernatant. This was the method initially used in this study, however three problems arose. Firstly, very little concentrate was obtained from one mouse which drastically limited the number of fibres, and their associated satellite cells, which could be tested per experiment (approximately 5 fibres/experiment). Secondly, all fibres cultured in the presence of CME turned black, usually by the third day in culture. This was suspected to be contamination of some sort. Thirdly, all culture wells containing CME developed a white precipitate which was spread throughout the media. The amount of white precipitate increased with increasing days in culture and obscured viewing of any round cell (satellite cell) growth that may have been occurring.

To improve the first problem we switched to a simpler method of CME preparation as described by Chen and Quinn (1992). This method allowed collection of a larger amount of CME, such that we were able to treat many multiwell plates of single fibres (the number of plates depending on the concentration of CME that was being tested). We realized that the white precipitate could be Ca^{2+}-phosphate. Ca^{2+} was probably released in large amounts from the crushed muscles used to make the CME, and was therefore free to bind with phosphate present in D-PBS. This was

confirmed when we observed that CME (simply the supernatant from crushed muscle extracted into D-PBS) left at 4°C for a few days also formed this precipitate. Use of Tris-buffered saline (TBS; Chen and Quinn, 1992) eliminated formation of the white precipitate, however the single myofibres still turned black after a few days in the presence of CME and yet another substance was seen forming in those cultures containing CME. This substance was also present in the D-PBS/CME cultures, however was often obscured by the white precipitate. The substance had a flattened, membranous, disc-like form (see Figure 4.4.1), appeared colourless, and increased in size with each day in culture. Many of these structures were seen in each well containing CME, and their growth was accompanied by an increase in "debris" covering the culture surface. By the end of 7 days in culture the "debris" covering the bottom of the culture well and the disc-like structures obscured satellite cell growth from view. Furthermore, the round cells that were seen often died by the end of the 7 day culture period. It was difficult to distinguish viable round-shaped cells and dead round-shaped cells from all of the "debris" surrounding them. This was the case even with cultures that had been fixed and stained for sarcomeric myosin expression and hematoxylin.

It was initially thought that the disk-like structures, "debris" and blackened fibres may be the result of some sort of bacterial or fungal infection. Various methods were tried to get rid of the "contamination"; such as boiling the CME (although it was quite likely that this would also eliminate any myogenic properties through denaturing the proteins), being very vigilant about using sterile culturing techniques and equipment, testing a different lot of sterile 0.22μm filters used for sterilizing liquids, irradiating the CME, using higher concentrations of penicillin-streptomycin and Fungizone than usual and using gentamycin, however none of these appraoches improved the "contamination" problem. CME from rat muscle was prepared and tested on single fibres from rat and mouse. The rat CME gave similar results to those obtained with the mouse CME. This eliminated the possibility that the mice being used to prepare the CME were suffering a systemic

infection which was somehow affecting the CME. A bacteriologist at the Children's Hospital of Eastern Ontario (CHEO) tested the CME, and the CME treated cultures; no bacterial, fungal or mold contamination was detectable. Further support for this conclusion was that cultures grown in the absence of CME (for example, in Basal Medium or CEE Medium), in wells adjacent to the CME wells, never showed any signs of the "contamination". One would expect a bacterial, fungal, mold or viral contamination to spread to adjacent culture wells.

The "contamination" was not a reaction between CME and the myofibre, since it developed with or without the presence of plated fibres. Nor was it a reaction with Matrigel, the substratum used to coat the culture surface of the wells, since it was also seen in untreated wells, wells coated with collagen, and wells coated with gelatin. There was no reaction between the horse serum contained within the medium, in fact with decreasing concentrations of horse serum there was an increase in the rate of "contamination" development.

Changing the medium every 2-3 days did not alleviate the problem as the "debris" clung to the Matrigel substratum, the single myofibre, and any round cells that were present.

It was possible that the "debris" and disk-like structures were clots, formed by various clotting agents found in blood. Blood was present in the muscles used for preparing the CME. In an attempt to eliminate this possiblity we tried to remove as much blood as possible from the mouse and its muscles by perfusing the mouse with saline solution prior to removal of the muscles for CME preparation. The mouse was perfused by a cardiac puncture with 1% heparin in D-PBS and 100 ml sterile D-PBS. Following the perfusion the muscles used for CME preparation were pale and did not appear to have any blood remaining in them. The cultures grown in CME prepared from perfused mice, however, still exhibited fibre blackening, and formation of "debris" and disk-like structures.

Various concentrations (according to protein content) of CME were tested, ranging from 25 μg/ml to 500 μg/ml. Chen and Quinn (1992) determined the

optimal concentration (results in the most myogenic cell proliferation) of mouse CME to be 240 μg/ml. As expected, increased CME concentration resulted in a larger amount of "contamination" present.

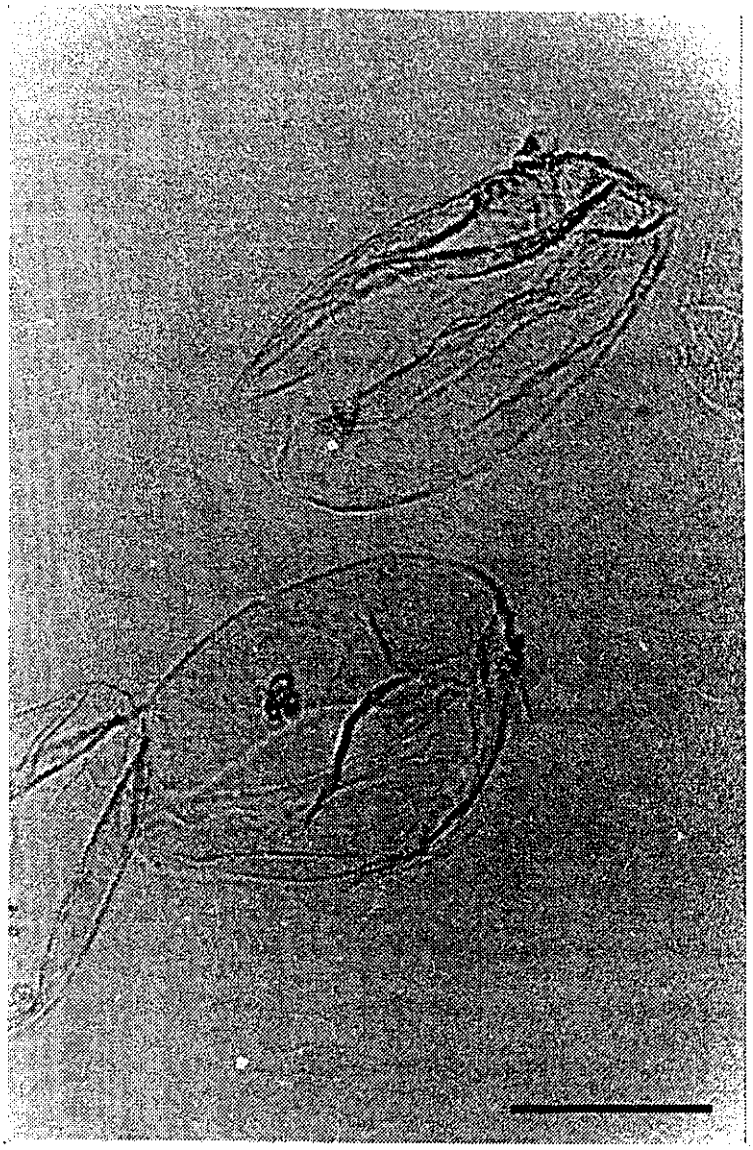


Figure 4.4.1 Photomicrograph detailing an aspect of the "contamination" seen in cultures treated with crushed muscle extract. Following 2-3 days in culture, flattened, membranous, disk-like structures were observed floating in the culture medium. They appeared colourless and increased in size over time. Bar = 100 μm.

CHAPTER V

DISCUSSION:

5.1 Development of single fibre-derived satellite cell cultures

This is the first report describing the isolation of a population of satellite cell progeny in a primary culture that was completely free of contaminating fibroblasts, macrophages or other non-myogenic cells. A pure colony of satellite cell progeny was obtained through the simple step of isolating and plating only a single viable myofibre per well. Viable myofibres were visually identified by their slender appearance, clear bumps of nuclei bulging from the fibre and tapered myofibre ends (Figure 4.1.3.2; 4.1.1.2, Panel B). Myofibres presenting this morphology always excluded trypan blue, indicating they were viable, while fibres with differing morphologies, such as those which were hypercontracted, always took up trypan blue indicating they were non-viable. Recent testing of the contractility of single fibres isolated in the described manner demonstrates that most fibres isolated, which exhibited this "viable" appearance, were able to contract following a maximal stimulation and had a resting membrane potential within the expected range for fibres from the FDB muscle (J.-M. Renaud, unpublished observations). By plating only a single myofibre per well the chances of plating fibroblasts (released into the

medium during the dissociation procedure) were decreased in three ways; by decreasing the amount of medium plated along with the myofibre, diluting the fibre suspension prior to plating also diluted any contaminating cells, and, finally, by not plating clumps of fibres we were able to eliminate the possibility of plating any fibroblasts that were trapped among the fibres.

The fibres were plated on a coat of dilute Matrigel, a gel composed of basement membrane components (including type IV collagen, laminin, heparan sulfate proteoglycan and entactin) extracted from the Engelbreth-Holm-Swarm mouse tumour. Matrigel also contains some growth factors, such as TGF-ß and bFGF, however these factors, supplied by Matrigel, did not have an overbearing influence on the culture growth. Despite the presence of Matrigel in all culture treatments there was significatly more growth of satellite cells in the presence of exogenously supplied growth factors (CEE, bFGF) than in Basal Medium (Figures 4.1.3.6, 4.2.1.1 and 4.2.2.2). Cultures had two possible sources of bFGF; Matrigel and exogenous bFGF added to the basal medium (horse serum does not contain bFGF). αbFGF elicited a dose-dependent inhibitory response in satellite cell cultures grown in the presence of 1.0 ng/ml bFGF (Figure 4.2.1.2). One would expect α-bFGF supplied at the optimal concentration to eliminate most, if not all, bFGF activity. Visual observations and absolute cell counts (not shown) suggested that αbFGF was inhibiting more than just the effect of exogenous bFGF that was added to the basal medium. Satellite cell colonies treated with more than 5μg/ml αbFGF were smaller than those in basal medium only, suggesting that the bFGF activity contained within the Matrigel was also being inhibited.

In most previous studies of satellite cells in culture the medium was changed every 1-2 days. Frequent changing of the culture medium was not desirable in the present study, since with each change there was the possibility of losing the single parent myofibre. The presence of the dying fibre in the culture was desirable to help maintain an environment similar to the true physiological condition. During trauma to muscle tissues <u>in vivo</u> the fibres start to degenerate (Komorowski et al.,

1990; Carpenter and Karpati, 1989). It has been shown that a saline extract from crushed muscle induces satellite cells to proliferate (Bischoff, 1986b; Chen and Quinn, 1992) therefore it is postulated that the injured degenerating myofibres release a mitogenic factor, or factors, responsible for triggering satellite cells to start the regeneration process. Bischoff (1990b) has also shown that viable myofibres exert an inhibitory effect on satellite cells in contact with the fibre plasmalemma. Therefore, it would seem that, during muscle regeneration in vivo, muscle fibres influence the growth and development of satellite cells. This relationship is maintained in the present culture system albeit between a single myofibre, its resident satellite cells and their progeny.

As described by Bischoff (1986b) 10% horse serum in Minimal Essential Medium was initially used as the Basal Medium in this study. Many studies investigating proliferation and differentiation of myogenic cells have used a Basal Medium with higher concentrations (15-20%) of serum (De Angelis et al., 1992; Alterio et al., 1990; Schultz and Lipton, 1982). In view of the fact that in the present study the culture medium was not changed throughout the culture period, we used a higher concentration of horse serum to maintain satellite cell proliferation for as long as possible (as cells proliferate they exhaust the supply of mitogenic factors present in the serum after which they differentiate). Higher amounts of horse serum did not give significantly more proliferation (Figure 4.1.3.5), however it did maintain proliferation of single satellite cells for longer. Visual observations showed that fusion was advanced by 5 days in culture with 10% horse serum and 0.5% CEE, however was not evident until 6-7 days in culture with 20% horse serum and 0.5% CEE.

The mouse satellite cells investigated in the present study (derived from isolated single myofibres) appear morphologically similar to those described in the literature (isolated by the mass satellite cell culture technique). More specifically the satellite cell progeny had a "round-cell" appearance which persisted for a number of days prior to the cells becoming bipolar in shape and fusing to form

multinucleated myotubes (Cossu and Molinaro, 1987; Colley et al., 1990). Similarly, the present study frequently demonstrated that mononucleated round-shaped cells are able to express sarcomeric myosin (Figure 4.1.2.1) indicating that the satellite cell progeny are able to differentiate prior to fusion. Similar expression by round-shaped cells of muscle-specific proteins (titin, myofibrillar MHC and zeugmatin) indicative of a differentiated state has also been observed by Colley and colleagues (1990).

Additional investigation of the satellite cell population isolated by the method described in this study remains to be done. For example, the expression of various muscle specific proteins (desmin, ACh receptors, adult MHC isoforms) and myogenic regulatory factors (myogenin, MyoD, myf-5, MRF4), as well as the temporal appearance and sequence of their expression.

A major limitation of the present study is the fact that there was so much variation in the satellite cell proliferation observed among single fibres, even when the fibres were derived from the same muscle from the same mouse. A large part of this variation could potentially be attributed to the fact that the number of satellite cells associated with a given myofibre may vary (see Discussion Section 5.3). Perhaps a more appropriate method for studying the proliferative capacity of satellite cells from mice would be to isolate the first satellite cell or satellite cell progeny which is observed on the plate adjacent to the adult myofibre (myofibres isolated as in the present study). All progeny that would result would then originate from a single parent cell. The obvious problem with this method, however, is that the isolated parent cell may not be the original satellite cell, but instead may be the result of one or more mitotic events which originated from the parent satellite cell.

5.2 Method of assessment of culture growth and differentiation

It was noted that within each experiment there were large variations in satellite cell activity from well to well, especially when using old normal, old dy^{2J} or old mdx mice. This may suggest that there is a large variation in the activity of satellite cells derived from different fibres from the same muscle within the same animal. However, the means from each experiment were similar. In this particular culture system (satellite cells derived from a single myofibre) there may be three possible explanations for this large variation; the number of satellite cells from each myofibre may vary (discussed in Discussion Section 5.7), satellite cells from different parent myofibres may start to proliferate at slightly different times, and, finally, each satellite cell may replicate at different rates. The latter explanation seems highly unlikely considering previous reports that myogenic precursor cells derived from the same muscle proceed through the cell cycle at the same rate (Bischoff and Holtzer, 1969; Yablonka-Reuveni and Rivera, 1994; reviewed by Johnston, 1990).

Initially, the method of assessment for culture growth employed in this study was by generation of cell proliferation curves (counts of mononucleated cells taken every 24 hours over a 7 day period) of each colony of satellite cells derived from a single myofibre. This method enabled us to assess the rate and onset of cell proliferation. As explained in the Methods Section 3.6 (Assessment of culture growth and development), however, this was a very inaccurate way of assessing any colonies consisting of more than 100 cells, so another more appropriate method of assessment was developed and used throughout most of the study. Despite the drawback of possible inaccuracy, the proliferation curves that were generated (Figures 4.1.3.4, 4.1.3.5) illustrate that with increasing CEE content (up to 0.5% CEE) there was a concomitant and significant increase in the number of cells induced to enter the cell cycle, and the start time for proliferation appeared similar in the presence of all media types tested. No proliferation curves were generated for satellite cells derived from old mice, however Schultz and Lipton (1982) reported that satellite cells

derived from rats demonstrate an increase in the onset time for mitoses (cell proliferation) with increasing age of the donor rat.

The method of assessment of culture growth and development used throughout much of this study was through accurate counts of the nuclei present in a standard area from the centre of satellite cell colonies, derived from single myofibres, that were fixed following a seven day culture period. The nuclei were visualized by staining with the nuclear stain, hematoxylin. Differentiation of the satellite cell cultures was assessed by staining for all those structures which expressed sarcomeric myosin heavy chain and subsequently comparing proportions of nuclei in myosin heavy chain-positive and -negative cells or myotubes. This method of assessment is similar to, although not exactly the same as, methods used by others in studying myogenic precursor cells in vitro.

In the literature concerned with primary cultures of myogenic cells there does not appear to be a single standard method for assessment of cell proliferation and/or differentiation, thus the following points were considered when deciding, for the present study, on a method to assess the satellite cell culture growth and development.

Indices of cell proliferation usually involve counts of the total number of cells or nuclei in a culture, or the uptake of [H^3]-thymidine (TdR) or a thymidine analogue, bromodeoxyuridine (BUdR) by proliferating cells (undergoing DNA replication). Absolute cell counts of entire colonies taken at various time periods are frequently presented (Schultz and Lipton, 1982; Cossu et al., 1989) as was initially the case in this study. As discussed with reference to the present culture system, this is very difficult to do accurately in colonies with more than 100-150 cells if using live cultures, rather than fixed ones. Similar to the method of assessment used throughout most of the present study, cell proliferation is frequently expressed as the total number of nuclei scored within a randomly selected field on a fixed culture at a single time point (Allen and Boxhorn, 1987, 1989; Allen et al., 1984; Cossu et al., 1980). In the present study it was not possible to randomly select a field as there was

only one colony covering only a small part of the culture surface per well (random selection would usually result in scoring an area with no cells present). Therefore, the central 1.70 mm^2 area of the satellite cell colony was selected in all cultures as the area scored. [H^3]-TdR uptake has been expressed as percent of the total nuclei with [H^3]-TdR uptake (Bischoff and Holtzer, 1969), counts per minute of [H^3]-TdR activity per plate (Hartley and Yablonka-Reuveni, 1990) or per 10^{-4} nuclei (Cossu et al, 1980), and as μmol [H^3]-TdR per mg protein (Alterio et al., 1990). A recent study assessed cell proliferation by measuring DNA content in the culture being tested relative to a control culture (Cook et al., 1993).

Indices of differentiation generally involve counts of the number of nuclei within myotubes (Bischoff and Holtzer, 1969; Allen and Boxhorn, 1987, 1989; Allen et al., 1984; Cossu et al., 1980) or within myosin-expressing structures (DeAngelis et al., 1992; Hartley et al., 1991) and are expressed in many different ways (such as percent of total nuclei, number per field or percent of maximum fusion). More recently, the accumulation of various muscle specific proteins has been used as an index of differentiation, such as μg myosin per culture plate (DeAngelis et al., 1992; Cossu et al., 1989) or units of creatine phosphokinase per mg protein (Alterio et al., 1990). Similar to assessment of cell proliferation the cell differentiation is usually assessed on fixed and stained cultures following a culture period (usually 6-7 days). Assessment of cultures using myotube formation as the indicator of differentiation is not a very appropriate method given the results of this and other studies. It has been demonstrated that muscle-specific protein expression, an indication of post-mitotic, terminally differentiated cells (Holtzer et al., 1957; Coleman and Coleman, 1968; Moss and Strohman, 1976; Jockusch and Jockusch, 1980; Konieczny et al., 1982; Hill et al., 1986; and others), sometimes occurs prior to cell fusion (Figure 4.1.2.1, this study; Clegg et al., 1987; Colley et al., 1990; Hill et al., 1986; Cossu et al., 1987).

5.3 Effects of growth factors on satellite cells derived from young and old mice

There were differences in the number of mitotically viable satellite cells, and in their proliferation potential, derived from single myofibres from young (2-4 months) versus old (12-24 months) mice.

Fewer myofibres derived from old mice (46.4% ± 11.5%) demonstrated myogenic precursor cell growth than myofibres derived from young mice (57.9% ± 10.6%; Figure 4.2.2.1, Panel B). This difference was not seen with Basal Medium treatment, however was clearly evident with the presence of CEE Medium (Figure 4.2.2.1, Panel A).

Satellite cells derived from old mice appear to have a lower proliferation potential (ie. a lower maximum number of cells) despite high levels of exogenously supplied bFGF (maximum: 314 ± 115 cells) or CEE (maximum: 456 ± 176 cells), as compared to satellite cells derived from young mice (bFGF maximum: 533 ± 211 cells; CEE maximum: 785 ± 278 cells). There was no age difference between young and old mice in the amount of proliferation seen in Basal Medium (Figure 4.2.2.2).

As expected, bFGF stimulated proliferation and inhibited differentiation of satellite cells derived from young mouse single muscle fibres in a dose dependent manner (Figure 4.2.1.1). However, satellite cells derived from old mice did not exhibit a significantly greater degree of proliferation in the presence of bFGF than they did with Basal Medium (Figure 4.2.2.3, Panel B). Despite this apparent lack of mitogenic effect, exogenously supplied bFGF still inhibited differentiation of satellite cells derived from old mice (Figure 4.2.2.4). This is further proof that proliferation and differentiation of myogenic precursor cells (in this case, adult mouse satellite cells) are two distinct processes that function independently of each other (Linkhart et al., 1982; Lathrop et al., 1985a,b; Spizz et al., 1986; Clegg et al., 1987), most likely through different mechanisms.

In summary, it was found that with increased age of donor mouse there were fewer fibres which contained viable satellite cells and those satellite cells which were viable had a lower proliferative capacity. It must be noted that the present study does not distinguish between fibres which contained differing amounts of satellite cells, therefore the proliferation potential that is scored in this study yields a value for the proliferative capacity of all the myogenic precursor cells in a given single fibre in a given medium, not of each individual satellite cell associated with that fibre.

The finding that fewer myofibres from old mice contain mitotically viable satellite cells in our cultures may simply be a product of this particular culture system. Perhaps the satellite cells on some myofibres are killed by the enzyme treatment used to isolate the single muscle fibres, or by a change in pH (becomes alkaline) of the enzyme solution during the isolation procedure. However, it is worth noting that this observation is consistent with the reports of Schultz and colleagues (1978) who demonstrated reduced numbers of satellite cells in old muscle by electron microscopy, coupled with autoradiography (Snow et al., 1977a,c; Schultz et al., 1978), and of Allen and colleagues (1980) who observed reduced numbers in culture.

The finding that fewer myofibres from old mice contain mitotically viable satellite cells is also relevant to the question of the origin of satellite cells. It is known that satellite cells arise during late embryogenesis (reviewed by Cossu and Molinaro, 1987; Hartley and Yablonka-Reuveni, 1992; Stockdale, 1992; Yablonka-Reuveni, 1994 as cited by Yablonka-Reuveni and Rivera, 1994). It is not clear, however, whether the satellite cells arise as a unique subpopulation of myoblasts, or if each population of developmental myoblasts (fetal and embryonic) contributes one or more myoblasts per myotube which would become the resident satellite cell, suspended in the G_0 phase of the cell cycle. Furthermore, it is unknown whether all fibres necessarily contain one or more satellite cells. One would expect that if one of the myonuclei derived from the fetal/embryonic myoblast populations within a

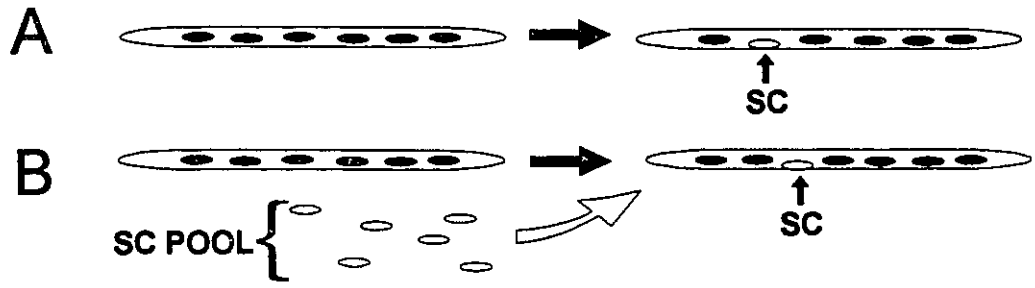


Figure 5.3.1: Possible origins of satellite cells.

forming myotube withdraws from the cell cycle (Figure 5.3.1 (A)) then all fibres may contain satellite cells. However, if satellite cells arise from a separate pool of myoblasts (the satellite cell pool) which subsequently fuse with the myotube then it is conceivable that some fibres may not have satellite cells (Figure 5.3.1 (B)).

Single fibres isolated from mouse flexor digitorum brevis muscle were approximately 350 μm in length and usually contained about 30 myonuclei, including satellite cells. As discussed in the Introduction, it is not possible to distinguish quiescent satellite cells from true myonuclei within live myofibres using light microscopy. We estimate, however, that the FDB myofibres contain between 0 and 3 satellite cells per fibre given that 4-6% of all myonuclei are satellite cell nuclei (reviewed by Mazanet and Franzini-Armstrong, 1986). Therefore, it is possible that some of the myofibres do not contain any satellite cells, as our results may suggest.

As pointed out in the Results (Section 4.2.2 - Influence of age of mice on the effect of bFGF) the decreased proliferative capacity seen in satellite cell cultures derived from old mice may be due to a decrease in the number of satellite cells per fibre in old mice. However, the significantly lower level of proliferation observed in the present study is in agreement with the report of Schultz and Lipton (1982) who monitored individual rat satellite cells, in culture, from each donor age

(6 days-30 months) to determine the number of progeny they were capable of producing. They concluded that satellite cells derived from old rats had a lower proliferation potential than those derived from young rat muscle. With increased donor age they observed a progressively longer latent period following cell plating before mitoses commenced and a reduction in the replication rate of the cells. Furthermore, the proliferation rate of cells from older rats started to decrease or plateau following 5 days in culture, whereas those from younger rats replicated at the same high rate for the duration of the study. The net effect was that by seven days in culture the colonies of satellite cells derived from older rats were much smaller than those derived from younger rats. Schultz and Lipton's (1982) findings may also apply to the present study although proliferation rates and time of onset of proliferation were not investigated.

The finding that cell proliferation is similar in Basal Medium but different in CEE Medium and bFGF Medium (>1.0 ng/ml bFGF) for young and old mouse satellite cells may indicate that satellite cells from old mice have an increased tendency to enter differentiation, despite having higher mitogen content in the medium. Alternatively, it may indicate that satellite cells derived from old mice are capable of entering mitosis similar to those derived from young mice, but the maximum number of mitotic events is lower in those derived from old mice. Satellite cells, like many other cell types, may only be capable of a finite number of mitotic divisions during the lifespan of the animal (Hayflick, 1973). Given the known role of satellite cells in normal growth, hypertrophy and regeneration one might expect satellite cells derived from old mice to be closer to this maximum than those from young mice. This could clearly result in fewer proliferative cycles in culture and hence fewer cells.

It must be noted that the fibre type composition of muscle has been reported to change with age, demonstrating a proportional decrease in the type IIB MHC-expressing myofibres with a corresponding increase in the more oxidative type IIA of IIX MHC-expressing myofibres (Kugelburg, 1976; Zardini and Parry,

submitted). It has been suggested that, rather than selective necrosis of the IIB fibres and their replacement by type IIA or IIX fibres, there may be a transformation from a IIB MHC to these other fibre types (Zardini, 1993). Therefore, it is conceivable that the present method of isolating single fibres could be biased if fibres of a particular type are more easily isolated than those of another type.

5.4 Replacement of single myofibres by a single myotube

A phenomenon was occasionally observed whereby single myofibres did not give rise to a colony of proliferating satellite cells on the substratum adjacent to the myofibre, but were replaced by a single myotube within the basal lamina sheath of the parent myofibre (Figure 4.1.3.2). This was observed most frequently in satellite cell cultures from old dystrophic mice of the mdx genotype (Figure 4.3.5). Although not statistically significant, satellite cell cultures from old dy^{2J} mice consistently demonstrated more single fibre replacement by a single myotube than satellite cell cultures from old and young normal mice. These findings are made more relevant by the fact that in both mdx and dy^{2J} mouse myofibre cultures a larger proportion of fibres demonstrated satellite cell proliferation which resulted in replacement of the parent myofibre with a single myotube, rather than satellite cell proliferation external to the myofibre as is usually seen in fibres derived from young and old mice (compare Figures 4.3.3 and 4.3.5). The majority of satellite cell proliferation in old and young mice was seen external to the myofibre (compare Figures 4.2.2.1 and 4.3.5). For example, in the presence of CEE Medium 69.0% ± 15.4% of single fibres from young mice give rise to satellite cell growth external to the myofibre, while 0.7% ± 1.9% of the fibres give rise to satellite cell growth within the fibre. For old mice, 51.6% ± 19.6% of single fibres give rise to external satellite cell growth while 3.0% ± 7.1% give rise to internal satellite cell growth. The pattern changes however with single fibres from old mdx mice; 17.3% ± 5.0% give rise to external growth while 15.5% ± 12.8% give rise to internal growth; and in old dy^{2J} mice 1.9% ± 3.2%

give rise to external growth while 10.2% ± 5.8% give rise to internal growth. It may be concluded that in this culture system satellite cells from both dystrophic mouse genotypes, dy^{2J} and mdx, are more likely to proliferate within the basal lamina sheath of the adult myofibre than those from young or old mice, although the reasons for this remain unclear.

It is worth noting that Bischoff (1986b) and Yablonka-Reuveni and Rivera (1994) have reported satellite cell proliferation occurring within viable rat single myofibres in culture. These studies maintained the viability of the myofibre, in the latter by using medium with CPSR-2 as a replacement for horse serum in the basal medium. We also found that use of CPSR-2 medium maintained viability of mouse single myofibres for a much longer period of time in culture (not shown). However, in the present study, no satellite cell proliferation was detected in these viable myofibres. Bischoff (1990b) has suggested that the plasmalemma of viable fibres appears to exert an inhibitory effect on the proliferation of satellite cells in contact with it. The finding that the presence of horse serum and/or CEE in the medium usually causes myofibre degeneration and supports proliferation of these satellite cells external to the myofibre (Figure 4.1.3.1) is in agreement with similar findings by Yablonka-Reuveni and Rivera (1994). In light of the fact that during the process of skeletal muscle regeneration in vivo the fibre undergoes necrosis and is replaced by new myotubes, formed following satellite cell proliferation, and their subsequent differentiation and fusion, the present study mimics the pathological situation in vivo more accurately. Furthermore, monitoring and quantitation of satellite cell growth is much simpler with growth that is external to the myofibre. No techniques to differentiate satellite cells from true myonuclei need to be employed as was the case in the above-mentioned studies (Yablonka-Reuveni and Rivera, 1994; Bischoff, 1986a, 1990b).

In all medium treatments on fibres from all mouse genotypes (except dy^{2J} myofibres in Basal Medium) the average number of nuclei scored following 7 days in culture was much greater in those cultures where satellite cell growth was

external to the myofibre (≥100 nuclei per satellite cell colony) than observed in those cultures where satellite cell growth was within the single parent myofibre (myofibre replaced by a single myotube; ≤40 nuclei per myotube). Cell density promotes myogenic fusion (Buckley and Konigsberg, 1974; Konigsberg, 1963, 1971, 1976; Konigsberg and Buckley, 1974), therefore the present results are not surprising. Within the confines of the basal lamina sheath the satellite cells are restricted as to the area in which they may proliferate (high density) and as such fuse early, whereas satellite cells growing external to the myofibre are not restricted in area for proliferation (low density) so cell proliferation occurs for longer and fusion is delayed.

It also interesting to note that the mean number of myonuclei observed in the myotube within the basal lamina sheath (approximately 30 myonuclei) was very similar to the number of myonuclei in the adult myofibres. It is difficult to speculate what this might indicate since the environment surrounding the proliferating satellite cells which form the myotube is quite different from that of the proliferating myoblasts which form the original myofibre during prenatal muscle growth and development; the latter do not have a basal lamina sheath formed prior to their proliferation and differentiation. However, given that myofibres appear to be programmed to maintain a specific cytoplasmic/nuclear ratio (Rosenblatt and Parry, 1993) it may be possible that the new myotube within the basal lamina sheath is merely maintaining this ratio. This is certainly a possibility since the volume of the new myotube looks similar (visual observations only) to that of the original adult myofibre. The new myotube is slightly longer than the original fibre, but it is also more slender.

It has been found (this study, visual observations not shown; Bischoff, 1990b; Yablonka-Reuveni and Rivera, 1994) that in vitro myogenic cells never fuse with isolated adult myofibres, however occasionally they were observed to fuse with myotubes which had "sprouted" from the ends of degenerating myofibres. Sprouting has also been observed in damaged muscle in vivo (Le Gros Clark, 1946; Hall-

Craggs, 1974a; Carpenter and Karpati, 1989; Shafiq and Gorycki, 1965) whereby the sprouts bridge the gap that resulted from myofibre degeneration following trauma to join myofibres from the opposite edge (Gay and Hunt, 1954; Hudgson and Field, 1973; Webb, 1973). These findings concerning myofibre sprouting may offer some support for the theory for continuous regeneration (Figure 1.1) in skeletal muscle.

5.5 Effects of growth factors on satellite cells derived from dystrophic mice.

Fewer myofibres derived from old dy^{2J} (2.5% ± 1.4%) and old mdx (22.6% ± 5.8%) mice demonstrated myogenic precursor cell growth than myofibres derived from old normal mice (46.4% ± 11.5%; Figure 4.3.3, Panel B). These differences were consistent in the presence of all media types tested. The proliferation potential of satellite cells derived from old dy^{2J} mice was similar to or greater than that of satellite cells derived from old normal mice. This is also the case for satellite cells derived from old mdx mice that are cultured in the presence of Basal Medium or 1.0 ng/ml bFGF. In the presence of higher concentrations of mitogenic substances (CEE Medium, 10.0 ng/ml bFGF), however, satellite cells derived from old mdx mice show a lower proliferation potential than those satellite cells derived from old normal mice and cultured in similar conditions. This discrepency between the genotypes of proliferation potentials within the same medium is due to the fact that medium has no effects on old mdx mouse satellite cells, but does on those from old normal and old dy^{2J} mice.

The finding that so few fibres from dy^{2J} mice gave rise to satellite cells capable of proliferation is consistent with the possibility that missing or faulty satellite cells are responsible for the reduced capacity for regeneration of dy^{2J} muscle. This, however, does not exclude the possibility that the mitogenic trigger is not present, or released, by degenerating muscle in dy^{2J} mouse (see Discussion Section 5.6) and could also be at fault.

It was somewhat surprising to find that myofibres derived from mdx mice also contain fewer mitotically viable satellite cells. As discussed in the Introduction, following a short phase of massive degeneration and regeneration in the first month of life, mdx muscle maintains a low level of degeneration and successful regeneration throughout the life of the animal (Coulton et al., 1988; Danguain and Vrbova, 1984; Torres and Duchen, 1987; Woo et al., 1987). DiMario and colleagues (1991) observed the level of regeneration in mdx mice to decrease following the phase of massive degeneration and regeneration to about 1% in mdx mice of one year of age. This was indicated by the frequency of fibres reacting with antibodies specific for embryonic and fetal myosin heavy chains (MHC). Embryonic and fetal MHC are coexpressed, albeit transiently, during the process of regeneration in normal mouse muscle (Matsuda et al., 1983; Saad et al., 1987; Whalen et al., 1990) prior to the expression of adult myosin isoforms. It was suggested that higher regenerative activity could simply be redundant in older mdx mice, perhaps because the newly regenerated muscles had acquired protein functions not present in the initial fibre population which would provide for sufficient fibre stability (DiMario et al., 1991). The present study would suggest that the low level of regeneration in old mdx muscle (Di Mario et al., 1991) is due to the absence in old mdx mouse myofibres of mitotically viable satellite cells.

It is important to note however that the present study made the assumption that the isolation of single myofibres from adult muscle required the same conditions for both normal and dystrophic mouse muscle. Therefore, the same batch of collagenase enzyme was used for all dissociation procedures. The possibility existed that the basal lamina of the myofibres derived from dystrophic mice was more susceptible to damage using that particular lot of collagenase. This may have led to the disruption of the basal lamina sheath and the loss of satellite cells during the dissociation procedure, which would have ultimately resulted in fewer myofibres from dystrophic mice which contained satellite cells.

Those satellite cells capable of proliferation demonstrated similar proliferation potentials for old normal and old dy^{2J} mice, except for in the presence of 1.0 ng/ml bFGF where it was significantly higher for old dy^{2J} mice (Figure 4.3.4). Mdx mice, however, appeared to have a lower proliferation potential and, furthermore, the maximum is attained with the addition of only 1.0 ng/ml bFGF. All three mouse genotypes exhibited similar morphology and patterns of growth (Figure 4.3.2). Others have observed defective growth in culture of satellite cells derived from other sources of dystrophic pathologies; dy/dy mouse (Hauschka et al., 1979; Wright 1985; Ontell et al., 1992) and DMD patients (Blau et al., 1983; Hurko et al., 1987; Webster and Blau, 1990). The present findings agree in part with these published observations in that fewer satellite cells are mitotically viable, however, unlike the other reports, the present study finds that those satellite cells derived from old dy^{2J} mice which are capable of mitosis are no different in their proliferation potential than satellite cells from old normal mouse. Similar to the present study however, Cossu and colleagues (1980) also observed that in culture satellite cells from dy/dy mouse exhibit similar morphology and proliferation potential to normal mouse satellite cells. The results obtained regarding satellite cell proliferation potential in the present study cannot be taken as conclusive, however, since there were so few satellite cell colonies from which nuclei counts could be taken. Similar numbers of fibres were plated for all genotypes (young normal: 425 fibres; old dy^{2J}: 601 fibres; old mdx: 571 fibres), with the exception of old normal (1653 fibres), yet since so few fibres gave rise to satellite cell proliferation in old dy^{2J} and mdx mice this restricted the number of fibres that could be scored.

5.6 Crushed muscle extract

We were unable to obtain a crushed muscle extract (CME) that was suitable for testing on myogenic cultures. As described in Results Section 4.4, every CME we prepared resulted in blackening of the single myofibre, and formation of

debris and disk-like structures in the culture medium. It was ascertained that this was not a result of bacterial, fungal or mould contamination, nor was it a reaction with any of the components in the culture system (for example, horse serum, Matrigel, MEM, myofibre). Saline perfusion of the mouse, prior to removal of the muscles used for CME preparation, to remove the blood and any clotting factors it contained, also did not improve the quality of the CME.

CME would be a useful tool for investigating regeneration in dy^{2J} and mdx murine muscular dystrophy if the described CME "contamination" problem was eliminated. As discussed in the Introduction and Review of the Literature there are two possible explanations for the reduced capacity for regeneration in dy^{2J} muscle. The mitogenic trigger may be absent in dy^{2J} muscle, or the satellite cells may be unable to respond to it even though it is present. CME from a normal mouse contains the mitogenic trigger responsible for regeneration in that mouse (Chen and Quinn, 1992). Therefore, by applying CME from dy^{2J} mouse to satellite cells derived from normal mouse it would be possible to check the viability of the mitogenic trigger in dy^{2J} muscle. By applying CME from normal mouse to satellite cells derived from dy^{2J} mouse it would be possible to check the regenerative capabilities of dy^{2J} muscle. Application of CME from mdx mouse to cultures of satellite cells and fibroblasts from dy^{2J} mouse, and the reverse experiment, application of CME from dy^{2J} mouse to mdx muscle satellite cells and fibroblasts may reveal some insight into the reasons why mdx mice experience much less fibrosis than their dy^{2J} counterparts.

5.7 Future studies:

Due to the fact that the populations of myogenic precursor cells are derived from isolated single adult myofibres, the method described in this thesis would be useful in determining myogenic lineage of satellite cells. Some questions that may possibly be answered using this technique are whether the satellite cells contained within a given myofibre give rise to new myofibres expressing the same

myosin isoform (fibre type) as that expressed by the parent myofibre; and, if clonal colonies are obtained , do the progeny of a single satellite cell give rise to similar myosin isoform expression? Preliminary work in this area appears to indicate that the progeny give rise to different isoforms despite being derived from the same parent satellite cell and the same parent myofibre of a given myosin isoform (J.D. Rosenblatt, personal communication).

The purity of adult myogenic precursor cell populations obtained by this isolated single fibre method is useful for many applications and reasons. First and foremost, analysis of cell growth and development is greatly simplified since the method eliminates any need to distinguish between non-myogenic and myogenic cells, as long as any cultures demonstrating stellate-shaped morphology are discarded from analysis. It has the potential for being a ready source of pure myoblasts, given the appropriate proliferation medium conditions, which many researchers believe are a good vehicle for various gene therapy strategies.

Muscle fibres are multinucleate syncytia with which myoblasts are capable of fusing (Karpati et al., 1989; Partridge et al., 1989) to contribute new myonuclei to the host myofibre and express their own genetic program. Satellite cells in culture have been stably transfected with a functional DMD gene construct (Klamut et al., 1992). Therefore if such satellite cells, transfected with the gene missing in DMD, could be injected into the muscles of DMD patients, then sufficient dystrophin may be expressed to protect the myofibres from necrosis, thereby slowing or halting the disease process. Two potential problems arise with this approach; a pure population of mitotically viable satellite cells is desirable in order to maximize the efficiency of the myoblast transplantion therapy, and maximal proliferative capacity is desired so the myoblasts don't fuse with each other prior to fusing with the defective myofibres which would decrease the efficiency of gene transfer.

Various techniques to purify satellite cells have been developed although there exist potential problems with all of them. Isolation of pure populations of human satellite cells is possible through labelling of satellite cells with

a monoclonal antibody, 5.1.H11 (Walsh and Ritter, 1981) against a human cell surface antigen following several days of culturing, and then sorting the labelled cells from unlabelled cells with a fluorescence-activated cell sorter (FACS; Webster et al., 1988). Recent development of a flow cytometry procedure, based on size separation, for the purification of human satellite cells taken directly from freshly dissociated muscle (Baroffio et al., 1993) involves fewer manipulations and decreases the risks of microbial contaminants. Rodent satellite cells are not labelled by the 5.1.H11 antibody, therefore the former method is not feasible for any cells other than human satellite cells. It is important to be able to purify rodent satellite cells so that experimental procedures can be tested on rodents prior to the use of the same procedures, but with human cells, in clinical trials on humans. The flow cytometry procedure has not yet been attempted on rodent satellite cells.

Purification of rodent satellite cells has been attempted by preplating and a modified panning technique (Jones et al., 1990a). Pre-plating selects for those myoblasts which take longer to attach to the culture surface as compared to fibroblasts, thereby allowing the unattached myoblasts to be easily harvested. Segregation of myogenic and fibroblastic populations by panning relies on differential expression of certain muscle specific proteins; for example, the expression of NCAM (neuronal cell adhesion molecule; Walsh and Moore, 1986). However, as discussed in the Introduction (Section 2.5), much evidence indicates that a subpopulation of fibroblasts also express NCAM (Gatchalian et al., 1989; Covault and Sanes, 1986; Grumet et al., 1982; Moore and Walsh, 1985).

Percoll density gradients are able to produce a fraction containing high proportions of myogenic, and another fraction consisting largely of non-myogenic cells (Morgan, 1988; Yablonka-Reuveni et al., 1988); however, the myogenic fraction always contains some additional non-myogenic cells.

The method described in the present study is the only method reported to date, whereby a pure population of myoblasts derived from rodent muscle can be obtained.

The gene transfer therapy problem remains concerning the finite mitotic capacity of satellite cells. This capacity is decreased even further if the myoblasts are cultured (ie. proliferate in culture) prior to their injection into the host muscle, however, this is an easy way of increasing the number of myogenic cells available for the gene transfer. While established cell lines have a virtually unlimited mitotic capacity, they also have a tumorogenic tendency in vivo (Partridge et al., 1988; Wernig et al., 1991; Morgan et al., 1992). Therefore, the availability of myogenic cells which combine the proliferative qualities of cell lines with the ability of primary cells to form normal muscle (Morgan et al., 1990), but not tumours, in vivo would benefit myoblasts transplantation research.

It would seem that if normal and healthy myoblasts were coupled with an inducible immortalizing gene then these criteria would be fulfilled. Such a gene has been isolated from H-2K^b-tsA58 transgenic mice (Jat et al., 1991). These mice express a tsA58 mutant of SV40 large T antigen, a thermolabile immortalizing gene (Tegtmeyer, 1975), which is under control of an inducible promotor, H-2K^b (David-Wattine et al., 1990). The expression of a functional tsA58 protein is induced by interferon-gamma. Cells grown in culture at 33°C in the presence of interferon-gamma proliferate for at least 80 generations (Morgan et al., 1994), while removal of interferon-gamma and increased temperature (39°C) resulted in the formation of myotubes by 24 hours after the change, and myosin expression within the next few days (Morgan et al., 1994). Following implantation into mdx mice these cells formed normal muscle, but not tumours. Furthermore, the host mdx muscle, normally missing dystrophin (Partridge et al., 1989), expressed dystrophin in a substantial proportion of the myofibres.

Both problems (myoblast purity and mitotic capacity) would theoretically be solved if this thermolabile immortalizing gene could be coupled with pure populations of myogenic precursor cells, obtained by the method described in the present study, by either transfecting the cells derived from normal mice with the gene, or, more efficiently, by simply isolating the satellite cells, also as described in

the present study, directly from transgenic H-2K^b-tsA58 mice which already expressed the desired gene.

The present satellite cell culture system presents a good model for observing satellite cell growth and development. As such, this system would be ideal for exploring the potential defects of satellite cells derived from dystrophic mice, as started in the present study, and for determining whether the mitogenic trigger is defective in, or absent from, muscle from dystrophic mice (discussed previously in Section 5.6 - Crushed muscle extract).

Further to the crushed muscle extract (CME) studies, the receptors for the myogenic factor(s) contained within the CME must be investigated. Downregulation, or the absence, of the receptors for the mitogenic trigger would result in a decreased response of satellite cells to the mitogenic trigger. One might expect from the present results that the receptors for the mitogenic trigger in dy^{2J} and mdx muscle are downregulated, or absent, since it would appear that the proliferative capacity of satellite cells from these two genotypes is the same as satellite cells from control mice (Figure 4.3.4). Downregulation, or absence, of the receptor might explain the decreased percentage of myofibres which result in satellite cell growth (Figure 4.3.3).

The growth factors responsible for triggering and maintaining skeletal muscle regeneration are not known, although there are many factors which show potential for their involvement. Two distinct processes are involved in skeletal muscle regeneration; proliferation of, and differentiation of, satellite cells.

LIF (leukemia inhibitory factor) is a growth factor that has, until recently, largely been overlooked in the literature concerned with skeletal muscle regeneration. LIF was found to be the most effective stimulator of mouse and human myoblast proliferation in culture (Austin et al., 1992), via direct receptor mediated mechanisms. LIF has no effect on fusion in culture (Vakakis et al., in press, as cited by Barnard et al., 1994). A recent study (Barnard et al., 1994) demonstrated that perfusion of LIF into crushed skeletal muscle in vivo had a significant positive effect

on the degree of regeneration in the muscle, by increased rate of regeneration and increased fibre size (rather than an increase in total number of myofibres). It is also suggested that some structure(s) in muscle binds LIF (Barnard et al., 1994). Furthermore, as early as 12 hours following the crush injury, and with an increase over (at least) the three days post crush, LIF mRNA synthesis was extremely high (Barnard et al., 1994); whereas, uninjured muscle does not express LIF mRNA (Metcalf and Gearing, 1989; Barnard et al., 1994). The source of endogenous LIF found in injured muscle is presently unknown, although preliminary work, in culture, would suggests that proliferating myoblasts express LIF mRNA (unpublished results, as cited by Barnard et al., 1994).

Localization of LIF expression, perhaps through immunohistochemistry, could be identified using the present culture system. Three possible sources of LIF in regenerating skeletal muscle exist; invading cells (such as macrophages), the degenerating adult myofibre or the proliferating myoblasts. The present culture system would show no LIF expression if invading cells were the source in vivo, yet would easily localize the expression to either, or both, the single adult myofibre and/or the satellite cell progeny if they were the source of LIF.

Further experiments to elucidate the role of LIF and other growth factors in crushed muscle extract could be performed using the myogenic cells obtained by the present culture system.

REFERENCES:

ADAL, M.N. (1969) The fine structure of the sensory region of cat muscle spindles. Journal of Ultrastructural Research. 26:332-354.

ALAMEDDINE, H.S., M. DEHAUPAS and M. FARDEAU (1989) Regeneration of skeletal muscle fibres from autologous satellite cells multiplied in vitro: an experimental model for testing cultured cell myogenicity. Muscle and Nerve. 12:544-555.

ALLBROOK, D.B. (1962) An electron microscopic study of regenerating skeletal muscle. Journal of Anatomy. 96:137-152.

ALLBROOK, D.B. (1981) Skeletal muscle regeneration. Muscle and Nerve. 4:234-245.

ALLEN, R.E. and L.K. BOXHORN (1987) Inhibition of skeletal muscle satellite cell differentiation by transforming growth factor-beta. Journal of Cellular Physiology. 133:567-572.

ALLEN, R.E. and L.K. BOXHORN (1989) Regulation of skeletal muscle satellite cell proliferation and differentiation by transforming growth factor-beta, insulin-like growth factor-I, and fibroblast growth factor. Journal of Cellular Physiology. 138:311-315.

ALLEN, R.E., M.V. DODSON and L.S. LUITEN (1984) Regulation of skeletal muscle satellite cell proliferation by bovine ptuitary fibroblast growth factor. Experimental Cell Research. 152:154-160.

ALLEN, R.E., M.V. DODSON, L.S. LUITEN and L.K. BOXHORN (1985) A serum-free medium that supports the growth of cultured skeletal muscle satellite cells. In Vitro. 21:636-640.

ALLEN, R.E., P.K. McALLISTER and K.C. MASAK (1980) Myogenic potential of satellite cells in skeletal muscle of old rats. A brief note. Mechanisms of Ageing and Development. 13:105.

ALLEN, R.E., P. McALLISTER, M. MASAK and G.R. ANDERSON (1982) Influence of ages on accumulation of alpha-actin in satellite cell-derived myotubes in vitro. Mechanisms of Ageing and Development. 18:89-95.

ALLEN, R.E., R.A. MERKEL and R.B. YOUNG (1979) Cellular aspects of muscle growth: myogenic cell proliferation. Journal of Animal Science. 49(1):115-127.

ALLEN, R.E., L.L. RANKIN, E.A. GREENE, L.K. BOXHORN, S.E. JOHNSON, R.G. TAYLOR and P.R. PIERCE (1991) Desmin is present in proliferating rat muscle satellite cells but not in bovine muscle satellite cells. Journal of Cellular Physiology. 149: 525-535.

ALTERIO, J., Y. COURTOIS, J. ROBELIN, B. BECHET and I. MARTELLY (1990) Acidic and basic fibroblast growth factor mRNA's are expressed by skeletal muscle satellite cells. Biochemical and Biophysical Research Communications. 166(3):1205-1212.

ANDERSON, J.E., L. LIU and E. KARDAMI (1991) Distinctive patterns of basic fibroblast growth factor (bFGF) distribution in degenerating and regenerating areas of dystrophic (mdx) striated muscles. Developmental Biology. 147:96-109.

ANDERSON, J.E., W.K. OVALLE and B.H. BRESSLER (1987) Electron microscopic and autoradiographic characterization of hindlimb muscle regeneration in the mdx mouse. Anatomical Record. 219:243-257.

AUSTIN, L., J. BOWER, J. KUREK and N. VAKAKIS (1992) Effects of leukemia inhibitory factor and other cytokines on murine and human myoblast proliferation. Journal of the Neurological Sciences. 112:185-191.

BADER, D. (1981) Density and distribution of α-bungarotoxin-binding sites in postsynaptic structures of regenerated rat skeletal muscle. Journal of Cellular Biology. 88:338-345.

BADER, D. , T. MASAKI and D.A. FISCHMAN (1982) Immunohistochemical analysis of myosin heavy chain during avian myogenesis in vivo and in vitro. Journal of Cell Biology. 95:763-770.

BAIRD, A. and N. LING (1987) Fibroblast growth factors are present in the extracellular matrix produced by endothelial cells in vitro: implications for a role of heparinase-like enzymes in the neovascular response. Biochemical and Biophysical Research Communications. 142:428-435.

BANKER, B.Q. (1960) The experimental myopathies. Res. Publ. Assoc. Res. Nervous and Mental Disorders. 38:197-233.

BANKER, B.Q. (1967) A phase and electron microscopic study of dystrophic muscle. I. The pathological changes in the two week old Bar Harbour 129 dystrophic mouse. Journal of Neuropathology and Experimental Neurology. 26:259-275.

BANKER, B.Q. and T.P. GIRVIN (1971) The ultrastructural features of the mammalian muscle spindle. Journal of Neuropathological and Experimental Neurology. 30:155-195.

BARNARD, W., J. BOWER, M.A. BROWN, M. MURPHY and L. AUSTIN (1994) Leukemia inhibitory factor (LIF) infusion stimulates skeletal muscle regeneration after injury: injured muscle expresses lif mRNA. Journal of the Neurological Sciences. 123:108-113.

BAROFFIO, A., J.-P. AUBRY, A. KAELIN, R.M. KRAUSE, M. HAMMANN and C.R. BADER (1993) Purification of human muscle satellite cells by flow cytometry. Muscle and Nerve. 16:498-505.

BAYNE, E.K., M.J. ANDERSON and D.M. FAMBOROUGH (1984) Extracellular matrix organization in developing muscle: correlation with acetylcholine receptor aggregates. Journal of Cell Biology. 99:1486-1501.

BECKER, D., C.B. MEIER and M. HERLYN (1989) Proliferation of human malignant melanoma is inhibited by antisenseoligodeoxynucleotidestargeted against basic fibroblast growth factor. EMBO Journal. 8:3685-3691.

BEKOFF, A. and W.J. BETZ (1977a) Physiological properties of dissociated muscle fibres obtained from innervated and denervated adult rat muscle. Journal of Physiology (London). 271:25-40.

BEKOFF, A. and W.J. BETZ (1977b) Properties of isolated rat muscle fibres maintained in tissue culture. Journal of Physiology (London). 271:537-547.

BENEZRA, R., R.L. DAVIS, D. LOCKSHON, D.L. TURNER and H. WEINTRAUB (1990) The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. Cell. 61:49-59.

BENGAL, E., L. RANSONE, R. SCHARFMANN, V.J. DWARKI, S.J. TAPSCOTT H. WEINTRAUB and I.M. VERMA (1992) Functional antagonism between c-jun and MyoD proteins: a direct physical association. Cell. 68:507-519.

BENNETT, M.R. (1983) Development of neuromuscular synapses. Physiological Reviews. 63:915-1048.

BINTLIFF, S. and B.E. WALKER (1960) Radioautographic study of skeletal muscle regeneration. American Journal of Anatomy. 106:233-246.

BISCHOFF, R. (1974) Enzymatic liberation of myogenic cells from adult rat muscle. Anatomical Record. 180:645-662.

BISCHOFF, R. (1975) Regeneration of single skeletal muscle fibres in vitro. Anatomical Record. 182:215-236.

BISCHOFF, R. (1986a) Proliferation of muscle satellite cells on intact myofibres in culture. Developmental Biology. 115:129-139.

BISCHOFF, R. (1986b) A satellite cell mitogen from crushed adult muscle. Developmental Biology. 115:140-147.

BISCHOFF, R. (1989) Analysis of muscle regeneration using single myofibres in culture. Medical Science and Sports Exercise. 21:S164-S172.

BISCHOFF, R. (1990a) Cell cycle commitment of rat muscle satellite cells. The Journal of Cell Biology. 111:201-209.

BISCHOFF, R. (1990b) Interaction between satellite cells and skeletal muscle fibres. Development. 109:943-952.

BISCHOFF, R. (1990c) Control of satellite cell proliferation. In Myoblast Transfer Therapy. (R. Griggs and G. Karpati, eds.) Plenum Press, New York. pp. 147-158.

BISCHOFF, R. and H. HOLTZER (1969) Mitosis and the process of differentiation of myogenic cells in vitro. The Journal of Cell Biology. 41:189-200.

BLAU, H.M., C. WEBSTER and G.K. PAVLATH (1983) Defective myoblasts identified in Duchenne muscular dystrophy. Proceedings of the National Academy of Science, USA. 80:4856-4860.

BLOCK, N.E. and J.B. MILLER (1992) Expression of MRF4, a myogenic helix-loop-helix protein, produces multiple changes in the myogenic program of BC3H1 cells. Molecular and Cellular Biology. 12:2484-2492.

BOBER, E., G.E. LYONS, T. BRAUN, G. COSSU, M.E. BUCKINGHAM and H.-H ARNOLD (1991) The muscle regulatory gene, myf-6 has a biphasic pattern of expression during early mouse development. Journal of Cell Biology. 113:1255-1266.

BRAUN, T., M.A. RUDNICKI, H.-H. ARNOLD and R. JAENISCH (1992) Targeted inactivation of the muscle regulatory gene myf-5 results in abnormal rib development and perinatal death. Cell. 71:1-20

BRAUN, T., E. BOBER, G. BUSCHHAUSEN-DENKER, S. KOTZ, K.-H. GRZESCHIK and H.H. ARNOLD (1989b) Differential expression of myogenic determination genes in muscle cells: possible autoactivation by the *myf* gene products. The EMBO Journal. 8:3617-3625.

BRAUN, T., E. BOBER, B. WINTER, N. ROSENTHAL and H.H. ARNOLD (1990a) Myf-6, a new member of the human gene family of myogenic determination factors: evidence for a gene cluster on chromosome 12. The EMBO Journal. 9:821-831.

BRAUN, T., G. BUSCHHAUSEN-DENKER, E. BOBER, E. TANNICH and H.H. ARNOLD (1989a) A novel human muscle factor related to but distinct from MyoD1 induces myogenic conversion in 10T1/2 fibroblasts. The EMBO Journal. 8:701-709.

BRAVO, R., R. FRANK, R.A. BLUNDELL and H. MACDONALD-BRAVO (1987) Cyclin/PCNA is the auxiliary protein of DNA polymerase-δ. Nature. 326:515-517.

BRAY, G.M. and B.Q. BANKER (1970) An ultrastructural study of degeneration and necrosis of muscle in the dystrophic mouse. Acta Neuropathologica. 15:34-44

BRENNAN, T.J., T. CHAKRABORTY and E.N. OLSON (1991a) Mutagenesis of the myogenin basic region identifies an ancient protein motif critical for activation of myogenesis. Proceedings of the National Academy of Sciences, USA. 88:5676-5679.

BRENNAN, T.J., D.G. EDMONDSON, L. LI and E.N. OLSON (1991b) Transforming growth factor ß represses the actions of myogenin through a mechanism independent of DNA binding. Proceedings of the National Academy of Sciences, USA. 88:3822-3826.

BRENNAN, T.J., D.E. EDMONDSON and E.N. OLSON (1990) Aberrant regulation of MyoD1 contributes to the partially defective myogenic phenotype of BC3H1 cells. Journal of Cell Biology. 110:929-937.

BRENNAN, T.J. and E.N. OLSON (1990) Myogenin resides in the nucleus and acquires high affinity for a conserved enhancer element on heterodimerization. Genes in Development. 4:582-595.

BRIDGES, L.R. (1986) The association of cardiac muscle necrosis and inflammation with the degenerative and persistant myopathy of mdx mice. Journal of Neurological Science. 72:147-157.

BROADLEY, K.N., A.M. AQUINO, S.C. WOODWARD, A. BUCKLEY-STURROCK, Y. SATO, D.B. RIFFKIN and J.M. DAVIDSON (1989) Monospecific antibodies implicate basic fibroblast growth factor in normal wound repair. Laboratory Investigations. 61:571-575.

BROCKDORFF, N., G.S. CROSS, J.S. CAVANNA, E.M.C. FISHER, M.F. LYON, K.E. DAVIES and S.D.D. BROWN (1987) The mapping of a cDNA from the human X-linked Duchenne muscular dystrophy gene to the mouse X-chromosome. Nature. 328:166-168.

BRUNETTI, A. and I.D. GOLDFINE (1990) Role of myogenin in myoblast differentiation and its regulation by fibroblast growth factor. Journal of Biological Chemistry. 265:5960-5963.

BUCKLEY, P.A. and I.R. KONIGSBERG (1974) Myogenic fusion and the duration of the post-mitotic gap (G1). Developmental Biology. 37:193-212.

BURGESS, W.H. and T. MACIAG (1989) The heparin-binding (fibroblast) growth factor family of proteins. Annual Review of Biochemistry. 58:575-606.

BUSKIN, J.N. and S.D. HAUSCHKA (1989) Identification of a myocyte specific nuclear factor which binds to the muscle-specific enhancer of the mouse muscle creatine kinase gene. Molecular and Cellular Biology. 9:2627-2640.

CAMPBELL, K.P. and S.D. KAHN (1989) Association of dystrophin and an integral membrane glycoprotein. Nature. 338:259-262.

CAMPION, D.R. (1984) The muscle satellite cell. International Review of Cytology. 87:225-251.

CARLSON, B.M. (1970) Histological observations on the regeneration of mammalian and amphibian muscle. In Regeneration of Striated Muscle and Myogenesis. (A. Mauro, S.A. Shafiq and A.T. Milhorat, eds.) Amsterdam, Excerpta Medica. pp. 38-72.

CARLSON, B.M. (1973) The regeneration of skeletal muscle. American Journal of Anatomy. 137:119-150.

CARLSON, B.M. and J.A. FAULKNER (1983) The regeneration of skeletal muscle fibres following injury: a review. Medicine and Science in Sports and Exercise. 15:187-198.

CARLSON, B.M. and J.A. FAULKNER (1989) Muscle transplantation between young and old rats: age of host determines recovery. American Journal of Physiology. 256:C1262-C1266.

CARLSON, B.M., A.F. FOSTER, D.M. BADER, P. HNIK and R. VEJSADA (1983) Restorationof full mass in nerve-intact muscle grafts after delayed reinnervation. Experimentia. 39:171-172.

CARLSON, B.M., F.M. HANSEN-SMITH and D.K. MAGON (1979) The life history of a free muscle graft. In Muscle Regeneration. (A. Mauro et al., eds.) Raven Press, New York. pp 493-507.

CARPENTER, S. and G. KARPATI (1989) Segmental necrosis and its demarcation in experimental micropuncture injury of skeletal muscle fibers. Journal of Neuropathology and Experimental Neurology. 48(2):154-170.

CHAKRABORTY, T., T.J. BRENNAN, L. LI, D. EDMONDSON and E.N. OLSON (1991a) Inefficient homooligomerization contributes to the dependence of myogenin on E2A products for efficient DNA binding. Molecular and Cellular Biology. 11:3633-3641.

CHAKRABORTY, T., T. BRENNAN and E.N. OLSON (1991b) Differential trans-activation of a muscle-specific enhancer by myogenic helix-loop-helix proteins is seperable from DNA binding. Journal of Biological Chemistry. 266:2878-2882.

CHEN, G. and L.S. QUINN (1992) Partial characterization of skeletal myoblast mitogens in mouse crushed muscle extract. Journal of Cellular Physiology. 153:563-574.

CHROMIAK, J.A., D.R. MULVANEY and C.H. RAHE (1991) L6 myoblast proliferation in response to muscle-derived extracts and serum from exercise-trained rats. Cell Biology International Reports. 15(4):335-344.

CHURCH, J. (1970) Cell populations in skeltal muscle after regeneration. Journal of Embryology and Experimental Morphology. 23:531-537.

CLEGG, C., T. LINKHART, B.B. OLWIN and S.D. HAUSCHKA (1987) Growth factor control of skeletal muscle differentiation: Commitment to terminal differentiation occurs in G_1 phase and is repressed by fibroblast growth factor. Journal of Cell Biology. 105:949-956.

COLEMAN, J.R. and A.M. COLEMAN (1968) Muscle differentiation and macromolecular synthesis. Journal of Cellular Physiology. 72: 19-34.

COLLEY, N.J., K.T. TOKUYASU and S.J. SINGER (1990) The eariy expression of myofibrillar proteins in round postmitotic myoblasts of embryonic skeletal muscle. Journal of Cell Science. 95:11-22.

CONDON, K., L. SILVERSTEIN, H.M. BLAU and W.J. THOMPSON (1990) Development of muscle fibre types in the prenatal rat hindlimb. Developmental Biology. 138:256-274.

CONSTANTINIDES, P.G., P.A. JONES and W. GEVERS (1977) Functional striated muscle cells from myoblast precursors following 5-azacytidine treatment. Nature. 267:364-366.

CONSTANTINIDES, P.G., S.M. TAYLOR and P.A. JONES (1978) Phenotypic conversion of cultures mouse embryo cells by azapyrinidine nucleosides. Developmental Biology. 66:57-71.

COOK, D.R., M.E. DOUMIT and R.A. MERKEL (1993) Transforming growth factor- beta, basic fibroblast growth factor, and platelet derived growth factor-BB interact to affect proliferation of clonally derived porcine satellite cells. Journal of Cellular Physiology. 157:307-312.

COSSU, G., M.G. CUSELLA-DE ANGELIS, M.I. SENNI, L. DE ANGELIS, E. VIVARELLI, S. VELLA, M. BOUCHE, C. BOITANI and M. MOLINARO (1989) Adrenocorticotropin is a specific mitogen for mammalian myogenic cells. Developmental Biology. 131:331-336.

COSSU, G., F. EUSEBI, F. GRASSI and E. WANKE (1987) Acetylcholine receptor channels are present in undifferentiated satellite cells but not in embryonic myoblasts in culture. Developmental Biology. 123:43-50.

COSSU, G. and M. MOLINARO (1987) Cell heterogeneity in the myogenic lineage. Current Topics in Developmental Biology. 23:185-208.

COSSU, G., M. MOLINARO and M. PACIFICI (1983) Differential response of satellite cells and embryonic myoblasts to a tumor promotor. Developmental Biology. 98:520-524.

COSSU, G., G. RANALDI, M.I. SENNI, M. MOLINARO and E. VIVARELLI (1988) 'Early' mammalian myoblasts are resistant to phorbol ester-induced block of differentiation. Development. 102:65-69.

COSSU, G., B. ZANI, M. COLLETTE, M. BOUCHE, M. PACIFICI and M. MOLINARO (1980) In vitro differentiation of satellite cells isolated from normal and dystrophic mammalian muscles. A comparison with embryonic myogenic cells. Cell Differentiation. 9:357-368.

COTE, C. and J.A. FAULKNER (1984) Motor unit function in skeletal muscle autografts of rats. Experimental Neurology. 84:292-305.

COULTON, G.R., N.A. CURTIN, J.E. MORGAN and T.A. PARTRIDGE (1988) The mdx mouse skeletal muscle myopathy. II. Contractile properties. Neuropathology and Applied Neurobiology. 14:299-314.

COVAULT, J. and J.R. SANES (1986) Distribution of N-CAM in synaptic and extrasynaptic portions of developing and adult skeletal muscle. The Journal of Cell Biology. 102: 716-730.

CRESCENZI, M., T.P. FLEMING, A.B. LASSAS, H. WEINTRAUB and S.A. AARONSON (1990) MyoD induces growth arrest independent of differentiation in normal and transformed cells. Proceedings of the National Academy of Science, USA. 87:8442-8446.

CUEVAS, P., J. BURGOS and A. BAIRD (1988) Basic fibroblast growth factor promotes cartilage repair in vivo. Biochemical and Biophysical Research Communications. 156:611-618.

DANGUAIN, J. and G. VRBOVA (1984) Muscle development in mdx mutant mice. Muscle and Nerve. 7:700-704.

DAVID-WATTINE, B., A. ISRAEL and P. KOURILSKY (1990) The regulation and expression of MHC class 1 genes. Immunology Today. 11:286-292.

DAVIS, R.L., P.-F. CHENG, A.B. LASSAR and H. WEINTRAUB (1990) The MyoD DNA binding domain contains a recognition code for muscle-specific gene activation. Cell. 60:733-746.

DAVIS, R.L. and H. WEINTRAUB (1992) Acquisition of myogenic specificity by replacement of three amino acid residues from MyoD into E12. Science. 256:1027-1030.

DAVIS, R.L., H. WEINTRAUB and A.B. LASSAR (1987) Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell. 51:987-1000.

DE ANGELIS, L., M.G. CUSELLA-DE ANGELIS, M. BOUCHE, E. VIVARELLI, C. BOITANI, M. MOLINARO and G. COSSU (1992) ACTH-like peptides in postimplantation mouse embryos: A possible role in myoblast proliferation and muscle histogenesis. Developmental Biology. 151:446-458.

DENNY-BROWN, D.E. (1960) Experimental studies pertaining to hypertrophy, regeneration, and degeneration. Res. Publ. Assoc. Res. Nervous and Mental Disorders. 38:147-196.

DIMARIO, J. and R.C. STROHMAN (1988) Satellite cells from dystrophic (mdx) mouse muscle are stimulated by fibroblast growth factor in vitro. Differentiation. 39:42-49.

DIMARIO, J., N. BUFFINGER, S. YAMADA and R.C. STROHMAN (1989) Fibroblast growth factor in the extracellular matrix of dystrophic (mdx) mouse muscle. Science. 244:688-690.

DIMARIO, J., A. UZMAN and R.C. STROHMAN (1991) Fibre regeneration is not persistant in dystrophic (mdx) mouse skeletal muscle. Developmental Biology. 148:314-321.

DODSON, M.V. R.E. ALLEN and K.L. HOSSNER (1985) Ovine somatomedin, multiplication-stimulating activity, and insulin promote skeletal muscle satellite cell proliferation in vitro. Endocrinology. 117:2357-2363.

DRIBIN, L.B. and S.B. SIMPSON (1977) Histochemical and autoradiographic study of injured and uninjured dystrophic (C57BL/6J dy^{2J}/dy^{2J}) and normal muscle. Experimental Neurology. 56:498-517.

DUSTERHOFT, S., Z. YABLONKA-REUVENI and D. PETTE (1990) Characterization of myosin isoforms in satellite cell cultures from adult rat diaphragm, soleus and tibialis anterior muscles. Differentiation. 45:185-191.

ECOB, M.S. (1983) The application of organotypic nerve cultures to problems in neurology with reference to their potential use in research into neuromuscular diseases. Journal of the Neurological Sciences. 58:1-15.

EDMONSON, D.G. and E.N. OLSON (1989) A gene with homology to the myc similarity region of MyoD1 is expressed during myogenesis and is sufficient to activate the muscle differentiation program. Genes Dev.. 3:628-640.

EMERSON, C.P., Jr. (1993) Skeletal myogenesis: genetics and embryology to the fore. Current Opinion in Genetics and Development. 3:265-274.

ENESCO, M. (1960) Increase in number of nuclei along with the enlargement of muscle fibres in the soleus of the growing rat. Proceedings of the Canadian Federation of Biological Soc. 3:20

ENESCO, M. and D. PUDDY (1964) Increase in the number of nuclei and weight in skeletal muscle of rats of various ages. American Journal of Anatomy. 114:235-244.

EWTON, D.Z. and J.R. FLORINI (1980) Relative effects of the somatomedins, multiplication-stimulating activity, and growth hormone on myoblasts and myotubes in culture. Endocrinology. 106:577-583.

FAERMAN, A., S. PEARSON-WHITE, C. EMERSON and M. SHANI (1993) Ectopic expression of MyoD1 in mice causes prenatal lethalities. Developmental Dynamics. 196:165-173.

FERNANDEZ, M.S., J.E. DENNIS, R.F. DRUSHEL, D.A. CARNINO, K. KIMATA, M. YAMAGATA and A.I. CAPLAN (1991) The dynamics of compartmentalization of embryonic muscle by extracellular matrix molecules. Developmental Biology. 147:46-61.

FINKELSTEIN, S.P., P.J. APOSTOLIDES, C.G. CADEY, J.PROSSER, M.F. PHILIPS and M. KLAGSBURN (1988) Increased basic fibroblast gorwth factor (bFGF) immunoreactivity at the site of focal brain wounds. Brain Research. 460:253-259.

FLORINI, J.R. and D.Z. EWTON (1990) Highly specific inhibition of IGF-I-stimulated differentiation by an antisense oligodeoxyribonucleotide to myogenin mRNA. The Journal of Biological Chemistry. 265:13435-13437.

FLORINI, J.R., D.Z. EWTON and K.A. MAGRI (1991) Hormones, growth factors and myogenic differentiation. Annual Reviews of Physiology. 53:201-216.

FLORINI, J.R., A.B. ROBERTS, D.Z. EWTON, S.L. FALEN, K.C. FLANDERS, M.B. STERN (1986) Transforming growth factor-beta: a very potent inhibitor of myoblast differentiation, identiacal to the differentiation inhibitor secreted by BRL cells. Journal of Biological Chemistry. 261:16509-16513.

FOLKMAN, J., M. KLAGSBRUN, J. SASSE, M. WADZINSKI, D. INGBER and I. VLODAVSKY (1988) A heparin-binding angiogenic protein - basic fibroblast growth factor - is stored within basement membrane. American Journal of Pathology. 130:393-400.

FRANCO, A. and J.B. LANSMAN (1990) Calcium entry through stretch-inactivated ion channels in mdx myotubes. Nature. 344:670-673.

FUCHTBAUER,E.-M. and H. WESTPHAL (1992) *MyoD* and *myogenin* are coexpressed in regenerating skeletal muscle of the mouse. Developmental Dynamics. 193:34-39.

FUJISAWA-SEHARA, A., Y. NABESHIMA, T. KOMIYA, T. UETSUKI, A. ASAKURA and Y.-I. NABESHIMA (1992) Differential trans-activation of muscle-specific regulatory elements including the myosin light chain box of chicken MyoD, myogenin and MRF4. Journal of Biologicla Chemistry. 267:10031-10038.

GATCHALIAN, C.L., M. SCHACHNER and J.R. SANES (1989) Fibroblasts that proliferate near denervated synaptic sites in skeletal muscle synthesize the adhesive molecules tenascin (J1), NCAM, fibronection and a heparan sulfate proteoglycan. Journal of Cell Biology. 108: 1873-1890.

GAY, A.J. and T.E. HUNT (1954) Reuniting of skeletal muscle fibres after transection. Anatomical Record. 120: 853-871.

GLASER, L. and B. WICE (1989) Control of differentiation in BC3H1 muscle cells. Environmental Health Perspectives. 80:143-148.

GOLDFARB, M. (1990) The fibroblast growth factor family. Cell Growth and Differentiation. 1:439-445.

GOLDSPINK, G. (1972) Postembryonic growth and differentiation of striated muscle. In The Structure and Function of Muscle (G.H. Bourne, ed.) Vol. 1. Academic Press Inc., New York. pp. 179.

GOSPODAROWICZ, D. (1974) Localization of a fibroblast growth factor and its effect alone and with hydrocortisone on 3T3 cell growth. Nature. 249:123-127.

GOSPODAROWICZ, D., J. CHENG, G. LUI, A. BAIRD and P. BOHLENT (1984) Isolation of brain fibroblast growth factor by heparin-Sepahrose affinity chromatography: Identity with pituitary fibroblast growth factor. Proceedings of the National Academy of Science (USA). 81:6963-6967.

GOSPODAROWICZ, D. and J. MORAN (1974) Effect of a fibroblast growth factor, insulin, dexamethasone, and serum on the morphology of BALB/c 3T3 cells. Proceedings of the National Academy of Science (USA). 71:4648-4652.

GOSPODAROWICZ, D., G. NEUFELD and L. SCHWEIGERER (1986a) Fibroblast growth factor. Molecular and Cellular Endocrinology. 46:187-206.

GOSPODAROWICZ, D., G. NEUFELD and L. SCHWEIGERER (1986b) Molecular and biological characterization of fibroblast growth factor: an angiogenic factor which also controls the proliferation and differentiation of mesoderm and ectoderm derived cells. Cellular Differentiation. 19:1-17.

GOSPODAROWICZ, D., F. WESEMAN and J. MORAN (1975) Presence in the brain of a mitogenic agent distinct from fibroblast growth factor that promotes the proliferation of myoblasts in low density culture. Nature. 256:216-220.

GOSPODAROWICZ, D., F. WESEMAN, J. MORAN and J. LINDSTROM (1976) Effect of fibroblast growth factor on the division and fusion of bovine myoblasts. Journal of Cell Biology. 70:395-405.

GOSSET, L., D. KELVIN, E. STERNBERG and E.N. OLSON (1989) A new myocyte-specific enhancer-binding factor that recognizes a conserved element associated with multiple muscle-specific genes. Molecular and Cellular Biology. 9:5022-5033.

GROUNDS, M.D. (1987) Phagocytosis of necrotic muscle in muscle isografts is influenced by the strain age and sex of host mice. Journal of Pathology. 153:71-82.

GROUNDS, M.D. (1991) Towards understanding skeletal muscle regeneration. Path. Res. Pract. 187:1-22.

GROUNDS, M.D. and J.K. McGEACHIE (1989) A comparison of muscle precursor replication in crush-injured skeletal muscle of Swiss and BALBc mice. Cell Tissue Research. 255:385-391.

GROUNDS, M.D. and Z. YABLONKA-REUVENI (1993) Molecular and cellular biology of muscle regeneration. In Molecular and Cell Biology of Muscular Dystrophy. (T. Partridge, ed.) Chapman and Hall, London. pp. 210-256.

GROUX-MUSCATELLI, B. Y. BASSAGLIA, D. BARRITAULT, J.-P. CARUELLE and J. GAUTRON (1990) Proliferating satellite cells express acidic fibroblast growth factor during in vitro myogenesis. Developmental Biology. 142:380-385.

GRUMET, M., U. RUTISHAUSER and G.M. EDELMAN (1982) neural cell adhesion molecule is on embryonic muscle cells and mediates adhesion to nerve cells in vitro. Nature. 295:693-695.

GURDON, J.B., N.D. HOPWOOD and M.V. TAYLOR (1992) Myogenesis in Xenopus development. Seminars in Developmental Biology. 3:255-266.

HALL-CRAGGS, E.C.B. (1974a) The regeneration of skeletal muscle fibers per continuum. Journal of Anatomy. 117:171-178.

HALL-CRAGGS, E.C.B. (1974b) Rapid degeneration and regeneration of a whole skeletal muscle following treatment with bupivacaine (Marcaine). Experimental Neurology. 43:349-358.

HAMBURGH, M., E. PETERSON, M.B. BORNSTEIN and C. KIRK (1975) Capacity of fetal spinal cord obtained from dystrophic mice (dy^{2J}) to promote muscle regeneration. Nature. 256:219-220.

HANNON, K., C.K. SMITH 2nd, K.R. BALES and R.F. SANTERRE (1992) Temporal and quantitative analysis of myogenic regulatory and growth factor gene expression in the developing mouse embryo. Developmental Biology. 151:137-144.

HARTLEY, R.S. and Z. YABLONKA-REUVENI (1990) Long-term maintenance of primary myogenic cultures on a reconstituted basement membrane. In Vitro Cellular and Developmental Biology. 26:955-961.

HARTLEY, R.S. and Z. YABLONKA-REUVENI (1992) Evidence for a distinct adult myogenic lineage in skeletal muscle. Comments on Developmental Neurobiology. 1:391-404.

HASTY, P., A. BRADLEY, J.H. MORRIS, D.G. EDMONDSON, J.M. VENUTI, E.N. OLSON and W.H. KLEIN (1993) Muscle deficiency and neonatal death in mice wtih a targeted mutation in the myogenin gene. Nature. 364:501-506.

HAUSCHKA, S.D., T.A. LINKHART, C. CLEGG and G. MERRILL (1979) Clonal studies of human and mouse muscle. In Muscle Regeneration. (A. Mauro, ed.) Raven Press, New York. pp.311-322.

HAYFLICK, L. (1973) The biology of human ageing. American Journal of Medical Science. 265:432-445.

HESS, A. and S. ROSNER (1970). The satellite cell bud and myoblast in denervated mammalian muscle fibres. American Journal of Anatomy. 129:21-40.

HILL, C.S., S. DURAN, Z. LIN, K. WEBER and H. HOLTZER (1986) Titin and myosin, but not desmin, are linked during myofibrillogenesis in postmitotic mononucleated myoblasts. Journal of Cell Biology. 103:2185-2196.

HINTERBERGER, T., D.A. SASSOON, S.J. RHODES and S.F. KONIECZNY (1991) Expression of the muscle regulatory factor MRF4 during somite and skeletal myofibre development. Developmental Biology. 147:144-156.

HOFFMAN, E.P., R.H. BROWN, Jr., L.M. KUNKEL (1987) Dystrophin: The protein product of the Duchenne muscular dystrophy locus. Cell. 51:919-928.

HOFFMAN, E.P., M.S. HUDSECKI, P.A. ROSENBERG, C.M. POLLINA and L.M. KUNKEL (1988) Cell and fibre-type distribution of dystrophin. Neuron. 1:411-420.

HOLTZER, H. and R. BISCHOFF (1970) Mitosis and myogenesis. In The Physiology and Biochemistry of Muscle as a Food, 2. (E.J. Brisky, R.J. Cassens and B.B. Marsh, eds.) The University of Wisconsin Press, Madison. pp. 29.

HOLTZER, H., J. MARSHALL and H. FINCK (1957) An analysis of myogenesis by the use of fluorescent antimyosin. Journal of Biophysical and Biochemical Cytology. 8:705-723.

HOWARD, A. and S.R. PELC (1951) Nuclear incorporation of P^{32} as demonstrated by autoradiographs. Experimental Cell Research. 2:178

HUDGSON, P. AND E.J. FIELD (1973) Regeneration of muscle. In Structure and Function of Muscle. Vol.II, 2nd edition. (G. Bourne, ed.) Academic Press, New York. pp.312-363.

HURKO, O., L. McKEE, J. ZUURVELD and H.M. SSWICK (1987) Comparison of myoblasts from a heterozygote. Neurology. 37:675-681.

IBRAGHIMOV-BESKROVNAYA, O., J.M. ERVASTI, C.J. LEVEILLE, C.A. SLAUGHTER, S.W. SERNETT and K.P. CAMPBELL (1992) Primary structure of dystrophin-associated glycoproteins linking dystrophin to the extracellular matrix. Nature. 355:696-702.

IRINTCHEV, A. and A. WERNIG (1987) Muscle damage and repair in voluntarily running mice: Strain and muscle differences. Cell and Tissue Research. 249:509-521.

JAT, P.S., M.D. NOBLE, P. ATALIOTIS, Y. TANAKA, N. YANNOUTSOS, L.LARSEN and D. KIOUSSIS (1991) Direct derivation of conditionally immortal cell lines from an *H-2K*^{b}-tsA58 transgenic mouse. Proceedings of the National Academy of Sciences, USA. 88:5096-5100.

JEN, Y., H. WEINTRAUB and R. BENEZRA (1992) Overexpression of Id protein inhibits the muscle differentiation program: in vivo association of Id with E2A proteins. Genes in Development. 6:1466-1479.

JOCKUSCH, H., and B.M. JOCKUSCH (1980) Stuctural organization of the Z-line protein, α-actinin, in developing skeletal muscle cells. Developmental Biology. 75:231-238.

JOHNSON, S.E. and R.E. ALLEN (1993) Proliferating cell nuclear antigen (PCNA) is expressed in activated rat skeletal muscle satellite cells. Journal of Cellular Physiology. 154:39-43.

JOHNSTON, L.H. (1990) Periodic events in the cell cycle. Current Opinion in Cell Biology. 2:274-279.

JONES, G.E., S.J. MURPHY and D.J. WATT (1990a) Segregation of the myogenic cell lineage in mouse muscle development. Journal of Cell Science. 97:659-667.

JONES, P.A., M. WOLOWICZ, W.M. RIDEOUT III, F.A. GONZALES, C.M. MARZIASZ, G.A. COETZEE and S.J. TAPSCOTT (1990b) *De novo* methylation of the MyoD1 CpG island during the establishment of immortal cell lines. Proceedings of the National Academy of Science, USA. 87:6117-6121.

JONES, P.H. (1982) In vitro comparison of embryonic myoblasts and myogenic cells isolated from regenerating adult rat skeletal muscle. Experimental Cell Research. 139:401-404.

JOSEPH-SILVERSTEIN, J., S.A. CONSIGLI, K.M. LYSER and C. VERPAULT (1989) Basic fibroblast growth factor in the chick embryo: Immunolocalization to striated muscle cells and their precursors. Journal of Cell Biology. 108:2459-2466.

KAHN, L.B. and J.S. MEYER (1970) Acute myopathy in chronic alcoholism: A study of 22 autopsy cases, with ultrastructural observations. American Journal of Clinical Pathology. 53:516-530.

KARDAMI, E., D. SPECTOR and R.C. STROHMAN (1985a) Myogenic growth factor present in skeletal muscle is purified by heparin-affinity chromatography. Proceedings of the National Academy of Science (USA). 82:8044-8047.

KARDAMI, E., D. SPECTOR and R.C. STROHMAN (1985b) Selected muscle and nerve extracts contain an activity which stimulates myoblast proliferation and which is distinct from transferrin. Developmental Biology. 112: 353-358.

KARDAMI, E., D. SPECTOR and R.C. STROHMAN (1988) Heparin modulates fibroblast growth factor efffects on myogenesis in vitro. Developmental Biology. 126:19-28.

KARLSSON, U. and E. ANDERSSON-CEDERGREN (1971) Satellite cells of the frog muscle spindle as revealed by electron microscopy. Journal of Ultrastructural Research. 34:426-438.

KARPATI, G., Y. POULIOT, E.E. ZUBRZYCKA-GAARN, S. CARPENTER, P.N. RAY and R.G. WORTON (1989) Dystrophin is expressed in mdx skeletal fibers after normal myoblast transplantation. American Journal of Pathology. 135:27-32.

KELLY, A.M. (1978) Satellite cells and myofibre growth in the rat soleus and extensor digitorum longus muscles. Developmental Biology. 65:1-10.

KELLY, A.M. (1979) Variations in satellite cell distribution in developing and mature muscles of the rat. In Muscle Regeneration (A. Mauro, ed.). Raven Press, New York. pp. 167-176.

KELLY, A.M. and S.I. ZACHS (1969) The histogenesis of rat intercostal muscle. Journal of Cell Biology. 41:135-153.

KELVIN, D.J., G. SIMARD and J.A. CONNOLLY (1989) FGF and EGF act synergistically to induce proliferation in BC3H1 myoblasts. Journal of Cellular Physiology. 138:267-272.

KLAMUT, H.J., L.O. BOSNOYAN, R.G. WORTON and P.N. RAY (1992) Expression of human dystrophin minigene in myogenic cell cultures. In Duchenne Muscular Dystrophy: Animal Models and Genetic Manipulations. (B.A. Kakulas, J. Howell and A.D. Roses, eds.) Raven Press, New York. pp.255-262.

KLIETSCH, R. J.M. ERVASTI, K.P. CAMPBELL and A. JORGENSEN (1993) Dystrophin-glycoprotein complex and laminin colocalize to the sarcolemma and transverse tubules of cardiac muscle. Circulation Research. 72:349-360.

KOMOROWSKI, T.E., B. SHEPARD, S. ØKLAND and B.M. CARLSON (1990) An electrom microscopic study of local anesthetic-induced skeletal muscle fibre degeneration and regeneration in the monkey. Journal of Orthopaedic Research. 8(4):495-503.

KONIECZNY, S.F. and C.P. EMERSON, Jr. (1984) 5-azacytidine induction of stable mesodermal stem cell lineages from 10T1/2 cells: evidence for regulatory genes controlling determination. Cell. 38:791-800.

KONIECZNY, S.F., J. McKAY and J.R. COLEMAN (1982) Isolation and characterization of terminally differentiated chick and rat skeletal muscle myoblasts. Developmental Biology. 91:11-26.

KONIGSBERG, I.R. (1979) Regeneration of single muscle fibres in vitro and in vivo. In Muscle Regeneration. (A. Mauro, ed.) New York, Raven Press. pp. 41-56.

KONIGSBERG, I.R. (1963) Clonal analysis of myogenesis. Science. 140:1273-1284.

KONIGSBERG, I.R. (1971) Diffusion-mediated control of myoblast fusion. Developmental Biology. 26:133-152.

KONIGSBERG, I.R. (1976) Clonal analysis of development. In: Tests of Teratogenesis In Vitro; 5th International Conference of the Institute de la Vie. (J.D. Ebert and M. Marois, eds.) North Holland, Amsterdam. pp.37-66.

KONIGSBERG, I.R. and P.A. BUCKLEY (1974) Regulation of the cell cycle and myogenesis by cell-medium interaction. In Concepts of Development. (J. Lash and J.R. Whittaker, ed.) Sinauer Associates, Stamford, Connecticut. pp.179-193.

KUGELBERG, E. (1976) Adaptive transformation of rat soleus motor units during growth. Journal of the Neurological Sciences. 27:269-289.

LASSAR, A.B., B.M. PATTERSON and H. WEINTRAUB (1986) Transfection of a DNA locus that mediates the conversion of 10T1/2 fibroblasts to myoblasts. Cell. 47:649-656.

LATHROP, B., E. OLSON and L. GLASER (1985a) Control by fibroblast growth factor of differentiation in the BC3H1 muscle cell line. Journal of Cell Biology. 100:1540-1547.

LATHROP, B., K. THOMAS and L. GLASER (1985b) Control of myogenic differentiation by fibroblast growth factor is mediated by position in the G_1 phase of cell cycle. Journal of Cell Biology. 101:2194-2198.

LeGROS-CLARK, W.E. (1946) An experimental study of the regeneration of mammalian striped muscle. Journal of Anatomy. 80:24-36.

LESLEY, J.F. and V.A. LENNON (1977) Nature. 268:163-165.

LEWIN, B. (1991) Oncogenic conversion by regulatory changes in transcription factors. Cell. 64:303-312.

LI, L., J.-C. CHAMBARD, M. KARIN and E.N. OLSON (1992) Fos and jun repress transcriptional activation by myogenin and MyoD: the amino terminus of jun can mediate repression. Genes in Development. 6:676-689.

LI, L. and E.N. OLSON (1992) Regulation of muscle cell growth and differentiation by the MyoD family of helix-loop-helix proteins. Advances in Cancer Research. 58:95-119.

LIN, H., K. YUTZEY and S.F. KONIECZNY (1991) Muscle-specific expression of the troponin I gene required interactions between helix-loop-helix muscle regulatory factors and ubiquitous transcription factors Molecular and Cellular Biology. 11:267-280.

LINKHART, T.A., C.H. CLEGG and S.D. HAUSCHKA (1980) Control of mouse myoblast commitment to terminal differentiation by mitogens. Journal of Supramolecular Structure. 14:483-498.

LINKHART, T.A., C.H. CLEGG, R.W. LIM, G.F. MERRILL, J.S. CHAMBERLAIN and S.D. HAUSCHKA (1982) Control of mouse myoblast commitment to terminal differentiation by mitogens. In Molecular and Cellular Control of Muscle Development. (M.L. Pearson and H.F. Epstein, eds.) Cold Springs Harbor, New York. pp. 877-882.

MACPIKE, A.D. and H. MEIER (1976) Comparison of dy and dy^{2J}, two alleles expressing forms of muscular dystrophy in the mouse. Proceedings of the Society for Experimental Biology and Medicine. 151:670.

MARECHAL, G. (1986) Regeneration of striated muscle. Biomed. Biochem. Acta. 45:S125-S130.

MASSAGUÉ, J., S. CHEIFETZ, T. ENDO and B. NADAL-GINARD (1986) Type beta transforming growth factor is an inhibitor of myogenic differentiation. Proceedings of the National Academy of Science (USA). 83:8206-8210.

MATSUDA, R., D. SPECTOR and R.C. STROHMAN (1983) Regenerating adult chicken skeletal muscle and satellite cell cultures express embryonic patterns of myosin and tropomyosin isoforms. Developmental Biology. 100:478-488.

MATSUMURA, K. and K. CAMPBELL (1994) Dystrophin-glycoprotein complex: its role in the molecular pathogenesis of muscular dystrophies. Muscle and Nerve. 17:2-15.

MATSUMURA, K. J.M. ERVASTI, K. OHLENDIECK, S.D. KAHL and K.P CAMPBELL (1992) Association of dystrophin-related protein with dystrophin-associated proteins in mdx mouse muscle. Nature. 360:588-591.

MAURO, A. (1961) Satellite cells of skeletal muscle fibres. Journal of Biophysical and Biochemical Cytology. 9:493-495.

MAURO, A. (ed.,1979) Muscle Regeneration. Raven Press, New York.

MAURO, A., S.A. SHAFIQ and A.T. MILHORAT (eds., 1970) Regeneration of Striated Muscle and Myogenesis. Amsterdam, Excerpta Medica.

MAYNE, R. and R.D. SANDERSON (1985) The extracellular matrix of skeletal muscle. Collagen Related Research. 5:449-468.

MAZANET,R. and C. FRANZINI-ARMSTRONG (1986) The satellite cell. In Myology (A.G. Engel and B.Q. Baker, eds.) McGraw-Hill Book Co., New York. pp. 285-307.

MESSIER, B. and C.P. LEBLOND (1960) Cell proliferation and migration as revealed by radioautography after injection of thymidine-H^3 into male rats and mice. American Journal of Anatomy. 106:247-265.

METCALF, D. and D.P. GEARING (1989) A myeloslerotic syndrome in mice engrafted with cells producing high levels of leukemia inhibitory factor (LIF). Leukemia. 3:847-852.

MICHELSON, A.M., E.S. RUSSELL and P.J. HARMAN (1955) Dystrophin muscularis: a hereditary primary myopathy in the house mouse. Proceedings of the National Academy of Science. 41:1079.

MILLER, J.B. (1990) Myogenic programs of mouse muscle cell lines: Expression of myosin heavy chain isoforms, MyoD1 and myogenin. Journal of Cell Biology. 111:1149-1159.

MILLER, J.B. (1992) Myoblast diversity in skeletal myogenesis: how much and to what end? Cell. 69:1-3.

MILLER. J.B., M.T. CROW and F.E. STOCKDALE (1985) Slow and fast myosin heavy chain content defines three types of myotubes in early muscle cell cultures. Journal of Cell Biology. 101:1643-1650.

MILLER, J.B. and F.E. STOCKDALE (1986a) Developmental origins of skeletal muscle fibers: Clonal analysis of myogenic cell lineages based on fast and slow myosin heavy chain expression. Proceedings of the National Academy of Science (USA). 83:3860-3864.

MILLER, J.B. and F.E. STOCKDALE (1986b) Developmental regulation of the multiple myogenic cell lineages of the avian embryo. Journal of Cell Biology. 103:2197-2208.

MILLER, J.B. and F.E. STOCKDALE (1987) What muscle cells know that nerves don't tell them. Trends in Neuroscience. 10(8):325-329.

MINER, J.H. and B. WOLD (1990) Herculin, a fourth member of the MyoD family of myogenic regulatory genes. Proceedings of the National Academy of Sciences, USA. 87:1089-1093.

MOORE, J.W., C. DIONNE, M. JAYE, J.L. SWAIN (1991) The mRNA's encoding acidic FGF, basic FGF and FGF receptor are coordinately downregulated during myogenic differentiation. Development. 111:741-748.

MOORE, M.J. (1975) A myogenic malfunction in adult dystrophic mice studied *in vitro*. Journal of Neurological Science. 24: 77-93.

MOORE, R., G. CASEY, S. BROOKS, M. DIXON, G. PETERS and C. DICKSON (1986) Sequence, topography and protein coding potential of mouse int-2: a putative oncogene activated by mouse mammary tumour virus. EMBO Journal. 5:919-924.

MOORE, S.E. and F.S. WALSH (1985) Specific regulation of N-CAM/D2-CAM cell adhesion molecule during skeletal muscle development. EMBO Journal. 4:623-630.

MORGAN, J.E. (1988) Myogenicity in vitro and in vivo of mouse muscle cells separated on discontinuous Percoll gradients. Journal of the Neurological Sciences. 85:197-207.

MORGAN, J.E., G.R. COULTON and T.A. PARTRIDGE (1989) Mdx muscle grafts retain the mdx phenotype in normal hosts. Muscle and Nerve. 12:401-409.

MORGAN, J.E., E.P. HOFFMAN and T.A. PARTRIDGE (1990) Normal myogenic cells from newborn mice restore normal histology to degenerating muscles of the mdx mouse. Journal of Cell Biology. 111:2437-2499.

MORGAN, J.E., S.E. MOORE, F.S. WALSH and T.A. PARTRIDGE (1992) Formation of skeletal muscle in vivo from the mouse C2 cell line. Journal of Cell Science. 102:779-787.

MORGAN, J.E., J.R. BEAUCHAMP, C.N. PAGEL, M. PECKHAM, P. ATALIOTIS, P.S. JAT, M.D. NOBLE, K. FARMER and T.A. PARTRIDGE (1994) Myogenic cell lines derived from transgenic mice carrying a thermolabile T antigen: A model system for the derivation of tissue-specific and mutation-specific cell lines. Developmental Biology. 162:486-498.

MORROW, N.G., W.E. KRAUS, J.W. MOORE, R.S. WILLIAMS and J.L. SWAIN (1990) Increased expression of expression of fibroblast growth factors in a rabbit skeletal muscle model of exercise conditioning. Journal of Clinical Investigation. 85:1816-1820.

MOSS, F.P. and C.P. LEBLOND (1970) Nature of dividing nuclei in skeletal muscle of growing rats. Journal of Cell Biology. 44:459-461.

MOSS, F.P. and C.P. LEBLOND (1971) Satellite cells as the source of nuclei in muscles of growing rats. Anatomical Record. 170:421-436.

MOSS, F.P. and R. STROHMAN (1976) Myosin synthesis by fusion-arrested chick embryo myoblasts in cell culture. Developmental Biology. 48:431-437.

MRAK, R.E. (1985) Muscular dystrophy. In Muscle Membranes in Diseases of Muscle. (R.E. Mrak, ed.) CRC Press Inc., Florida. pp. 23-80.

MUELLER, P.R. and B. WOLD (1989) In vivo footprinting of a muscle specific enhancer by ligation mediated PCR. Science. 246:780-786.

MUIR, A.R. (1970) The structure and distribution of satellite cells. In Regeneration of Striated Muscle and Myogenesis. (A. Mauro, S.A. Shafiq and A.T. Milhorat, eds.) Excerpta Medica, Amsterdam. pp. 91-100.

MURRE, C., P.S. McCAW and D. BALTIMORE (1989a) A new DNA binding and dimerization motif in immunoglobin enhancer binding *daughterless, MyoD* and *myc* proteins. Cell. 56:777-783.

MURRE, C., P.S. McCAW, H. VAESSIN, M. CAUDY, L.Y. JAN, Y.N. JAN, C.V. CABRERA, J.N. BUSKIN, S.D. HAUSCHKA, A.B. LASSAR, H. WEINTRAUB and D. BALTIMORE (1989b) Interactions betweeb heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. Cell. 58:537-544.

MURRE, C., A. VORONOVA and D. BALTIMORE (1991) ß-cell- and myocyte-specific E2-box-binding factors contain E12/E47-like subunits. Molecular and Cellular Biology. 58:537-544.

NABESHIMA, Y., K. HANAOKA, M. HAYASAKA, E. ESUMI, S. LI, I. NONOKA and Y.-I. NABESHIMA (1993) Myogenin gene disruption results in perinatal lethality because of severe muscle defect. Nature. 364:532-535.

NAPARSTEK, Y., I.R. COHEN, Z. FUKS and I. VLODASKY (1984) Activated T-lymphocytes produce a matrix-degrading heparin sulfate endoglycosidase. Nature. 310:241-244.

OLSON, E.N. (1990) MyoD family: a paradigm for development? Genes in Development. 4:1454-1461.

OLSON, E.N., E. STERNBERG, J.S. HU, G. SPIZZ and C. WILCOX (1986) Regulation of myogenic differentiation by type beta transforming growth factor. Journal of Cell Biology. 103:1799-1806.

OLSON, E.N., T.J. BRENNAN, T. CHAKRABORTY, T.-C. CHENG, P. CSERJESI, D. EDMONDSON, G. JAMES and L. LI (1991) Molecular control of myogenesis: antagonism between growth and differentiation. Molecular and Cellular Biochemistry. 104:7-13.

OLWIN, B.B. and S.D. HAUSCHKA (1986) Identification of the fibroblast growth factor receptor of Swiss 3T3 cells and mouse skeletal muscle myoblasts. Biochemistry. 25:3487-3492.

OLWIN, B.B. and S.D. HAUSCHKA (1988) Cell surface fibroblast growth factor and epidermal growth factor receptors are permanently lost during skeletal muscle terminal differentiation in culture. Journal of Cell Biology. 107:761-769.

OLWIN, B.B. and S.D. HAUSCHKA (1989) Cell type and tissue distribution of the fibroblast growth factor receptor. Journal of Cellular Biochemistry. 39:443-454.

ONTELL, M. and K. KOZEKA (1984) The organogenesis of murine striated muscle: a cytoarchitectural study. American Journal of Anatomy. 171:133-148.

ONTELL, M., D. HUGHES, S.D. HAUSCHKA and M. ONTELL (1992) Transient neonatal denervation alters the proliferative capacity of myosatellite cells in dystrophic (129ReJ$^{dy/dy}$) muscle. Journal of Neurobiology. 23(4):407-419.

OSAWA, E. and Y. HAGIWARA (1981) Avian and mammalian transferrins are required for chick and rat myogenic cell growth in vitro, respectively. Proceedings of the Japanese Academy. 57:406-409.

OTT, M.-O., E. BOBER, G. LYONS, H. ARNOLD and M. BUCKINGHAM (1991) Early expression of the myogenic regulatory gene, myf-5, in precursor cells of skeletal muscle in the mouse embryo. Development. 111:1097-1107.

PARSONS, R. (1974) Expression of the dystrophia muscularis (dy) recessive gene in mice. Nature. 251:621-622.

PARTRIDGE, T.A., J.E. MORGAN, S.E. MOORE and F.S. WALSH (1988) Myogenesis in vivo from the mouse C2 muscle cell-line. Journal of Cell Biochemistry, Suppl.. 12C:331.

PARTRIDGE, T.A., J.E. MORGAN, G.R. COULTON, E.P. HOFFMAN, L.M. KUNKEL (1989) Conversion of mdx myofibers from dystrophin-negative to positive by injection of normal myoblasts. Nature. 337:176-179.

PAUL, C.V. and J.A. POWELL (1974) Organ culture studies of coupled fetal cord and adult muscle from normal and dystrophic mice. Journal of Neurological Science. 21:365-379.

PETERSON, C., H. GORDON, Z. HALL, B. PETERSON and H. BLAU (1990) Negative control of helix-loop-helix family of myogenic regulators in NFB mutant. Cell. 62:493-502.

PIN,C.L. and P.A. MERRIFIELD (1993) Embryonic and fetal rat myoblasts express different phenotypes following differentiation in vitro. Developmental Genetics. 14:356-368.

POWNALL, M.E. and C.P. EMERSON, Jr. (1992) Sequential activation of three myogenic regulatory genes during somite morphogenesis in quail embryos. Devlopmental Biology. 151:67-79.

REIF, A.E. (1989) The discovery of thy-1 and its development. In Cell Surface Antigen Thy-1: Immunology, Neurology and Therapeutic Applications. (A.E. Reif and M. Schlesinger, eds.) Marcel Dekker, Inc., New York. pp 3-45.

REZNIK, M. (1969) Origin of myoblasts during muscle regeneration: Electron microscope observations. Laboratory Investigations. 20:353-363.

RHODES, S.J. and S.F. KONIECZNY (1989) Identification of MRF4: A new member of the muscle regulatory factor gene family. Genes in Development. 3:2050-2061.

ROBERTS, A.B. and M.B. SPORN (eds.) (1990) The transforming growth factor-betas. In Handbook of Experimental Pharmacology. 95:419-472. Heidelberg: Springer-Verlag.

ROBERTS, P. and J.K. McGEACHIE (1992) Propranolol retards revascularization and impedes early myogenesis in regenerating skeletal muscle transplants: an autoradiographic and morphometric study in mice. Journal of Anatomy. 181(1): 101-111.

ROSENBLATT, J.D. and D.J. PARRY (1992) Gamma irradiation prevents compensatory hypertrophy of overloaded mouse extensor digitorum longus muscle. Journal of Applied Physiology. 73(6):2538-2543.

ROSENBLATT, J.D. and D.J. PARRY (1993) Adaptation of rat extensor digitorum longus muscle to gamma irradiation and overload. Pflugers Archives. 423:255-264.

ROSENBLATT, J.D., D. YONG and D.J. PARRY (1994) Satellite cell activity is required for hypertrophy of overloaded adult rat muscle. Muscle and Nerve. 17:608-613.

RUBIN, J.S., H. OSADA, P.W. FINCH, W.G. TAYLOR, S. RUDIKOFF and S.A. AARONSON (1989) Purification and characterization of a newly identified growth factor specific for epithelial cells. Proceedings of the National Academy of Science (USA). 86:802-806.

RUBINSTEIN, N.A., and A.M. KELLY (1981) Development of muscle fibre specialization in the rat hindlimb. Journal of Cell Biology. 90:128-144.

RUDNICKI, M.A., T. BRAUN, S. HINUMA and R. JAENISCH (1992) Inactivation of MyoD in mice leads to up-regulation of the myogenic HLH gene myf-5 and results in apparently normal muscle development. Cell. 71:383-390.

RUMPELT, H.J. and H. SCHMALBRUCH (1969) On the structural elements of muscle spindles in man and rat. Zeitshrift fur Zellforschung und Mikroskopishe Anatomie. 102:601 (abstract).

SAAD, A.D., T. OBINATA and D.A. FISCHMAN (1987) Immunochemical analysis of protein isoforms in thick myofilaments of regenerating skeletal muscle. Developmental Biology. 119:336-349.

SAKAMOTO, H., M. MORI, M. TAIRA, T. YOSHIDA, S. MATSUKAWA, K. SHIMIZU, M. SEKIGUCHI, M. TEREDA and T. SUGIMURA (1986) Transforming gene from stomach cancers and a noncancerous portion of stomach mucosa. Proceedings of the National Academy of Science, USA. 83:3997-4001.

SARTORELLI, V., K.A. WEBSTER and L. KEDES (1990) Muscle-specific expression of the cardiac actin gene requires MyoD1, CArG-box binding factor, and SP1. Genes and Development. 4:1811-1822.

SASSOON, D., G. LYONS, W.E. WRIGHT, V. LIN, L.LASSAR, H. WEINTRAUB and M. BUCKINGHAM (1989) Expression of two myogenic regulatory factors, myogenin and MyoD1 during mouse embryogenesis. Nature. 341:303-307.

SCHIAFFINO, S., L. GORZA and D. PARRY (1993) Age-related failure of muscle regeneration in the dystrophic dy^{2J}/dy^{2J} mouse. Rend. Fis. Acc. Lincei. 9:269-277.

SCHIAFFINO, S., L. GORZA, S. SARTORE, L. SAGGIN, S. AUSONI, M. VIANELLO, K. GUNDERSEN and T. LOMO (1989) Three myosin heavy chain isoforms in type II skeletal muscle fibres. Journal of Muscle Research and Cell Motility. 10:197-205.

SCHIAFFINO, S., L. SAGGIN, A. VIEL, S. AUSONI, S. SARTORE and L. GORZA (1986) Muscle fibre types identified by monoclonal antibodies to myosin heavy chains. In Biochemical Aspects of Physical Exercise. (G. Benzi, L. Packer and N. Siliprandi, eds.). Elsevier, Amsterdam. pp. 27-34.

SCHULTZ, E. (1974) A quantative study of the satellite cell population in postnatal mouse lumbrical muscle. Anatomical Record. 180:589-596.

SCHULTZ, E., M.C. GIBSON and T. CHAMPION (1978) Satellite cells are mitotically quiescent in mature mouse muscle: An EM and autoradiographic study. Journal of Experimental Zoology. 206:451

SCHULTZ, E., D.L. JARYSZAK, M.C. GIBSON and D.J. ALBRIGHT (1986) Absence of exogeneous satellite cell contribution to regeneration of frozen skeletal muscle. Journal of Muscle Research and Cell Motility. 7:361-367.

SCHULTZ, E. and B.H. LIPTON (1982) Skeletal muscle satellite cells: Changes in proliferation potential as a function of age. Mechanism of Aging and Development. 20:377-383.

SCHMALBRUCH, H. (1986) Muscle regeneration: Fetal myogenesis in a new setting. Bibl. Anat.. 29:126-153.

SCHMALBRUCH, H. and U. HELLHAMMER (1977) The number of nuclei in adult rat muscles with special reference to satellite cells. Anatomical Record. 189:169-176.

SCHWEIGERER, L., G. NEUFELD, J. FRIEDMAN, J.A. ABRAHAM, J.C. FIDDES and D. GOSPODAROWICZ (1987) Capillary endothelial cells express basic fibroblast growth factor, a mitogen that promotes their own growth. Nature. 325:257-259.

SEED, J. and S.D. HAUSCHKA (1988) Clonal analysis of vertebrate myogenesis. VIII. Fibroblast growth factor (FGF)-dependent and FGF-independent muscle colony types during chick wing development. Developmental Biology. 128:40-49.

SHAFIQ, S.A. and M.A. GORYCKI (1965) Regeneration in skeletal muscle of mouse, some electron-microscope observations. Journal of Pathology and Bacteriology. 90:123-127.

SLATER, C.R. (1976) Control of myogenesis in vitro by chick embryo extract. Developmental Biology. 50:264-284.

SMITH, T.H., N.E. BLOCK, S.J. RHODES, S.F. KONIECZNY and J.B. MILLER (1993) A unique pattern of expression of the four muscle regulatory factor proteins distinguishes somitic embryonic, fetal and newborn mouse myogenic cells. Development. 117:1125-1133.

SMITH, T.H. and J.B. MILLER (1992) Distinct myogenic programs of embryonic and fetal mouse muscle cells: Expression of the perinatal myosin heavy chain isoform in vitro. Developmental Biology. 149:16-26.

SNOW, M.H. (1977a) The effects of aging on satellite cells in skeletal muscles of mice and rats. Cell and Tissue Research. 185:399.

SNOW, M.H. (1977c) Myogenic cell formation in regenerating rat skeletal muscle injured by mincing. II. An autoradiographic study. Anatomical Record. 188:201-218.

SNOW, M.H. (1979) Origin of regenerating myoblasts in mammalian skeletal muscle. In Muscle Regeneration (A. Mauro, ed.) Raven Press, New York. pp. 91-100.

SORRENTINO, V., R. PEPPERKOK, R.L. DAVIS, W. ANSORGE and L. PHILIPSON (1990) Cell proliferation inhibited by MyoD1 independently of myogenic differentiation. Nature. 345:813-815

SPEIDEL, C.C. (1938) Studies of living muscles. I. Growth, injury and repair of striated muscle, as revealed by prolonged observations of individual fibres in living frog tadpoles. American Journal of Anatomy. 62:179-235.

SPIZZ, G., J.S. HU and E.N. OLSON (1987) Inhibition of myogenic differentiation by fibroblast growth factor or type ß transforming growth factor does not require persistant c-*myc* expression. Developmental Biology. 123:500-507.

SPIZZ, G., D. ROMAN, A. STRAUSS and E.A. OLSON (1986) Serum and fibroblast growth factor inhibit myogenic differentation through a mechanism dependent on protein synthesis and independent of cell proliferation. Journal of Biological Chemistry. 261:9483-9488.

STERN, P.L. (1973) Θ alloantigen on mouse and rat fibroblasts. Nature New Biology. 246:76-78.

STOCKDALE, F.E. (1992) Myogenic cell lineages. Developmental Biology. 154:284-298.

STOCKDALE, F.E. and J.L. FELDMAN (1990) Satellite cells and the concept of cell lineage in avian myogenesis. In The Dynamic State of Muscle Fibres. (D. Pette, ed.) Walter de Gruyter and Co., Berlin. pp. 641-649.

STOCKDALE, F.E. and H. HOLTZER (1961) DNA synthesis and myogenesis. Experimental Cell Research. 24:508.

STROHMAN, R.C., J.X. DIMARIO and N. BUFFINGER (1990) The control of satellite cell growth in skeletal muscle during hypertrophy and regeneration. In The Dynamic State of Muscle Fibres (D. Pette, ed.) Walter de Gruyter, New York. pp. 707-717.

STUDITSKY, A.N. (1964) Annals of the New York Academy of Science. 120:789-801. (as cited by Marechal, 1986)

SUMMERS, P.J., C.R. ASHMORE, Y.B. LEE and S. ELLIS (1985) Stretch-induced growth in chicken wing muscles: role of soluble growth-promoting factors. Journal of Cellular Physiology. 125:288-294.

TAKEMITSU, M., ISHIURA, R. KOGA, K. KAMAKURA, K. ARAHATA, I. NONAKA and H. SUGITA (1991) Dystrophin-related protein in the fetal and denervated skeletal muscles of normal and *mdx* mice. Biochemical and Biophysical Research Communications. 180(3):1179-1186.

TAUBMAN, M.B., C.W.J. SMITH, S. IZUMO, J.W. GRANT, T. ENDO, A. ANDREADIS and B. NADAL-GINARD (1989) The expression of sarcomeric muscle-specific contractile protein genes in BC3H1 cells: BC3H1 cells resemble skeletal myoblasts that are defective for commitment to terminal differentiation. Journal of Cell Biology. 108:1799-1806.

TAYLOR, S.M. and P.A. JONES (1979) Multiple new phenotypes induces in 10T1/2 and 3T3 cells treated with 5-azacytidine. Cell. 17:771-779.

TAYLOR, S.M. and P.A. JONES (1982) Changes in phenotypic expression in embryonic and adult cells treated with 5-azacytidine. Journal of Cellular Physiology. 111:187-194.

TEGTMEYER, P. (1975) Function of simian virus 40 gene A in transforming infection. Journal of Virology. 15:613-618.

TERAVAINEN, H. (1970) Satellite cells of striated muscle after compression injury so slight so as not to cause degeneration of the muscle fibres. Zeitschrift fur Zellforschung und Mikroskopische Anatomie. 103:320-327.

THAYER, M.J., S.J. TAPSCOTT, R.L. DAVIS, W.E. WRIGHT, A.B. LASSAR and H. WEINTRAUB (1989) Positive autoregulation of the myogenic determination gene MyoD1. Cell. 58:241-248.

THAYER, M.J. and H. WEINTRAUB (1990) Activation and repression of myogenesis in somatic cell hybrids: evidence for *trans*-negative regulation of MyoD in primary fibroblasts. Cell. 63:23-32.

TORRES, L.F.B. and L.W. DUCHEN (1987) The mutant mdx: inherited myopathy in the mouse. Brain. 110:269-299.

TROUCHE, D., M. GRIGORIEV, J.-L. LENORMAND, P. ROBIN, S.A. LEIBOVITCH, P. SASSONE-CORSI and A. HAREL-BELLAN (1993) Repression of c-*fos* promotor by MyoD on muscle cell differentiation. Nature. 363:79-82.

TURNER, P.R., T. WESTWOOD, C.M. REGEN and R.A. STEINHARDT (1988) Increased protein degradation results from elevated free calcium levels found in muscle from mdx mice. Nature. 335:735-738.

VACA, K., S.S. STEWART and S.H. APPEL (1989) Identification of basic fibroblast growth factor as a cholinergic growth factor from human muscle. Journal of Neuroscience Research. 23:55-63.

VAIDYA, T.B., S.J. RHODES, E.J. TAPAROWSKY and S.F. KONIECZNY (1989) Fibroblast growth factor and transforming growth factor-ß repress transcription of the myogenic regulatory gene MyoD1. Molecular and Cellular Biology. 9:3576-3579.

VAKAKIS, N., J. BOWER and L. AUSTIN (1994) In vitro myoblast to myotube transformation in the presence of LIF. Neurochem. Int.. In press.

VANDENBURGH, H.H., S. HATFALUDY, P. KARLISCH and J. SHANSKY (1991b) Mechanically induced alterations in cultured skeletal muscle growth. Journal of Biomechanics. 24:91-99.

VANDENBURGH, H.H., M.F. SHEFF and S.I. ZACKS (1984) Soluble age-related factors from skeletal muscle which influence muscle development. Experimental Cell Research. 153:389-401.

VANDENBURGH, H.H., S. SWASIDON and P. KARLISCH (1991a) computer-aided mechanogenesis of skeletal muscle organs from single cells in vitro. FASEB Journal. 5:2860-2867.

VENABLE, J.H. (1966) Morphology of the cells of normal, testosterone-deprived and testosterone stimulated levator ani muscles. American Journal of Anatomy. 119:263.

VIVARELLI, E., W.E. BROWN, R.G. WHALEN and G. COSSU (1988) The expression of sloww myosin during mammalian somatogenesis and limb bud differentiation. Journal of Cell Biology. 107:2191-2197.

VLODAVSKY, I., J. FOLKMAN, R. SULLIVAN, R. FRIEDMAN, R. ISHAI-MICHAELI, J.SASSE and M. KLAGSBRUN (1987) Endothelial cell-derived basic growth factor: synthesis and deposition into subendothelial extracellular matrix. Proceedings of the National Academy of Science (USA). 85:2292-2296.

VOYTIK, S.L., M. PRZYBORSKI, S.F. BADYLAK and S.F. KONIECZNY (1993) Differential expression of muscle regulatory factor genes in normal and denervated adult rat hindlimb muscles. Developmental Dynamics. 198:214-224.

WALKER, B.E. (1972) Skeletal muscle regeneration in young rats. American Journal of Anatomy. 133:369-378.

WALSH, F.S. and MOORE (1986) Expression of muscle cell surface antigen 5.1H11 in infantile or juvenile spinal muscular atrophy. Neurology. 36(8):1140-1142.

WALSH, F.S. and M.A. RITTER (1981) Surface antigen differentiation during human myogenesis in culture. Nature. 289:60-64.

WANG, Y.-C. and P.A. RUBENSTEIN (1988) Epidermal growth factor controls smooth muscle a-isoactin expression in BC3H1 cells. Journal of Cell Biology. 106:797-803.

WEBB, P. (1973) The effect of innervation, denervation and muscle type on the reunion of skeletal muscle. British Journal of Surgery. 60:180-182.

WEBSTER, C. and H.M. BLAU (1990) Accelerated age related decline in replicative life-span of Duchenne muscular dystrophy myoblasts: implications for cell and gene therapy. Somat. Cell Mol. Genetics. 16:557-565.

WEBSTER, C., C.K. PAVLATH, D.R. PARKS, F.S. WALSH and H.M. BLAU (1988) Isolation of human myoblasts with the fluorescence-activated cell sorter. Experimental Cell Research. 174:252-265.

WEINER, H.L. and J.L. SWAIN (1989) Acidic fibroblast growth factor mRNA is expressed by cardiac myocytes in cultures and the protein is localized to the extracellular matrix. Proceedings of the National Academy of Science (USA). 86:2683-2687.

WEINTRAUB, H. S.J. TAPSCOTT, R.L. DAVIS, M.J. THAYER, M.A. ADAM, A.B. LASSAR and A.D. MILLER (1989) Activation of muscle-specific genes in pigment, nerve, fat, liver, and fibroblast cell lines by forced expression of MyoD. Proceedings of the National Academy of Science, USA. 86:5434-5438.

WERNIG, A., A. IRINTCHEV, A. HARTLING, G.STEPHAN, K. ZIMMERMAN and A. STARIZNSKI-POWITZ (1991) Formation of new muscle fibres and tumours after injection of cultures myogenic cells. Journal of Neurocytology. 20:982-997.

WEST, W.T. and E.D. MURPHY (1960) Histopathology of hereditary progressive muscular dystrophy in inbred strain 129 mice. Anatomical Record. 137:279-295.

WHALEN, R.G., J.B. HARRIS, G.S. BUTLER-BROWNE and S. SESODIA (1990) Expression of myosin isoforms during Notexin-induced regeneration of rat soleus muscles. Developmental Biology. 141:24-40.

WHITE, N.K., P.H. BONNER, D.R. NELSON and S.D. HAUSCHKA (1975) Clonal analysis of vertebrate myogenesis. IV. Medium-dependent classification of colony-forming cells. Developmental Biology. 44:346-378.

WHITE, N.K. and S.D. HAUSCHKA (1971) Muscle development in vitro: A new conditioned medium effect on colony differentiation. Experimental Cell Research. 67:479-482.

WOO, M., Y. TANABE, H. ISHII, J. NONAKA, M. YOKOYAMA and K. ESAKI (1987) Muscle fibre growth and necrosis in dystrophic muscles: a comparative study between dy and mdx mice. Journal of Neurological Sciences. 82:111-122.

WRIGHT, W.E. (1985) Myoblast senescence in muscular dystrophy. Experimental Cell Research. 157:343-354.

WRIGHT, W.E. (1992) Muscle basic helix-loop-helix proteins and the regulation of myogenesis. Current Opinions in Genetics and Development. 2:243-248.

WRIGHT, W.E., D.A. SASSOON and V.K. LIN (1989) Myogenin, a foctor regulating myogenesis, has a domain homologous to MyoD1. Cell. 56:607-617.

YABLONKA-REUVENI, Z., S.K. ANDERSON, D.F. BOWEN-POPE and M. NAMEROFF (1988) Biochemical and morphological differences between fibroblasts and myoblasts from embryonic chicken skeletal muscle. Cell and Tissue Research. 252:339-348.

YABLONKA-REUVENI, Z., D.F. BOWEN-POPE and R.S. HARTLEY (1990) Proliferation and differentiation of myoblasts: the role of platelet-derived growth factor and the basement membrane. In The Dynamic State of Muscle Fibres. (D. Pette, ed.) Walter de Gruyter, New York. pp. 693-706.

YABLONKA-REUVENI, Z. and M. NAMEROFF (1985) Desmin is expressed in dividing cells in skeletal muscle mass cultures and clones. Journal of Cell Biochemistry (Supplement) 9B:43.

YABLONKA-REUVENI, Z. and M. NAMEROFF (1987) Skeletal muscle cell populations: separation and partial characterization of fibroblast-like cells from embryonic tissue using density centrifugation. Histochemistry. 87:27-38.

YABLONKA-REUVENI, Z. and A.J. RIVERA (1994) Temporal expression of regulatory and structural muscle proteins during myogenesis of satellite cells on isolated adult rat fibres. Developmental Biology. In press.

YAMADA, S., N. BUFFINGER, J. DI and R.C. STROHMAN (1989) Fibroblast growth factor is stored in fibre extracellular matrix and plays a role in regulating muscle hypertrophy. Medicine and Science in Sports and Exercise. 21:S173-S180.

YUTZEY, K.E., S.J. RHODES and S.F. KONIECZNY (1990) Differential *trans* activation associated with the muscle regulatory factors MyoD1, myogenin and MRF4. Molecular and Cellular Biology. 10:3934-3944.

ZARDINI, D. (1993) Characterization of Type IIX Muscle Fibres in the Mouse. (Ph.D. Thesis - University of Ottawa)

ZARDINI, D. and D.J. PARRY (submitted) Identification, distribution and myosin subunit composition of type IIX fibres in mouse muscles. Muscle and Nerve.

ZHAN, X., A. CULPEPPER, M. REDDY, J. LOVELESS and M. GOLDFARB (1987) Human oncogenes detected by a defined medium culture assay. Oncogene. 1:369-376.