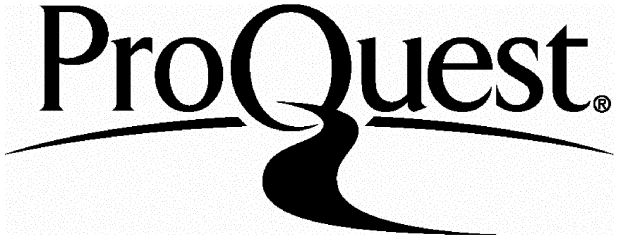
Cell cycle re-entry and the plasticity of myotubes in newt limb regeneration

Cristiana Paranhos Velloso

Thesis submitted to the University of London
for the degree of Doctor of Philosophy

Department of Biochemisty and Molecular Biology,
University College London



Abstract

Urodele amphibians regenerate various structures including the limb, tail, lens, and heart. Underlying this regenerative ability may be the plasticity of cells at the site of injury. Muscle is a key tissue to study these issues because myogenesis proceeds by fusion of mononucleate precursor cells into a multinucleate syncytium which is in a state of stable post-mitotic arrest. Following implantation into a regenerating limb, newt myotubes re-enter the cell cycle and give rise to proliferating mononucleate progeny. In culture, generation of mononucleate cells has never been observed, but the myotube nuclei re-enter the cell cycle and arrest in G2. This is in contrast to their mammalian counterparts, which are refractory to growth factor stimulation, and suggests that cell cycle re-entry may be one aspect of myotube plasticity.

In initial experiments mouse and newt myoblasts were fused to create hybrid myotubes. In these hybrids, DNA synthesis was observed in both mouse and newt nuclei demonstrating that the post-mitotic arrest of mammalian nuclei can be destabilised.

Other experiments addressed the hypothesis that mononucleate cells are generated from multinucleate myotubes by fragmentation of the syncytium, as the nuclei progress through mitosis and cytokinesis. I pursued several strategies to overcome the G2 arrest of cultured myotubes. Transfection with SV-40 large T antigen induced endoreplication of DNA in myotubes, but did not lead to mitosis. Treatment with caffeine resulted in the appearance of fragmented nuclei, which are indicative of aberrant mitosis. This response was dependent on traversal of S-phase by the myotube nuclei. These observations suggested that the block to mitosis is stable in cultured myotubes.

A critical question is whether cell cycle re-entry is required for generation of mononucleate cells following implantation of myotubes into regenerating limbs. Cell-cycle re-entry was blocked in myotubes by X- irradiation or expression of the cdk4/6 inhibitor p16. These myotubes were fluorescently labelled and implanted. Both arrested and control myotubes gave rise to mononucleate progeny, demonstrating that cell cycle re-entry is not required for generation of mononucleate cells.

To my family
and the Brockes lab,

You have inspired more than this

Acknowledgements

I would like to thank my colleagues in this laboratory, past and present, for their exceptional generosity, particularly Mónica Dias, András Simon, Nennaya Kanu, and Phillip Gates.

I will never be able to express the depth of my gratitude and admiration for Elly Tanaka, Anoop Kumar, and Jeremy Brockes.

I have been very fortunate, and I shall remember you fondly.

I would also like to thank Dr. Parmjit Jat for the use of his tissue culture facilities, Dr. John Scholes for help with X-irradiation, and those researches mentioned in Chapter 2 for providing plasmids and antibodies. Finally, I would like to acknowledge CAPES, Brazil for the financial support of my studies.

Contents

List of Figures

List of Tables

Abbreviations

A1	ankle 1 newt cell line
AEC	apical epidermal cap
AER	apical ectodermal ridge
APC	anaphase promoting complex
ATP	adenosine triphosphate
bHLH	basic helix loop helix
BMP	bone morphogenetic protein
BrdU	5-bromo-2-deoxyuridine
CAK	cdk activating kinase
cdc	cell division cycle
CDI	cyclin dependent kinase inhibitor
cdk	cyclin dependent kinase
cDNA	complementary deoxyribonucleic acid
C/EBP	CCAAT/enhancer binding proteins
DNA	deoxyribonucleic acid
Dlx	newt homologue of *Drosophila distalless* gene
ECM	extracellular matrix
EGF	epidermal growth factor
FBS	foetal bovine serum
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
G1	gap 1 phase of the cell cycle
G2	gap 2 phase of the cell cycle
eGFP	enhanced green fluorescent protein
H3	histone H3
HLH	helix loop helix
IGF	insulin-like growth factor
M	mitotic phase of the cell cycle
MADS	*MCM1, agamous, deficiens, serum response* superfamily of DNA binding proteins
MAPK	mitogen activated protein kinase
M cadherin	muscle cadherin
MCM	minichromosome maintenance proteins
MEF	myocyte enhancer factor
MHC	myosin heavy chain
MMP	matrix metalloproteinases
MRF	muscle regulatory factor
Msx	newt homologue of *Drosophila muscle segment homeobox* gene
N cadherin	neuronal cadherin
NEB	nuclear envelope breakdown
PCNA	proliferating cell nuclear antigen
PDGF	platelet derived growth factor
PI3	phosphatidylinositol-1,4,5-triphosphate
pRb	retinoblastoma protein
Rb	retinoblastoma gene
S	DNA synthesis phase of the cell cycle
TGF	transforming growth factor

UV	ultraviolet
WE	wound epithelium

Single letter code for nucleotide bases

A	adenine
C	cytosine
G	guanine
T	thymine

Single and three letter code for amino acids

Amino acid	Three letter	Single letter
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Chapter 1

Introduction

1.1 Introduction

Multicellular organisms are composed of cells which are phenotypically and physiologically distinct from one another, and these differences enable them to carry out their specific functions (Alberts et al., 1994). The fertilised egg gives rise to all of the different cell types in the organism, and it is therefore a totipotent cell. Differentiation is a process which occurs gradually during development and is associated with changes in gene expression patterns (Davidson, 1986). As cells follow certain differentiation pathways, they become restricted in their developmental potential and express a subset of genes. For example, once a cell enters the mesodermal lineage, it will only give rise to mesodermal cell types, but not to neurons, which are ectodermal in origin; after a cell enters the muscle lineage, it does not form cartilage or connective tissue (Buckingham, 1992; Ordhal and LeDouarin, 1992; Tajbakhsh and Buckingham, 2000). For any organism, maintenance of the differentiated state must be stable for the proper functioning of tissues and organs. In most tissues, proliferative activity is low and expression of markers of differentiation in cells is associated with cell cycle withdrawal (Alberts et al., 1994). How cells differentiate, and how the differentiated state is maintained are fundamental questions for biologists.

The alteration in gene expression patterns associated with differentiation is not the result of the loss of genetic material. All the differentiated somatic cells of the vertebrate animal possess the same genome as the egg - with the exception of the B lymphocyte, in which chromosomal rearrangements leading to the loss of DNA are important for the generation of antibody diversity (Tonegawa, 1983; Yancopoulos and Alt, 1986; Alt et al., 1987). This was first shown by nuclear transplantation experiments carried out with amphibian eggs and oocytes in the 1950s and 1960s (Briggs and King, 1952, 1960; Gurdon, 1962 a, b; Gurdon and Uehlinger, 1966). The most relevant example for this thesis is the experiment of Gurdon and colleagues (1984) in which post-mitotic nuclei of somitic myocytes of stage 26 *Xenopus* embryos (post neurula) were shown to sustain development to the swimming tadpole stage in 2% of eggs in which the nuclei had been inactivated by UV irradiation. Nuclear transplantation is now a viable technique

for the production of fertile mammals from the nuclei of differentiated adult cells (Wilmut et al., 1997). These nuclear transplantation experiments also illustrate the importance of the cytoplasm and cellular environment in regulating gene expression in the nucleus, and show that cell differentiation is not intrinsically irreversible, at least at the nuclear level. This contrasts with the stability of the differentiated state as generally observed.

Plasticity, as referred to in this thesis, is the ability of a cell to undergo reversible changes in its differentiation program. Although reversal of the differentiated state is not considered to be a 'normal' option for a cell, it does occur in the context of adult and larval regeneration. This thesis is concerned with a striking example – the production of proliferating mononucleate cells from the post-mitotic syncytial skeletal myotube, which occurs experimentally in the context of limb regeneration in urodele amphibians. I will first describe the events of urodele regeneration and the evidence for plasticity. In view of the importance of the skeletal myotube as a target cell, the regulation of vertebrate myogenesis is considered next. A major question in the thesis concerns the role of cell cycle re-entry in plasticity, and I have therefore reviewed the cell cycle in relation to muscle differentiation.

1.2 Amphibian limb regeneration

1.2.1 The context of urodele regeneration

As first described by Spallanzani in his *Prodromo* (1768), urodele amphibians (which retain the tail after metamorphosis) are remarkable in their regenerative abilities, both as larvae and adults. The anuran amphibians, such as frogs and toads, posses extensive regenerative abilities as larvae, but not after metamorphosis (Wallace, 1981; Thouveny and Tassava, 1998). Newts and axolotls can regenerate their limbs, tails, jaws, lens, iris, and retina, as well as various internal organs including the central nervous system and large sections of the heart (Figure 1.1; reviewed by Brockes 1997; Eguchi et al., 1974; Ghosh et al., 1994;

Figure 1.1. Urodele amphibians such as the North American Red Spotted Newt, *Notophthalmus viridescens*, are able to regenerate various body parts as adults (shown here). These include the limbs (1), the tail (2), the jaws (3), ocular structures, (4) and internal organs such as the heart.

Oberpriller and Oberpriller, 1974; Oberpriller et al., 1995; Okada, 1991). A long standing and difficult question has been why regeneration on this scale is curtailed or absent in other vertebrates, including mammals. One hypothesis is that the extensive cell proliferation and reversal of differentiation that are associated with regeneration might increase the risk of tumour development and malignant transformation, but this is unlikely since urodeles are very resistant to tumour formation in the regenerative territories (reviewed by Brockes, 1998). There are no compelling answers to these questions, but the widespread occurrence of regenerative phenomena among invertebrate phyla would seem to suggest that regeneration is the 'ground state', and that the response may be lost or inhibited in most vertebrates (Thouveny and Tassava, 1998). Support for this theory comes from the fact that when regeneration does occur in certain invertebrate species, it is often not observed in closely related ones (Goss, 1969). The hypothesis that regeneration is suppressed in some species is more appealling than the hypothesis that regeneration would have appeared independently several times in the course of evolution.

A key aspect of regeneration in urodeles, and perhaps the underlying reason for their regenerative capabilities, is the local plasticity of differentiated cells. The complex events of limb regeneration, which will be discussed in detail, do not allow us to rule out a contribution from a stem cell population, but in the somewhat simpler case of lens regeneration such a contribution is not detectable. Lens regeneration has been an attractive model for study since it was first described by Wolff (1895), because it is a simple system involving the conversion between two cell types. The regenerated lens arises from the dedifferentiation and transdifferentiation of cells from the pigmented dorsal iris epithelium. Dedifferentiation is the loss of differentiated characteristics and may occur by a reversal of differentiation pathways or through separate pathways. Transdifferentiation is the process by which one differentiated cell type gives rise to another. In normal conditions, the cells of the dorsal iris epithelium have a distinct and stable phenotype, and possess pigment granules. Following lentectomy, the cells begin to proliferate and lose their pigment granules, forming

a vesicle of depigmented cells that is continuous with the dorsal iris (reviewed by Reyer, 1954). The depigmented cells lengthen, synthesise lens specific crystallins, and the vesicle detaches from the dorsal iris to replace the lens (Yamada, 1977). *In vivo*, the lens can also regenerate from the pigmented retinal epithelium when the iris is removed, since the iris regenerates from this epithelium. Formation of lens cells was observed in culture from dorsal iris explants (Yamada et al., 1974), but the critical demonstration of transdifferentiation of pigmented epithelial to lens cells came from clonal cultures of pigmented dorsal iris and retinal epithelial cells (Eguchi et al., 1974; Okada et al., 1979). The pigmented epithelium of the iris or retina of other vertebrates, including chicks and humans, is also able to dedifferentiate and transdifferentiate into lens cells under the appropriate conditions; these include treatment with phenylthiourea to inhibit melanogenesis and use of fibroblast growth factor 2 (FGF2) to promote proliferation (Eguchi and Okada, 1973; Itoh and Eguchi, 1986; Eguchi, 1988; Okada ,1991; Agata et al., 1993). Plasticity of these pigmented epithelial cells is therefore widespread under the defined culture conditions. It is possible that these extracellular cues are present in the urodele amphibians but not in other vertebrates, and/or that there is an intrinsic difference between the iris cells of urodeles and other animals. Proof of either hypothesis is lacking.

The response of newt cardiomyocytes to injury also illustrates the importance of plasticity in the regenerative response and the contrast with the mammalian response. Newt cardiomyocytes are able to proliferate after transection of the heart muscle, and this proliferation leads to growth and functional repair of the heart (Oberpriller and Oberpriller, 1974; Oberpriller et al., 1995). Importantly, the proliferative response is localised to the site of injury. Adult newt cardiomyocytes are also able to proliferate in culture (Tate et al., 1989). In mammals, cardiomyocyte proliferation ceases perinatally and cardiac growth is achieved after birth by hypertrophy; cultured neonatal and adult cardiomyocytes do not proliferate (MacLellan and Schneider, 1999; Brodsky, et al., 1980). In consequence, cardiac lesions in mammals result in fibrosis and minimal restoration of heart function.

The focus of this thesis is the plasticity of the skeletal muscle myotube in the context of limb regeneration, and the mechanisms underlying this plasticity. A second theme is to understand to what extent urodele myotubes are intrinsically different from their mammalian counterparts.

1.2.2 Events of limb regeneration

The events of limb regeneration in the adult newt are shown in the series of photographs of Figure 1.2 (reviewed in Wallace, 1981; Stocum, 1995; Tsonis 1996, 2000). Following amputation, the wound surface is covered by cells which migrate from the circumference of the epidermis surrounding the stump, and form the wound epithelium (WE). In the adult newt, this process takes from 6 to 12 hours and occurs in the absence of cell division (Hay and Fischman, 1961; Hay, 1962; Schmidt, 1968; Repesh and Oberpriller, 1978, 1980). The role of the WE in cell proliferation during limb regeneration is discussed later. It also functions, together with the leucocytes which migrate to the injury, in eliminating cellular debris (Singer and Salpeter, 1961). The neutrophils and macrophages which invade the wound site may secrete proteases and protease activators that contribute to the extensive remodelling of the extracellular matrix and histolysis which occurs during this phase (Weiss and Rosenbaum, 1967; Mailman and Dresden, 1976; Gulati et al., 1983; Mescher and Cox, 1988).

Regeneration proceeds from a mesenchymal growth zone or blastema which forms beneath the WE. The progenitor cells of the blastema can be distinguished from the cells of a normal limb by a variety of antibody markers of which the first to be identified was the 22/18 antigen (Kintner and Brockes, 1984; Ferretti and Brockes, 1991). The origin of the blastema from differentiated cells is considered in detail in 1.2.5. After the blastema is formed, the patterning for the tissues and structures of the normal limb occurs, and apparently involves interactions between the blastemal cells (Bryant and Gardiner, 1992). The blastema is an autonomous, self-organising system, and gives rise to the structure appropriate for its site of origin, even after transplantation into neutral territories such as the ocular globe and the dorsal fin (Stocum, 1968; Thoms and Stocum, 1984).

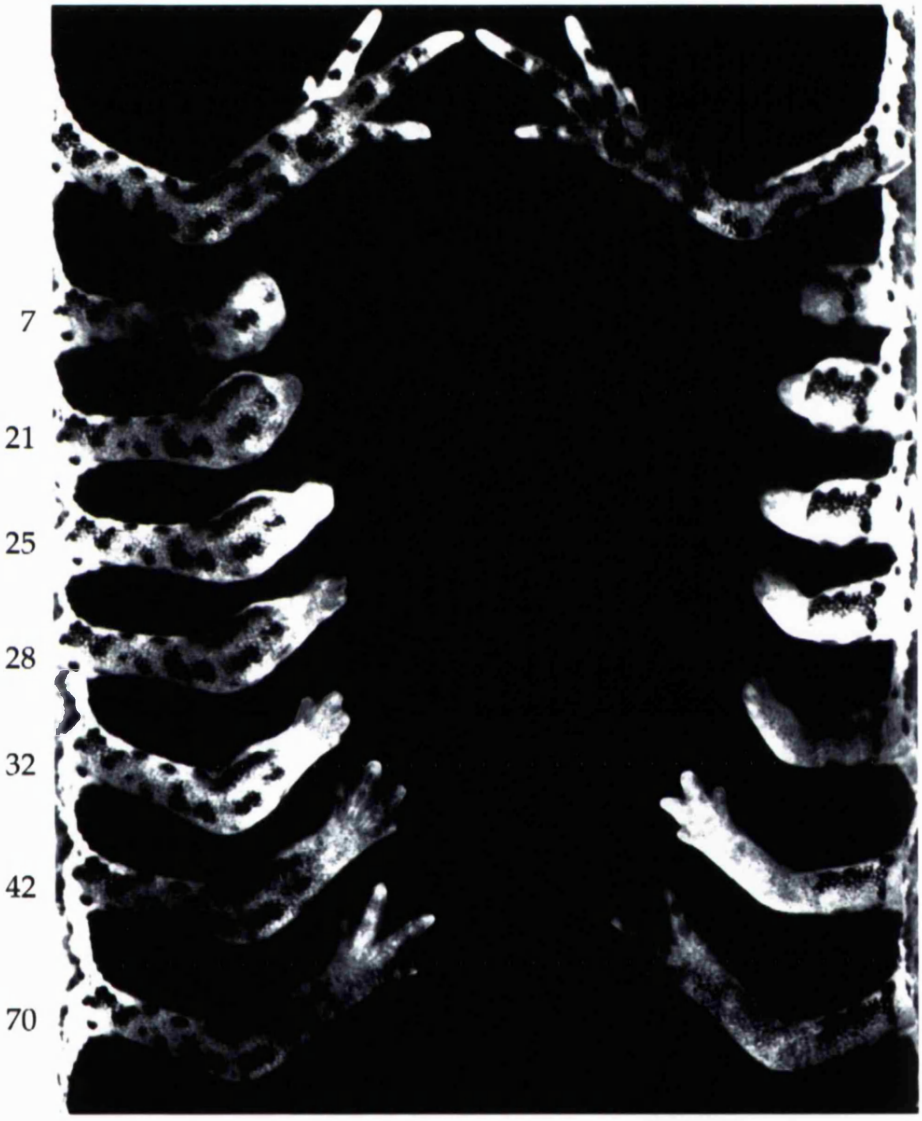


Figure 1.2. Regeneration of the adult newt forelimb. Following amputation at distal (mid radius/ulna, left), or proximal (mid humerus, right) sites, complete regeneration of the amputated structure is observed. At 21 days after amputation a mid bud stage blastema is readily visible. Proliferation and differentiation of the blastema proceed and give rise to a palette stage limb with digits at 28 days. Further growth and morphogenesis occurs and regeneration is complete at 70 days. The site of amputation is no longer distinguishable (modified from Goss, 1969). The numbers on the left indicate days after amputation.

Blastemal cells inherit or otherwise derive a memory of their positional identity and give rise only to the structures distal to the plane of amputation (Butler, 1955; Pescitelli and Stocum, 1980).

Both dedifferentiation and cell proliferation contribute to the growth of the blastema. Dedifferentiation occurs in the first 2-3 weeks after amputation until the blastema reaches a stage called the mid bud (Figure 1.2, 21 days) when it is still composed exclusively of mesenchymal cells. The dedifferentiated cells re-enter the cell cycle (Hay and Fischman, 1961) and proliferate intensively. Continuous labelling experiments with [^{3}H]-thymidine indicate that over 90% of blastemal cells eventually synthesise DNA (Tassava et al., 1987; Tomlinson and Barger, 1987). Other events at an early stage are important for regeneration. The cells of the wound epithelium begin to proliferate starting at about 5 days after amputation (Hay and Fischman, 1961), and the WE thickens into a structure called the apical epidermal cap (AEC). In addition, regenerating axons begin to reinnervate the mesenchymal and epithelial components of the limb blastema within 2-3 days after amputation (Singer, 1949), and it becomes revascularised by capillaries sprouting from the blood vessels at the stump.

Once the blastema has reached the late bud stage, it flattens distally (Figure 1.2, 25 days), and differentiation and morphogenesis begin. Differentiation occurs, as in the developing limb, in a proximal to distal (PD) direction. The first signs of histological differentiation are seen in the central part of the blastema where the cartilagenous condensations of the skeletal limb elements appear. Muscle differentiation then begins as elongated cells aggregate alongside the condensations. Subsequently, the outline of the digits at the distal extremity of the blastema becomes evident (Figure 1.2, 28 days), and this is followed by the gradual formation of the skeletal elements of the digits. In the adult newt, ossification succeeds chondrification, again in a proximal to distal direction. At this point, a complete but small regenerate is formed, and continues to grow until it reaches the size of the original limb (Chalkley, 1954; Iten and Bryant, 1973).

The critical processes for blastema formation are dedifferentiation of stump tissues and proliferation of the dedifferentiated cells. Blastema formation also has at least two additional requirements: the presence of the wound epithelium and the presence of nerves; simple skin wounds do not elicit a regenerative response unless the proximal stump of a peripheral nerve is inserted (Egar, 1988). The role of the wound epithelium and the role of the nerve supply will now be considered.

1.2.3 The role of the wound epithelium

Various experiments suggest that the accumulation of mesenchymal cells in the blastema occurs as a result of interactions that are established between these cells and the WE. If the WE is removed or if its formation is prevented by grafting of a skin flap over the stump, the blastema does not form (Thornton, 1957). Association of an irradiated epidermis with healthy stump tissues precludes regeneration; the stump cells dedifferentiate, but do not proliferate (Lhereux and Carey, 1988). Displacement of the wound epithelium to an ectopic site, leads to ectopic blastema formation underneath the WE (Thornton, 1960).

The WE is in close communication with the mesenchymal cells during the period of blastema formation. The basal stratum of the wound epithelium is in direct contact with the internal tissues of the stump as both the dermis and the basement membrane of the epidermis end at the wound borders (Singer and Salpeter, 1961; Schmidt, 1968). During regeneration the basement membrane is not completely reconstituted until digit formation begins (Neufeld and Aulthouse, 1986).

It is not known how the cells of the wound epithelium exert their influence on the cells of the blastema but several observations suggest that it is through secretion of diffusible factors. Chapron (1974) observed that a glycoprotein synthesised in the wound epithelium is transported to the blastemal cells. In cultured blastemas, cells only remain undifferentiated and proliferate rapidly in the presence of the WE (Globus et al. 1980); AEC extracts can sustain blastema cell proliferation in culture (Boilly and Albert, 1988; Hondermarck and Boilly, 1992; Boilly et al., 1991).

One potentially important molecule secreted by the WE in culture is 9-cis retinoic acid, as it may have a role in establishing the positional identity of cells in the limb (Viviano et al., 1996).

Other observations also suggest that the WE is a secretory epithelium, although it does not contain glands. Tassava and co-workers isolated a monoclonal antibody, WE3, which reacts to the wound epithelium but not with normal epidermis; it is present in the glands of the skin (Tassava et al. 1986). The WE3 antigen is observed in epithelia specialised in active transport and secretion such as endothelium and the epithelia of the gastrointestinal tract (Goldhamer et al., 1989).

1.2.4 The nerve supply

A requirement for nerves in limb regeneration has been known since the experiments of Todd in the 19th century (Todd, 1823) involving denervation of regenerating limbs, but it was only in the middle of the 20th century that the quantitative nature of this requirement was established. Marcus Singer and his co-workers performed a series of partial denervations followed by careful quantitation of the number of fibers in regenerating versus non regenerating limbs. Their observations demonstrated that there is a threshold level of innervation, equivalent to 30% of normal limb innervation, below which regeneration does not occur (Singer, 1952, Singer et al., 1967). This threshold is independent of the type of innervation (motor or sensory) and of connection to the central nervous system, since implanted ganglia and spinal chord segments can sustain blastema formation on denervated limbs (Thornton 1956; Kamrin and Singer, 1959).

According to Singer's neurotrophic theory (Singer, 1952), the nerve fibres exert their action by releasing a factor which promotes blastema cell proliferation and DNA synthesis (Singer and Craven 1948; Dresden 1969; Singer and Caston 1972; Kelly and Tassava, 1973; Mescher and Tassava, 1975; Geraudie and Singer, 1978; Tassava and McCullough, 1978; Maden, 1979; Boilly et al., 1985; Munaim and

Mescher, 1986). Experiments in culture suggested that the neurotrophic 'factor' is diffusible, since it can act through micropore membranes (Globus and Vethamany-Globus, 1977; Carlone and Foret, 1979), and various candidate factors have been proposed which both have mitogenic activity on blastemal cells and are found in the blastema (Munaim and Mescher, 1986; Anand et al., 1987). Glial growth factor, a neuregulin, is one likely candidate as it is specifically mitogenic for a subpopulation of blastemal cells which is observed to proliferate only in innervated limbs (Brockes and Kintner, 1986; Wang et al., 2000). Beads soaked in FGF2 have been shown to support the regeneration of denervated limbs (Mullen et al., 1996). It has also been suggested that the nerves may exert their action indirectly by causing the release of FGFs and other growth factors from their sites of binding on the extra-cellular matrix (Zenjari et al., 1997).

An alternative proposal for the role of the nerve is that after denervation, the denervated Schwann cells release an inhibitor of proliferation, for example a member of the transforming growth factor β (TGFβ family (Ferretti and Brockes, 1991). Production of the inhibitor would then be turned off after reinnervation by the regenerating axons; an example of this mechanism in the rodent peripheral nerve was given by Scherer et al. (1993).

Not all stages of regeneration are nerve–dependent. Denervation prior to amputation or before blastema cells start to proliferate precludes blastema formation, and denervation during the proliferative phase of regeneration abrogates further growth. Once differentiation and morphogenesis begin, however, a small, but morphologically and histologically normal regenerate is formed regardless of the presence of nerve fibres (Singer and Craven, 1948; Powell 1969; Kelly and Tassava, 1973).

The only instance where regeneration has been observed in the complete absence of nerves is in the aneurogenic limb - a limb that develops in the absence of nerves. It is thought that newt limbs acquire nerve-dependency during development as a result of innervation; innervation of a nerve-independent,

aneurogenic limb causes it to become nerve-dependent (Yntema, 1959; Thornton and Thornton, 1970).

The elucidation of the molecular basis of nerve dependence has proven notoriously difficult. In the 1980s most studies were directed at identifying the elusive neurotrophic factor. One important finding was the identification of the regeneration associated antigen 22/18, which recognises a conformational change in an intermediate filament protein and identifies a subpopulation of blastemal cells that is nerve-dependent for proliferation (Kintner and Brockes, 1984, 1985; Ferretti and Brockes, 1990). The appearance and down-regulation of the antigen coincide with the nerve-dependency status of the different stages of the blastema (Fekete and Brockes, 1988). Significantly the aneurogenic limb does not contain 22/18 positive cells, but the antigen appears following innervation and establishment of nerve-dependence (Fekete and Brockes, 1987). Other blastemal antigens label cells that are not nerve-dependent for proliferation. It is possible, therefore, that responsiveness to mitogenic stimuli may also be regulated in the responsive population.

One recently identified gene seems to play a role in the transition from nerve dependence to nerve independence in regenerating limbs: *Dlx* is the newt homologue of *Drosophila* distalless (*Dll*) which is required for distal outgrowth of appendages (Mullen et al., 1996; Panganiban et al., 1997). *Dlx* is expressed in the apical epidermis and reaches its maximal expression levels when the blastema becomes nerve-independent. Denervation at early nerve-dependent stages, but not late stages, results in loss of *Dlx* expression. In denervated limbs, implantation of beads soaked in FGF2 rescues expression of *Dlx* and results in normal regeneration (Mullen et al., 1996). The nerve supply may therefore maintain the expression of genes associated with limb outgrowth.

Denervation at around the time of amputation does not affect either cell cycle re-entry or dedifferentiation of stump tissues, and it has been proposed that innervation is required for traversal of G2/M by the dedifferentiated cells

(Tassava and McCullough, 1978) Other researchers, however, have proposed that innervation is required in G1, and this point remains unresolved (Olsen and Tassava, 1984).

1.2.5 Origin of the limb blastema

The local origin of blastemal cells was first demonstrated by experiments involving X-irradiation of limbs prior to amputation. Grafting of triploid larval limbs onto irradiated diploid host larvae resulted in the formation of blastemas and regenerates containing triploid cells (Butler, 1935). The origin of the cells in the regenerate had to be the grafted limb as the host limb was unable to regenerate. Blastemal cells arise locally within about 100 μm of the plane of amputation, as shown by other experiments involving inhibition of limb regeneration by X-irradiation. Exposure of a limited portion of the limb to X-rays followed by amputation through the irradiated region did not lead to regeneration, whereas amputation through regions immediately proximal or distal to the irradiated zone did elicit regeneration (Butler and O'Brien, 1942; Wolff and Wey Schué, 1952).

Several studies have used markers such as trinucleolar nuclei or [^{3}H]-thymidine to label differentiated tissue or cells which were implanted into normal or X-irradiated blastemas. In an early example, [^{3}H]-thymidine labelled cartilage was implanted into normal blastemas and careful analysis established the purity of the donor population as differentiated chondrocytes (Steen, 1968). These gave rise to labelled cartilage, bone and connective tissue, but not to muscle. Similar conclusions were reached in respect of transplantation of triploid cartilage into irradiated diploid host limbs (Namenwirth 1974). If instead of cartilage, triploid muscle was transplanted, the regenerate contained triploid cartilage, muscle cells, and fibroblasts, but not labelled melanocytes or glands of the dermis (Namenwirth, 1974). In this case, however, the transplanted tissue may have contributed myofibres, connective tissue fibroblasts, and possibly post-satellite cells (see below), so it is not possible to interpret the result. Other studies have

underlined the importance of the contribution to the blastema from dermal fibroblasts (Muneoka et al., 1984; 1986).

The ability of resident muscle fibres to generate blastemal cells has not been demonstrated conclusively and remains controversial. The source of myogenic cells for muscle repair in other vertebrates is considered to be the satellite cell, which arises during muscle development and lies beneath the basal lamina of the myofibre (Mauro, 1961; Allen and Rankin, 1990). Although satellite cells as such do not exist in newt muscle, a similar cell type which is located outside the basement membrane of the myofibre, within its own basal lamina, has been described and termed the post-satellite cell (Popiela, 1976; Cameron et al., 1986). It has been reported that post-satellite cells in muscle explants are the only cell types to label with [^{3}H]-thymidine in the first six days of muscle explant cultures (Cameron et al., 1986). These labelled cells subsequently undergo myogenesis in culture, but their contribution to the blastema has not been demonstrated directly, and no cell marker has been described that would allow their unambiguous identification.

There are other observations which suggest that myofibres are able to dedifferentiate. Electron microscopy studies established that mononucleate cells are not present in uninjured muscle but appear within the myofibre external lamina following amputation (Hay, 1979). It was suggested that these mononucleate cells arise as a result of the formation of a membrane between the nucleus and the sarcoplasm derived from vesicles of the endoplasmic reticulum. Subsequently, the muscle basement membrane disappears and the mononucleate cells are free to participate in blastema formation. The possibility that these cells are post-satellite cells cannot be discounted, and it is very difficult to rigorously infer lineage relationships in a complex tissue context by direct observation, without the use of cell markers.

Studies with molecular markers have suggested that some blastema cells have a muscle origin. Mononucleate cells that label with the antibodies 22/18, the regeneration associated antigen, and 12/101, a muscle marker, have been

observed in the blastema 14 days after amputation (Kintner and Brockes, 1984; Griffin et al., 1987). The authors of this study argued that these double-labelled cells arise from myofibre dedifferentiation. An equally probable scenario is that these cells are indicative of myogenesis, as they are also observed with the same time course in muscle that is re-implanted into a normal limb after mincing, a context that does not involve cell dedifferentiation and blastema formation (Griffin et al., 1987).

Another study used a heritable molecular marker associated with skeletal muscle, the hypomethylation of a site in the cardioskeletal myosin gene. The hypomethylation of this site was detected at higher levels in DNA of the cartilage of normal regenerates and regenerates from which the humerus had been removed, compared to DNA from the cartilage of unamputated limbs (Casimir et al., 1988). The authors concluded that as much as 10% of cartilage cells in the regenerate had a muscle origin, and therefore that the muscle contributes to the blastema, although the precise type of muscle cell is not identified by this study. A subset of blastemal cells express the myogenic regulatory factor *Myf5*, which may indicate their muscle origin (Simon et al., 1995).

One striking feature of myofibre nuclei close to the amputation plane is that they re-enter the cell cycle and synthesise DNA (Hay, 1959; Kumar et al., 2000). This may be indicative of the plasticity of these cells *in vivo*, since DNA synthesis is also observed in multinucleate myotubes which give rise to mononucleate progeny after implantation into a regenerating limb (Kumar et al., 2000 see below); myotubes, although differentiated, are not as structurally specialised as myofibres.

The only instance where a direct contribution of cells of muscle origin to the blastema, and subsequently to the regenerate was observed involved the implantation of myotubes injected with a lineage tracer or expressing an integrated retrovirus marker (Lo et al., 1993, Kumar et al., 2000). In view of the

importance of these studies for my work, they are now considered in more detail.

1.2.6. Implantation of cultured myotubes

An important approach to the problems of limb regeneration has come from the derivation of tissue culture cells with an indefinite replicative life span from the newt limb (Ferretti and Brockes, 1988). It is difficult to dissociate adult limb or limb blastema tissue and retain viability, but it was possible to explant fragments of mesenchymal tissues from the normal limb and the limb blastema, and passage the cells migrating onto the substrate. Cultured cells from all tissues expressed to different extents the regeneration-associated antigen 22/18. This was true for normal limb mesenchyme and it appears that the circumstances of explantation mimic some of the early events after amputation. Although the resulting populations are not clonal, and it has been very difficult to propagate colonies from a single cell, they have been grown successfully without any indications of crisis or senescence, in some cases for more than 200 generations (P. Ferretti, unpublished results).

One population, termed A1, was isolated by P. Ferretti from an explant of ankle level mesenchyme. When the serum concentration in the medium is lowered to 0.5% for several days, these cells fuse to form contractile multinucleate myotubes expressing sarcomeric myosin heavy chain, and the muscle specific antigen 12/101 (Lo et al., 1993; Tanaka et al., 1997). The myotubes also enter a state of post mitotic arrest, such that they are completely refractory to several mitogenic growth factors, for example platelet derived growth factor (PDGF), which act on the mononucleate A1 cells (Tanaka et al., 1997). The myotubes can be purified from most remaining mononucleate cells by sequential sieving through 100 μm and 35 μm nylon meshes – the myotubes are retained on the second mesh (Lo et al., 1993). If the rigorous removal of all mononucleate cells is required, these can be ablated with a micropipette under control of a micromanipulator (Kumar et al., 2000). The relatively large myotubes are also convenient for transfection or labelling by microinjection.

In the initial experiments, A1 myotubes in low density culture were selectively microinjected with the established lineage tracer, rhodamine conjugated lysinated dextran (Lo et al., 1993). This tracer was not taken up from the medium by cultured newt cells or transferred from labelled to unlabelled cells, even in very dense cultures. The selectivity of microinjection was checked by fluorescence microscopy, and most injected preparations contained no labelled mononucleate cells (Lo et al., 1993). The myotubes were pelleted and implanted under the wound epidermis of the limb blastema at 3-7 days after amputation (a detailed account of the current protocol is given in the Materials and methods, Figure 2.4). At 7-10 days after implantation, about 100 strongly labelled mononucleate cells (15-25% of implanted nuclei) were observed in the blastema, but not in mock implanted controls (Figure 1.3 A). Myotubes were also double labelled by exposing the mononucleate cells to [^{3}H]-thymidine prior to fusion and microinjecting the tracer after fusion. After implantation, double-labelled mononucleate cells were observed. The number of labelled cells increases such that by 2-3 weeks, hundreds to thousands of cells were observed. By 4-6 weeks the staining intensity of labelled cells was weaker, presumably due to dilution of the tracer by cell division. Interestingly, a small number of cases were reported of clones of 2-4 clearly labelled cells in cartilage nodules in the regenerate (Figure 1.4, Lo et al., 1993). Therefore, transdifferentiation from muscle to cartilage might occur at a low level. In summary, the experiments of Lo et al. (1993) provided the first compelling demonstration of reversal of the mononucleate to multinucleate transition.

This approach was significantly extended by the experiments of Kumar et al. (2000), who used two additional and distinct methods to label the A1 myotubes. The first was to introduce a genetic marker by stably infecting the dividing mononucleate cells with a pseudotyped retrovirus expressing human placental alkaline phosphatase (Figure 1.3 C). After fusion the myotubes were rigorously purified from mononucleate cells and implanted as before. The results were comparable to those of Lo et al. (1993), in that the virally labelled myotubes were

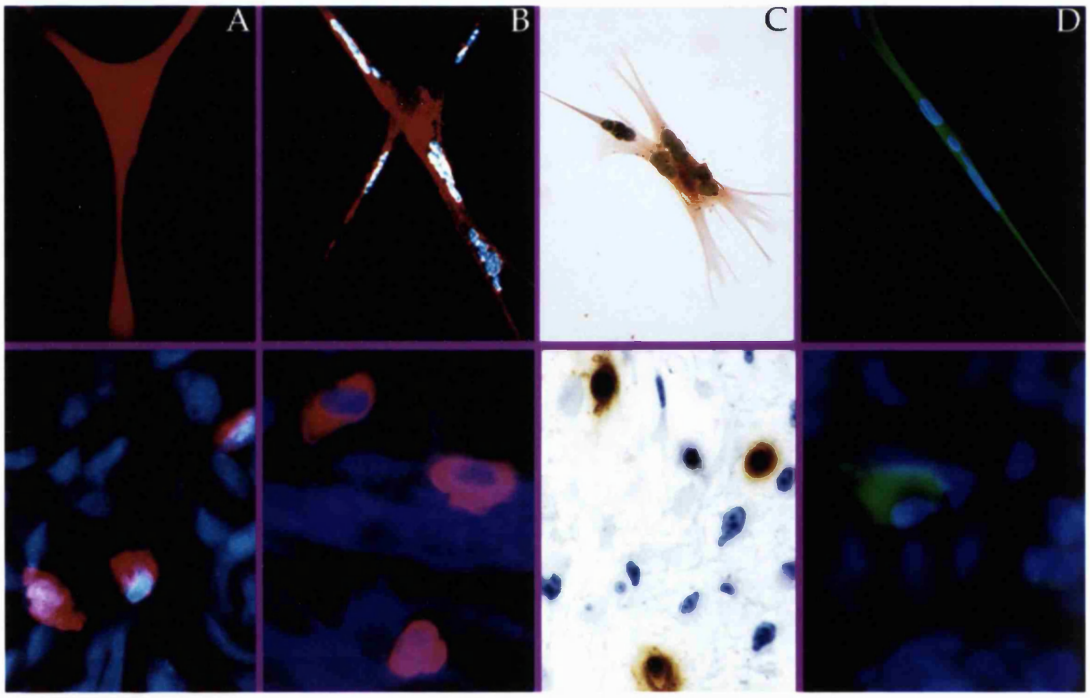


Figure 1.3. Multinucleate myotubes give rise to mononucleate cells after implantation into a regenerating limb. Top panels - myotubes in culture labelled by a lineage tracer, rhodamine-dextran (**A**), a lipophilic cell tracker dye PKH-26 (**B**), by an integrated provirus expressing the markers human placental alkaline phosphatase (**C**), or green fluorescent protein (**D**). Bottom panels - mononucleate cells observed 7-10 days after implantation of the corresponding labelled myotubes. Nuclei in A, B and D are stained with Hoechst dye while those in C are stained with hematoxylin (modified from Lo et al., 1993; Kumar et al., 2000; C. P. Velloso, unpublished).

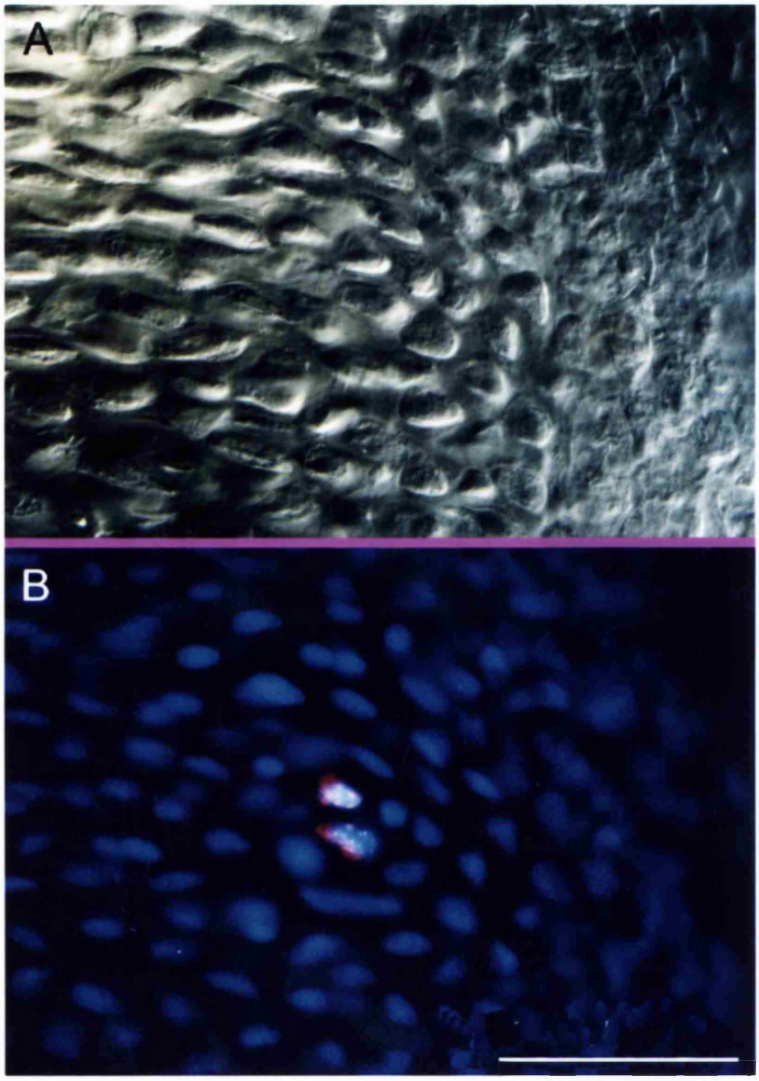


Figure 1.4. Rhodamine-dextran labelled myotubes give rise to some labelled nuclei in cartilage at 26 days after implantation into a blastema. (**A**) Interference microscopy of section of a cartilage nodule showing the regular arrangement of cells in the matrix. (**B**) Hoechst staining of nuclei in (**A**). Note the two rhodamine-labelled nuclei within the cartilage matrix. The scale bar is 100 μm (modified from Lo et al., 1993)

effectively converted to labelled mononucleate cells. The second method was to use a cell tracker dye, called PKH 26 (Figure 1.3 B), to label the myotube. This lipophilic dye partitions into cell membranes and is an effective marker; the labelled myotubes behaved after implantation in the same way as the dextran or virus labelled cells. When a viable nuclear stain (Hoechst 33342) was used in conjunction with PKH 26, double-labelled mononucleate cells were found after implantation. This is a second demonstration of the thymidine/dextran double label result of Lo et al (1993). I have also used green fluorescent protein (GFP) expressed either from an injected plasmid or from an integrated provirus to label myotubes and have obtained comparable results (Figure 1.3 D, C. P. Velloso unpublished, and Chapter 5).

In addition to verifying that implanted myotubes give rise to mononucleate cells, it was also found that nuclei within implanted myotubes undergo extensive re-entry to S-phase (Kumar et al., 2000). Thus, when virally labelled myotubes were implanted, and the newts were injected with 5-bromo-2-deoxyuridine (BrdU) as a DNA precursor, many labelled nuclei were observed in residual myotubes at 10 days after implantation. In some cases, all of the nuclei in the myotubes were labelled (Kumar et al., 2000). Nuclei in endogenous myofibres were also labelled in confirmation of the earlier result of Hay in larval *Ambystoma maculatum* (Hay, 1959). It seems therefore that both indices of myogenic differentiation – the change in cytology upon fusion, and the post-mitotic arrest – are reversed after implantation into the blastema. Although inferences regarding the fate of the endogenous myofibres during regeneration cannot be made from these experiments, they have established the A1 myotube as an appropriate target cell for investigating the mechanisms of plasticity.

1. 3 Myofibre structure and development

In vertebrates, skeletal muscles are formed by bundles of myofibres, the functional cells of the muscle, which are orientated in a parallel configuration and sheathed by connective tissue. When these myofibre bundles contract in unison

they generate a considerable directional force at their attachment sites. This is the basis of voluntary movement in animals.

The generation of mononucleate cells from newt myotubes implanted into regenerating limbs is impressive because of the structure and the stability of the differentiated myofibre in other vertebrates.

1.3.1 Myofibre structure

The myofibre is highly specialised for contraction. The bulk of the myofibre cytoplasm contains contractile myofibrils formed by actin, myosin and various muscle-specific proteins. These proteins are organised into sarcomeres, the actual contractile units of the myofibrils. Each sarcomere is delimited by a Z line, an alpha actinin rich region where the plus ends of actin filaments are anchored. In the centre of the sarcomere is the M line (midline) where other structural proteins, such as myomesin, anchor myosin filament tails. The heads of the myosin filaments extend towards both ends of the sarcomere. Actin and myosin filaments are arranged in a parallel and overlapping configuration such that, when contraction is stimulated, the myosin filament heads "walk" along the actin filaments toward the Z lines. This results in shortening of the sarcomere, which multiplied over the length of the myofibre, leads to myofibril contraction. The contraction of many myofibrils generates myofibre contraction and ultimately, contraction of the muscle (reviewed in Stockdale, 1992; Buckingham, 1992).

The various skeletal muscles in the body are composed of diverse fibre types distributed in distinctive patterns. The fibre type and its physiological behaviour are determined by the isoforms of muscle specific proteins that it contains; practically no two fibres in the body are identical (Stockdale, 1992). The final phenotype of the myofibre is determined during embryogenesis, and it is modulated throughout the life of the organism by stimuli such as innervation (Sohal, 1995; Hughes and Salinas, 1999; Miller et al., 1999; Olson and Williams, 2000).

1.3.2 Myofibre differentiation during development

The skeletal myofibre is a superb example of structural specialisation in a cell and its formation is one of the most dramatic phenotypical switches seen in cell differentiation during development. In most tissues, cells differentiate at an individual level, but in skeletal muscle, mononucleate myoblasts, muscle precursor cells, fuse with other myoblasts to produce a single differentiated cell, the myofibre. Once formed, myofibres persist for the entire life of the organism and growth, adaptation, and repair must be achieved by mechanisms that do not depend on myofibre proliferation. One such mechanism is hypertrophy, a process whereby the cells increase in size and protein content. Another is fusion of additional myoblasts into pre-existing muscle fibres (Moss and Leblond, 1971).

The first step in myofibre formation is withdrawal of myoblasts from the cell cycle. The myoblasts then assume a characteristic spindle shape and begin to fuse with one another creating a tube-like syncytium called a myotube. Once myoblasts fuse they are in a state of post-mitotic arrest and do not proliferate (Okazaki and Holtzer, 1966). There follows the coordinated activation of a large number of muscle specific genes which code for proteins of the myofibrillar apparatus, as well as other regulatory proteins and enzymes. Myofibril assembly begins, and the mature myofibre is formed when its nuclei migrate from the centre toward the periphery (Fischman 1967; Peng et al., 1981; Furst et al., 1989, Flucher et al., 1992; Gregorio et al., 1999). In the mouse embryo, multinucleate fibres begin to form around day 13 post coitum (pc). These are called primary fibres. Secondary fibres begin to form around day 16 pc adjacent the primary fibres. Initially, the primary and secondary fibres are distinguishable based on size differences, and are contained within the same basal lamina. Postnatally, the fibres become morphologically indistinguishable and possess individual basal lamina. Underneath the basal lamina, are myogenic mononucleate cells that participate in myofibre repair and regeneration.

The myofibres that form in culture from myoblasts obtained from muscles at different stages of development have different growth requirements and

morphologies, and express different structural protein isoforms (Hauschka, 1974; Hauschka et al., 1979; Cusella de Angelis et al., 1994). Based on these differences, myoblasts can be designated as embryonic, foetal and adult or primary, secondary and satellite, respectively (Stockdale and Miller, 1987; Stockdale, 1992). The term satellite cell derives from the location of these mononucleate cells, juxtaposed between the plasma membrane (sarcolemma) and the basal lamina of the myofibre (Mauro, 1961). Embryonic myoblasts obtained from the pre-muscle masses form short myotubes with few nuclei. Foetal and adult myoblasts on the other hand, form long, highly nucleated myotubes in culture, and express cytoskeletal protein isoforms that are distinct from those of embryonic myoblasts (Miller et al., 1985; Kaufman et al., 1991; Hartley et al., 1991). In addition, satellite cells isolated from different muscles can produce clonal progeny that give rise to fibres of distinctive phenotypes, depending on the muscles from which they derive (Feldman and Stockdale, 1991; Hartley et al., 1991; Baroffio et al., 1995; Rosenblatt et al., 1996). These satellite cells are committed to their phenotype, and fuse into the appropriate fibres types when implanted in a developing embryo (Di Mario et al., 1993).

Satellite cells, already present in foetal stages, play an important role in new fibre formation and fibre growth during this period. Depending on the age of the muscle, satellite cells may account for between 2 and 30% of the nuclei in a myofibre. They are characterised by a heterochromatic nucleus, sparse cytoplasm with few organelles, and an absence of myofibrils. In adult muscle, satellite cells are not mitotically active, but can be induce to re-enter the cell cycle by stressful stimuli such as increased workload, stretch, or injury. They proliferate, and may both fuse with existing myofibres or remain in the satellite cell compartment, ready in reserve to participate in another round of proliferation and fusion (Allen and Rankin, 1990; Grounds, 1998; Miller et al., 1999).

1.3.3 Origin of the muscle lineage

During embryogenesis, the myogenic progenitor cells or myoblasts arise within the somites and give rise to the skeletal muscle of the trunk and limbs; myoblasts arising at different locations will give rise to different muscles (Buckingham, 1992;

Lassar and Munsterberg, 1994; Ordahl et al., 2000; Tajbakhsh and Buckingham, 2000). Somites are formed by the condensation of cells in the paraxial mesoderm located alongside the neural tube and notochord and are compartmentalised into sclerotome, myotome, and dermamyotome. Cells of the sclerotome give rise to chondrogenic and fibroblastic lineages, and migrate from the sclerotome to surround the neural tube and form the vertebrae and ribs (Christ and Ordahl, 1995; Huang et al., 1996). Cells from the myotome and dermamyotome give rise to the limb and body musculature (Ordahl and LeDouarin, 1992; Christ and Ordahl, 1995; Denetclaw et al., 1997). The tongue also has a somitic origin, but the remaining facial muscles derive from cranial paraxial mesoderm which is not overtly segmented (Couly et al., 1993). Ectopic transplantation experiments involving grafting of quail half somites onto chick half somites suggest that the cell fate within the somite is plastic and responsive to extrinsic signals. Ventral medial cells, which would have given rise to the sclerotome, when transplanted dorsally will participate in the formation of the myotome giving rise to the muscles of the body and the dermis (Ordahl and LeDouarin, 1992). These experiments suggest that non-myogenic cells can be induced to adopt a myogenic fate by external cues.

Limb muscle precursors derive from lateral somites at the level of the limb bud, from where they migrate to their final location in the limb (Williams and Ordahl, 1994). Somitic myoblasts are committed to a myogenic fate, but do not express the phenotypic markers of muscle differentiation until they receive the appropriate environmental cues in the limb. In the trunk, these signals originate in the neural tube and notochord and include *sonic hedgehog* (see section 1.3.7); the signals that induce muscle-specific gene expression in the limb are as yet undidentified (Brand-Saberi and Christ, 1999).

1.3.4. Molecular characterisation of the muscle lineage

Two families of mutually inducible transcription factors interact to regulate skeletal myogenesis in the developing embryo: muscle regulatory factors (MRFs) and myocyte enhancer factors (MEF2s) Their identification and isolation has led to considerable advances in our understanding of the mechanisms underlying

the commitment to the myogenic lineage and the regulation of muscle differentiation.

The MRFs were identified over 10 years ago and since then have been the subject of extensive and frequent reviews (Buckingham, 1992; Olson and Klein, 1994; Arnold and Braun, 1996; Arnold and Braun, 2000). Their distinguishing biochemical property is the ability to convert non-myogenic cells to the myogenic lineage (Davis et al., 1987; Tapscott et al., 1988; Choi et al., 1990; Aurade et al., 1994,Weintraub et al.,1991). The first myogenic regulatory factor, MyoD, was identified in a subtractive hybridisation screen exploiting the ability of 5-azacytidine, which affects DNA methylation patterns, to convert C3H10T1/2 fibroblasts to myoblasts (Davis et al., 1987). Subsequently, three related genes were identified in mammals: *myogenin* (Edmonson and Olson, 1989; Wright et al., 1989), Myf5 (Braun et al., 1989; Edmonson and Olson, 1989; Wright et al., 1989), and *MRF4* (Rhodes and Konieczny, 1989; Miner and Wold, 1990).

The MRFs are members of the basic helix loop helix (bHLH) family of transcription factors and are expressed exclusively in the skeletal muscle lineage, as described below. The bHLH factors contain an evolutionarily conserved 70 amino acid segment of homology, the helix loop helix (HLH), and an adjacent basic domain. The HLH region mediates dimerisation among family members and this brings together the basic regions to form a DNA binding domain. The basic region of the MRFs is essential for muscle specific transcription (Lassar et al., 1989; Brennan et al., 1991, Weintraub et al., 1991). MRFs bind to the consensus sequence CANNTG, also known as the E-box or 'myocyte specific enhancer binding nuclear factor 1' motif (MEF1; Buskin and Hauschka, 1989). E-box binding motifs have been identified in the regulatory regions of most skeletal muscle specific genes including the MRF genes themselves.

The first evidence for the existence of muscle regulatory factors in addition to

the MRFs came from studies involving the fusion of myoblasts with other cell types (Blau et al., 1985). Not all cells were equally susceptible to conversion suggesting that either inhibitors of muscle differentiation were present or additional positive regulatory factors were missing. A second muscle specific regulatory sequence, distinct from the E-box, was first observed in the muscle creatine kinase enhancer (Buskin and Hauschka, 1989), and designate myocyte enhancer element 2. This is an A-T rich region bound by the MEF2 factors, which are members of the MADS family of transcription factors (reviewed in Olson et al., 1995; Black and Olson, 1998). There are 4 vertebrate MEF2s, MEF2A-D. Unlike the MRFs, MEF2 proteins are expressed in non-myogenic tissues, but MEF2 DNA binding activity is restricted to muscle (skeletal, cardiac, and smooth) and to the brain; transcriptional activity of MEF2C in cultured cells has been observed specifically in the skeletal muscle line C2C12, although DNA binding activity is present in other cell types (Ornatsky and McDermott, 1996). MEF2 elements are found in the regulatory regions of many muscle specific genes, including *MyoD*, *myogenin*, and *MRF4*. MEF2s and MRFs interact directly, through the bHLH and MADS domains respectively, to confer muscle-specific gene expression (Molkentin et al., 1995). In addition, MEF2 can confer muscle specific transcription in the absence of an E box (Gosset et al., 1989; Molkentin et al., 1995), suggesting that it can act in transcriptional regulation of MRFs by protein to protein interactions. Although myogenic conversion of fibroblasts by MEF2 has been reported (Kaushal et al., 1994), it has not been confirmed (Molkentin et al., 1995;Ornatsky et al., 1997). Nevertheless, a dominant negative MEF2A lacking transcriptional activation domain is able to inhibit myogenic conversion of fibroblasts by MyoD (Ornatsky et al., 1997), demonstrating a requirement for MEF2 activity in myogenesis.

Regulatory hierarchies *in vivo*

The expression of the muscle regulatory factors (MRFs), *MyoD*, *Myf 5*, *myogenin*, and *MRF4*, is the first evidence of commitment to the skeletal myogenic lineage in the developing embryo. *MyoD*, *myogenin*, and *MRF4* are expressed exclusively in skeletal muscle cells, but expression of *Myf*5 has been observed in neural tissues as well (Tajbakhsh and Buckingham, 1995).

Analyses of the spatio-temporal expression patterns of the MRFs and MEFs both *in vivo* and *in vitro*, as well as of the phenotypes of knockout mice and cultured myogenic cells have led to the conclusion that *MyoD* and *Myf*5 function in the determination and commitment of cells to the muscle lineage, and that *myogenin* and *MRF4* regulate the more overt aspects of muscle differentiation and maintenance. Nevertheless, there are distinctions as well as redundancies in MRF functions.

In mammals, MRF genes are expressed in the somites just prior to segmentation. In the mouse, *Myf* 5 is the earliest expressed MRF at day 8 post coitum (E8), and although downregulated, *Myf* 5 expression may continue after birth (Ott et al., 1991; Smith et al., 1994; Tajbakhsh et al., 1996a). In the myotome, *myogenin* expression is detected next, 12 hours after *Myf*5 expression and prior to the appearance of *MyoD* (Sassoon et al., 1989; Smith et al., 1994). *Myogenin* is expressed throughout embryonic development, but its levels decline after birth. *MRF4* is expressed transiently in the somite between E9.0 and 11.5, a few hours after *myogenin* expression is first observed. It is then re-expressed after E16 to become the most abundant MRF after birth (Bober et al., 1991; Hinterberger et al., 1991). This late expression of *MRF4* coincides with formation of secondary fibres and with the onset of motor innervation. *MyoD* is the last MRF to appear in the somites at E10.5, but is then present throughout embryonic development (Sassoon et al., 1989; Smith et al., 1994).

In the developing limb the pattern of MRF expression is different from that observed in the somite, with *Myf*5 coming on first at E8.5, followed concomitantly by *myogenin* and *MyoD* at E10.5, and finally *MRF4* after E16. In addition to the MRFs, the sequence of expression of structural muscle proteins also differs between fibres that form in the somites and in the limb bud (Sassoon et al., 1989; Bober et al., 1991; Ott et al., 1991).

Several observations suggest that *Myf*5 and *MyoD* have important roles in determining axial and limb musculature respectively. The two proteins are expressed in different subdomains in the somite (Smith et al., 1994; Braun and

Arnold, 1996). *Myf*5 is first expressed in the dorsal somite whereas *MyoD* first appears in the ventral somites; as the somite matures, their expression overlaps. In the *Myf*5 knockouts, *MyoD* expression comes on in the somites at the normal time at day 10.5 and it is only subsequent to its expression that *myogenin* expression comes on. In *MyoD* -/- embryos, the development of skeletal muscle in the limb is delayed by 2.5 days (Kablar et al., 1997), but *MRF4* and *myogenin* expression are normal. Thus, *Myf*5 and *MyoD* are expressed independent of each other.

The above observations and the phenotype of mice null for *Myf*5 and/or *MyoD* show that these genes define two temporally distinct points of entry to the muscle lineage (Arnold and Braun, 2000). *MyoD* and *Myf*5 single knockouts both have normal muscle, but the double knockout is totally devoid of muscle, suggesting that the expression of either *MyoD* or *Myf*5 is required for myogenic determination or for the survival of cells of the muscle lineage (Braun et al., 1992; Rudnicki et al., 1992, 1993). In the absence of *Myf*5, cells fated to give rise to muscle migrate to adopt chondrogenic and epidermal fates and *Myf* 5 is thus required for pluripotential precursor cells of the somite to adopt a myogenic fate (Tajbakhsh et al., 1996b).

Myogenin is only expressed subsequent to *Myf*5 or *MyoD* and thus lies downstream of both these genes in the regulatory hierarchy. Nevertheless, it is essential for normal differentiation. *Myogenin* null mice die at birth with a reduction of skeletal muscle throughout the body, but the presence of mononucleate myocytes in the muscle forming regions shows that muscle cells are determined, survive, and differentiate to the extent that they express some muscle markers (Hasty et al. 1993, Nabeshima et al., 1993). The late expression of *MRF4* (after E16) is not observed in these mutants, but the transient early expression at E9.0 is observed. There are sparse myofibres in *myogenin* null embryos (as well as in the *myogenin/MyoD* and *myogenin/Myf5* double knockouts) and these myofibres express *MRF4*, so it was proposed that they formed as a result of the transient early expression *MRF4* in the somite (Rawls et al., 1995). This model was disproven by the observation that the *MRF4/myogenin* double knockout exhibits a similar muscle phenotype, with only a few myofibres (Rawls et al., 1998).

From the phenotypes of the knockout mice described above, it is clear that the function of *myogenin* does not overlap with that of either *MyoD* or *Myf*5. This was further demonstrated by targeting myogenin to the *Myf*5 locus. These *myogenin/Myf*5 knockin mice are viable, but show reduced skeletal muscle. Crosses of *myogenin/Myf*5 knockins with *MyoD* null mice are not viable (Wang and Jaenish, 1997), demonstrating again the non-redundancy of *MyoD* and *Myf* 5 and showing that the protein products of *Myf*5 and *myogenin* are not interchangeable.

In *MRF4* knockouts, muscle is essentially normal, but disruption of *MRF4* may also disrupt *Myf* 5 transcription. *Myf*5 lies 8.5 kb downstream of *MRF4* on the same chromosome, but sequences that effect its regulation are present far upstream of the open reading frame (Braun and Arnold, 1995; Yoon et al., 1997; Zweigerdt et al., 1997). *Myf*5 transcription may be effected in the MRF4 knockout and it would be reasonable then to expect that MyoD and myogenin together can sustain normal muscle development. The *MyoD/MRF4* double knockout, however, has a phenotype similar to the *myogenin* knockout (Rawls et al., 1998); this demonstrates that in *MRF4* knockout some *Myf 5* activity must be present otherwise the phenotype should be the same as that of the *MyoD/Myf*5 double knockout. The phenotype of the *MRF4 /MyoD* double knockout also suggests that these genes may function in the same pathway and cooperate with *myogenin*.

A triple null mutant expressing only *Myf* 5 shows a more severe phenotype with almost no myofibres in the somites and no desmin expression in the muscle masses. Desmin detects both undifferentiated myoblasts and muscle (Valdez et al., 2000), and it is not clear whether the cells in the muscle forming regions in the triple knockout are determined.

In addition to MRF expression, there is also a requirement for *MEF2* expression in normal muscle development. Activation of *myogenin* gene transcription *in vivo* is dependent on both MEF2 and the E-box, which mediates MRF binding (Cheng et al., 1993; Buchberger et al., 1994). The expression of *MEF2* transcripts in the myotome occurs after that of *Myf*5 and *myogenin*, suggesting that it lies downstream

of these genes in the muscle lineage (Black and Olson, 1998). In the triple MRF knockout expressing only *Myf5*, *MEF2C* is expressed (Valdez et al., 2000), suggesting that *Myf5* is sufficient to induce MEF2s. In *Drosophila*, the single MEF2, *D-Mef2* is required for muscle formation (Lilly et al., 1995), and ectopic expression of D-Mef2 can lead to ectopic myotube formation (Lin et al., 1997). Interestingly, the single MRF *Nautilus* is not required for normal development in all of the muscles of the fly (Abmayr et al., 1998; Keller et al., 1998). This observation lends importance to the function of MEF2 in muscle development and suggests that alternative mechanisms for induction of myogenesis may exist even in vertebrates.

Other genes may play a role in muscle determination. In the *Pax3* mutant Splotch, body muscle is normal, but the limb musculature is defective (Bober et al., 1994; Daston et al., 1996). In *Pax3-Myf5* double mutants there is no body muscle and *MyoD* is not expressed in the body (Tajbakhsh et al., 1997). Ectopic *Pax3* expression in lateral mesoderm and neural tube can induce *MyoD* and *Myf5* expression (Maroto et al., 1997). *Pax3* is expressed in migrating myoblasts and is downregulated after the cells reach their final destination, and the MRFs are then expressed. It is possible that in the absence of the MRFs during migration, *Pax3* maintains the cells in the muscle lineage, alternatively it could act upstream of *MyoD* as a muscle determinant. Another gene, which is expressed in migrating myoblasts, is *Mox2* (Mankoo et al., 1999). *Mox2* inactivation by homologous recombination results in the reduction of muscle mass specifically in the limb, but there is no defect in the body muscles. In this situation, *Pax3* expression is reduced suggesting that *Mox2* may have a role in the maintenance of *Pax3*. Nevertheless, *Mox2* is not a primary muscle determinant like the MRFs, and perhaps *Pax3*.

Regulatory hierarchies *in vitro*

The MRFs are expressed in a distinct temporal sequence during differentiation of muscle cell lines (Aurade et al., 1994; Miner and Wold, 1990; Andres and Walsh, 1996); *MyoD* and *Myf5* are expressed in proliferating myoblasts prior to differentiation (Tapscott et al., 1988; Kitzmann et al., 1998; Yoshida et al., 1998), *myogenin* is expressed in all cell lines after they are induced to differentiate (Wright et al., 1989; Edmonson and Olson, 1989; Andres and Walsh, 1996), and *MRF4* is not

expressed until after myotubes have formed (Miner and Wold, 1990; Montarras et al., 1991; Aurade et al., 1994). In fact, *MRF4* is activated in non-muscle cells by other MRFs but not by its own gene product (Naidu et al, 1995).

Cells that are null for the expression of a combination of any two MRFs can differentiate *in vitro*, and this differs from their *in vivo* phenotypes. Thus, *myogenin* and *MRF4* null cells fuse *in vitro* to form myotubes as do *MyoD-MRF4* null myoblasts. In contrast, myoblasts derived from a triple mutant that expresses only *Myf*5 do not fuse (Valdez et al., 2000). In another report, *MyoD-/-* myoblasts were shown to be defective in myotube formation and were rescued by the expression of MyoD. These cells were of early passage and this study indicates the importance of using early passage cells that may retain a memory of their 'in vivo' conditions, which may be lost after long term passaging (Sabourin et al., 1999).

In muscle cell lines, MEF2 is upregulated during differentiation and its expression can be induced by *MyoD* and *myogenin*, which suggests that MEF2s lie downstream of the MRFs in the regulatory cascade (Cserjesi and Olson, 1991). The MEF2 sites on the promoters of *myogenin*, *MRF4* and *MyoD* are, however, known to be essential for the transcription of these genes, and the co-expression of MEFs and MRFs during skeletal myogenesis increases the efficiency of myogenic conversion of non muscle cell lines (Molkentin et al., 1995). *Myogenin* and MEF2, for example, activate *MRF4* transcription in a synergistic manner (Naidu et al., 1995). Interestingly, MEF2C can activate myogenesis in concert with a mutated myogenin that lacks transcriptional activation domain (Molkentin et al., 1995), but MyoD cannot initiate myogenesis in fibroblasts if a dominant negative MEF2A which lacks the transcriptional activation domain is expressed (Ornatsky and et al., 1997). Additionally, MEF2A did not potentiate the myogenic conversion by MyoD, suggesting that the different MEF2 members may have different functions.

The mutual inducibility of MRFs and MEFs has led to the suggestion that the differentiated state is maintained by autoregulatory loops that are established among these transcription factors during myogenesis. *MRF4* in particular is thought to have a role in stabilising and maintaining the differentiated phenotype because of

its late expression both in culture and *in vivo* (Stockdale, 1992; Lassar and Munsterberg, 1994; Olson and Black, 1998; Arnold and Braun, 2000).

1.3.5 Generation of satellite cells

Quiescent satellite cells express c-met receptor and M-cadherin but not late markers of muscle differentiation or detectable MRFs (Smith et al., 1994; Yablonka-Reuveni and Rivera, 1994; Koishi et al., 1995; Cornelison and Wold, 1997). Upon activation and entrance to the cell cycle, *MyoD* or *Myf5* is rapidly upregulated and *myogenin* is expressed before satellite cells start to fuse (Smith et al., 1994, Yablonka-Reuveni and Rivera, 1994; Rantanen et al., 1995). *MRF4* transcripts are also found in mononucleate satellite cells. *In vivo*, activated satellite cells contribute both to myofibres and to satellite cell pools (Schultz, 1996). In culture, the clonal progeny of a single satellite cell can both form differentiated myotubes, and contribute to a reserve pool that remains undifferentiated, but is still myogenic (Baroffio et al., 1996; Kitzmann et al., 1998; Yoshida et al., 1998). In addition, cultured myogenic cells are able to fuse into existing myofibres when injected into regenerating muscle and may also give rise to satellite cells (Blaveri et al., 1999).

A mechanism for generation of precursors in culture has recently been proposed involving *MyoD* regulation (Kitzmann et al., 1998; Yoshida et al., 1998). Serum deprivation in culture leads to generation of two different populations, one that maintains *MyoD* expression and fuses into myotubes and another that downregulates *MyoD* and can subsequently be expanded clonally to give rise to myogenic progeny. This correlates well with the cell cycle distribution of *MyoD* and *Myf5* in myoblasts, since continued *Myf5* expression is associated with commitment to proliferate whereas *MyoD* expression is maintained as cells differentiate. Furthermore, *Myf5* is not detected in myotubes whereas *MyoD* is elevated in myotubes. If this mechanism operates *in vivo*, then the cells that stay in the satellite compartment must downregulate *Myf5* expression subsequent to their activation.

The importance of *MyoD* in muscle regeneration is demonstrated by the fact that it is required for efficient muscle regeneration (Megeney et al., 1996). Muscle

regeneration is impaired in *MyoD* deficient mice and the activated satellite cells fail to exit the cell cycle and enter the differentiation program efficiently (Sabourin et al., 1999; Yablonka-Reuveni et al., 1999). A requirement for MyoD could occur during development to establish the satellite cell or could be necessary only subsequent to its activation for entry into the myogenic pathway.

Satellite cells may constitute a different population from the migratory myoblasts that colonise the limb (reviewed in Miller et al., 1999; Seale and Rudnicki, 1999). In *splotch* mutants, in which myoblasts do not migrate from the somite, satellite cells are nevertheless present in the limb. It is becoming increasingly unclear to what extent satellite cells are committed *in vivo* to the myogenic lineage. Muscle derived stem cells can repopulate the hematopoietic compartment following intravenous injection into irradiated mice (Gussoni et al., 1999). These experiments indicate that there are stem cell populations in muscle other than satellite cells or that the differentiation of satellite cells can be modulated by external signals. The fact that they do not express MRFs may be indicative of a pluripotential state.

Hematopoietic stem cells have been shown to contribute nuclei to muscle fibres (Ferrari et al., 1998; Gussoni et al., 1999). It is not known whether these cells can contribute to the satellite cell pool. The existence of pluripotential stem cells in different tissue compartments raises questions about how their 'stem cell' status is maintained and how their differentiation along different pathways is induced by their microenvironment.

1.3.6 The mononucleate to multinucleate transition

Fusion of myoblasts is separable from biochemical differentiation. Cultured myocytes prevented from fusing are still able to withdraw stably from the cell cycle, express muscle specific proteins of the myofibrillar apparatus, and achieve some degree of sarcomeric organisation. There are at least three cell systems in which biochemical differentiation is uncoupled from fusion: BC3H1 cells

(Strauch et al., 1989); C2C12 cells transformed by v-myc or c-myc (Crescenzi et all, 1994); and NIH3T3 fibroblasts converted by MRF expression (Russo et al., 1998). The mechanism that inhibits fusion in the first two cell types may be related to defects in MRF function, since *MyoD* or *MRF4* expression rescues fusion in BC3H1 cells (Block and Miller, 1992), and *c-myc* downregulates MRF expression when expressed in C2C12 myoblasts (LaRocca et al., 1994).

The case of converted 3T3 fibroblasts is particularly interesting, since converted 10T1/2 fibroblasts are fusion competent (Davis et al., 1987; Novitch et al., 1996). Converted 3T3 and 10T1/2 fibroblasts express many of the same regulatory and structural muscle specific genes, independent of which MRF initiated conversion. The only differences found between these cell lines were in the expression of a muscle specific MEF2D splice variant and integrin β1. Nevertheless, expression of β1 integrin or the MEF2D splice variant failed to rescue myotube formation (Russo et al., 1998).

Myoblast fusion is not a cell autonomous phenomenon, and MRF4-3T3 myoblasts are able to fuse with L8 myoblasts (Russo et al., 1998). This suggests that events leading to fusion are activated by cell to cell contact, and highlights the importance of interactions present in a tissue context, in the regulation of muscle differentiation.

The cell surface receptors which mediate interaction between cells, and between cells and proteins such as integrins, N-Cams, cadherins and disintegrins are thought to participate in myoblast fusion. Incubation of myoblasts with peptides antagonistic to M or N-cadherin or expression of antisense RNA inhibits both myoblast fusion and cell cycle withdrawal in conditions that normally promote differentiation (Zeschnigk et al., 1995; Goichberg and Geiger, 1998). In C2C12 myoblasts, integrin α5 subunits, which form a functional receptor with the β1 subunit, mediate proliferation and inhibit differentiation, whereas the α6β1 receptor promotes differentiation even in high serum conditions (Sastry et al., 1999), which normally inhibits myogenesis.

The function of growth factor receptors can be regulated by integrin-mediated cell adhesion and, although there are cases where receptor function is independent of adhesion, the signalling pathways downstream of the receptors are adhesion dependent (reviewed in Schwartz and Baron, 1999). Both adhesion and growth factors activate signalling through Rho family GTPases, the Ras/Raf-MAPK pathway, PI3 kinase, ribosomal S6 kinase (RSK), and Jun amino-terminal kinase (JNK). Numerous physical interactions between integrins or focal adhesion components and mitogenic signalling proteins have been demonstrated (reviewed in Howe et al., 1998). For example, focal adhesion kinase can interact with PI-3 kinase (Chen et al., 1996). This highlights the importance of considering multiple signals in induction of differentiation.

The extracellular environment and differentiation

The ability of the extra-cellular environment to regulate muscle differentiation during development is evident in the regulation of MRF expression in the myoblasts that migrate from the somite to the limb bud and give rise to the limb musculature. In these cells, *Pax3* but not MRFs are expressed (Bober et al., 1994; Williams and Ordahl, 1994; Daston et al., 1996). MRF expression does not occur until the myoblasts reach the limb bud where *Pax3* expression is downregulated as MRF expression is initiated (Bober et al., 1994; Williams and Ordahl, 1994). In these migrating myoblasts, bone morphogenetic protein (BMP) signalling may play a role in restricting the myogenic activity of *Pax3*. BMP4 inhibits myogenesis in cocultures of somite/ectoderm and is expressed in the lateral plate mesoderm through which the *Pax3*-expressing myoblasts migrate (Reshef et al., 1998).

Myogenesis is inhibited *in vivo* by cell and tissue interactions. This is demonstrated by the differentiation of chick epiblast cells into myotubes after dissociation in culture. These cells do not undergo myogenesis in their normal developmental context, and it has been suggested that this inhibition may involve signalling through the Notch receptor (George-Weinstein et al., 1996). In

neurogenesis, Notch signalling, which is activated by Delta ligands, antagonises neuronal bHLH factors and prevents differentiation, thereby maintaining cells as neuronal precursors (Weinmaster, 1997). There is evidence to suggest that Notch signalling may also inhibit myogenesis. *MyoD* expression and differentiation can be inhibited reversibly in culture by Notch signalling (Shawber, 1996; Kato et al., 1997). In *Xenopus* gastrula, MyoD stimulates *Delta-1* transcription and activation of an endogenous Delta regulated gene, *ESR1*, in presumptive myoblasts (Wittenberger et al., 1999). *ESR1* is related to the *Drosophila enhancer of split* which antagonises bHLH function.

1.3.7 The reversal of the mononucleate to multinucleate transition in newt myotubes

Developmental programs may be reactivated in the context of limb regeneration. Pathways that function in the patterning of the limb during development, for example, also operate in the patterning of the limb regenerate (Bryant and Gardiner, 1992). Presumably, some of the signalling pathways, such as the *Delta-Notch* pathway, that act during development to regulate differentiation of muscle and other cell types are also reactivated in the blastema environment and may play a role plasticity. In the blastema, a low level of *Notch* expression is observed from 9 days after amputation. In cultured newt A1 cells *Notch* expression is observed in both mononucleate cells and in myotubes (Stark and Brockes, unpublished). It is possible that notch signalling is activated in the context of the blastema. This consideration is highly conjectural, but it is possible that some of the signals discussed above or others are important in invoking tissue plasticity after amputation.

The trauma produced by amputation is sufficient to activate wound closure and dedifferentiation of the stump tissue (Tassava and Mescher, 1975), but the molecular mechanism underlying these processes remains unclear. Not surprisingly, second messenger activity is detected very early in the regeneration blastema (Tabin and Cathieni, 1989, Cathieni and Tabin 1992) and there is also evidence for PI3 kinase signalling at this time (Tsonis et al., 1991).

Several studies have shown the upregulation of genes in the first week after amputation, a time when dedifferentiation occurs. Expression of *Msx2* and *MMPs* (matrix metalloproteinases) is upregulated in the first 24 hours after amputation (Carlson et al., 1998; Koshiba et al., 1998; Yang and Bryant 1994; Yang et al., 1999). *Hox D* transcripts are also expressed during these very early time points, but it is thought that their expression is associated the establishment of positional identity (Torok et al., 1998). Upregulation of these genes is also observed after infliction of superficial wounds, and it has been suggested that at these early stages, the regeneration and wound healing pathways may be common (Gardiner et al., 1999). The recent finding of Tanaka and co-workers (1999) implicating enzymes of the clotting cascade in the cell cycle re-entry response of cultured newt myotubes is also suggestive of a link between wound healing and the earliest events of dedifferentiation.

Msx1 and *Msx2* were originally identified based on their homology to the *Drosophila muscle segment homeobox* (*Msh*) gene (Hill et al., 1989). In the developing chick limb, *Msx1* expression requires the influence of the apical ectodermal ridge (AER), a thickened epithelium at the extremity of the limb bud which functions to maintain the underlying cells in an undifferentiated, proliferative state (Davidson et al., 1991). The AER is analogous to the AEC of the wound epithelium. Interestingly, expression of Msx1 in mammalian myoblasts inhibits their fusion (Song et al., 1992). Newt *Msx1* genes are found in normal limb and tail as well as their respective blastemas (Crews et al., 1995; Simon et al., 1995), and it has been suggested that its continued expression may underlie the ability to regenerate limbs (Muneoka and Sassoon, 1992). The *Msx2* gene is upregulated after amputation and expressed in the cells of the wound epithelium before the wound is closed (Carlson et al., 1998; Koshiba et al., 1998). Later it is also found in the mesenchyme.

Other genes are expressed at slightly later times, but at stages where dedifferentiation and proliferation are active in the blastema, and may also have interesting functions. The expression of *Dlx* begins between three and five days

after amputation. Its regulation by FGF2 has been discussed in relation to the onset of nerve-dependence (Mullen et al., 1996). *Inhibitor of differentiation (Id)* expression was also found to be up regulated in the blastema relative to the normal limb of the Japanese newt, and its expression was observed both in the mesenchyme (*Id3*) and the wound epidermis (*Id2 and Id3*, Shimizu-Nishikawa et al., 1999). The *Id* gene is a member of the helix loop helix (HLH) family of transcription factors, whose members include muscle and neural determination genes. It functions by heterodimerising with other HLH family members and inhibiting their DNA binding by virtue of the fact that it does not contain the basic region that forms the DNA binding domain upon dimerisation of the bHLH factors.

Sonic and *banded hedgehog* are members of a family of vertebrate homologues of the *Drosophila* segment polarity gene *Hedgehog* (Ingham and Fietz, 1995). *Sonic hedgehog* (*shh*) is expressed in the blastema mesenchyme one week after amputation (Imokawa et al., 1997). Newt *banded hedgehog* is expressed beneath the wound epithelium and around the muscle fibres at a time when blastemal cells arise; it continues to be expressed in the mesenchyme throughout phases associated with blastema growth, but is downregulated at the onset of differentiation (Stark et al., 1998).

In the developing chick limb, *shh* forms a signalling loop with FGF4: it requires FGF4 for its expression and induces BMP2 which in turn maintains FGF4 expression in the AER (Niswander et al., 1994). There is substantial evidence in developing limbs that FGF2 and FGF4 maintain limb outgrowth in the absence of the AER (Niswander et al., 1994). Recently, the signalling pathway involving *shh* and FGF4 has been shown to be more complex (Sun et al., 2000), but the above observations are interesting in light of the pattern of FGFs and FGF receptor expression in the blastema. FGF1 and FGF2 are present in the wound epithelium (Boilly et al., 1991; Mullen et al., 1996). Transcripts of FGFR2, a receptor for FGF4 as well as FGF1 and 2, are present in the wound epithelium at all stages of

regeneration, and FGFR1 is present in the mesenchymal cells during the phases of blastema growth (Poulin et al., 1993, 1995).

The up regulation of MMPs is particularly interesting, in light of the extensive remodelling of the ECM that occurs after amputation. The ECM of the early blastema is characterised by the presence of molecules which favour cell proliferation and migration, and by the absence of those which inhibit these processes. Thus, hyaluronate and fibronectin synthesis are greatly intensified (Mescher and Cox, 1988; Repesh et al., 1982; Gulati et al., 1983), and collagenolytic activity and matrix metalloproteinase (MMP) expression are upregulated (Miyazaki et al., 1996; Yang and Bryant, 1994; Yang et al., 1999). When differentiation begins, fibronectin, tenascin, and the MT2 and 9G1 antigens of the extracellular matrix are downregulated (Gulati et al., 1983; Onda et al., 1990, 1991; Klatt et al., 1992; Onda and Tassava, 1991). Conversely, collagen, laminin and the ST1 antigen reappear (Mailman and Dresden, 1976; Gulati et al., 1983; Yang et al., 1992).

In chick myoblast cultures, differentiation is inhibited by culturing the cells on hyaluronate, whereas it is promoted when the cells are transferred onto collagen substrate (Kujawa and Tepperman, 1983; Kujawa et al., 1986). In C2C12 myoblasts, laminin binding may be sufficient to override the mitogenic stimulus of soluble growth factors because activation of the laminin receptor $\alpha 6\beta 1$ results in differentiation, even in the presence of mitogens (Sastry et al., 1999). Thus lack of laminin signalling in the blastema combined with signalling through the fibronectin receptor may be sufficient to induce cell cycle re-entry, generation of mononucleate cells, or both in implanted newt myotubes.

The medusa of the jellyfish *Podocoryne*, is an interesting example of plasticity in striated muscle. The body wall of the animal is composed of three cell layers separated by a thick layer of extracellular matrix which gives the animal its gelatinous appearance. The innermost layer is composed of mononucleate striated cells. When isolated in culture, these cells undergo phenotypic conversion into

smooth muscle and nerve cells. ECM degrading enzymes are effective at destabilising the striated muscle phenotype but the stability of striated muscle is also influenced by drugs that act upon the cytoskeleton (Schmid, 1992; Brockes, 1994). If cultured on cell free ECM, the muscle cells maintain their commitment to the striated muscle phenotype, although they downregulate muscle structural genes transiently during the process of migration (Yanze et al., 1999) .

Recently, reversal of the mononucleate to multinucleate transition was reported to be induced in C2C12 mouse myotubes by treatment with a molecule called myoseverin. Myoseverin is a substituted purine derivative isolated from a combinatorial library, that induces scission of multinucleate myotubes into proliferating mononucleate cells. This transition is associated with changes in the microtubule cytoskeleton and also upregulation of a number of genes associated with wound healing, among which is the FGF4 receptor(Rosania et al., 2000). Also effected are genes involved in extracellular matrix remodelling; the 3/10 metalloproteinase inhibitor is downregulated for example. The authors suggest that myoseverin may activate a programme that is related to the one observed in newt myotubes during regeneration, and this is a hypothesis that could be tested in future.

In the blastema environment, myotubes encounter many signals which are not present in culture. One or more of these might stimulate the generation of mononucleate cells from these myotubes through a process that may or may not require DNA synthesis. Whatever the mechanism, the generation of mononucleate cells very likely involves cytoskeletal modifications. It is possible to envisage signalling pathways that would concomitantly, but independently result in cytoskeletal modifications and cell cycle re-entry. Alternatively, cell cycle re-entry and cytoskeletal modifications might be the result of activation of the same enzyme. The most obvious candidate is the cdc2 kinase which activates mitosis, but there are others.

The cultured newt myotube offers the unique opportunity for effecting manipulations before implantation, and thereby to begin to dissect some of the extrinsic and intrinsic factors regulating its plasticity.

1.4 Establishment and maintenance of the post-mitotic state

In mammalian skeletal myogenesis, cell cycle withdrawal is an effectively irreversible process and is tightly linked to the onset of differentiation. Signalling pathways driving proliferation are suppressed in differentiated myotubes and must be inactivated for differentiation to proceed (Olson, 1992; Lassar et al., 1994). The mechanisms involved in establishing and maintaining the post-mitotic arrest in myotubes will be discussed in sections 1.4.6 and 1.4.7, but an understanding of the molecules regulating cell cycle progression is required first.

1.4.1 An overview of the cell cycle

The cell division cycle is a series of precisely co-ordinated events that result in the generation of two daughter cells from a single parent. Progress through the cell cycle is unidirectional and the initiation of each cell cycle phase depends on the completion of the one immediately preceding it (Johnson and Rao, 1970; Rao and Johnson, 1970; Nasmyth, 1996).

The cell cycle is divided into four phases: G1, S, G2, and M. M-phase was the first to be recognised as the dramatic events of mitosis, namely chromosome condensation and segregation into daughter cells, were readily visible by microscopists in the late 19th century. At that time, G1, S, and G2 were grouped into a period called interphase, when the cell seemed to be at rest. The advance of isotopic labelling techniques in the mid 20th century led to the identification of a synthetic phase, termed S (Howard and Pelc, 1951). During S phase, DNA is synthesised and the genome is duplicated so that both daughter cells inherit a diploid genome. The interval following M and preceding S phase was termed gap1 or G1, and the interval following S and preceding M was called gap 2, G2. G1 is a phase of cell growth and it is during this period that external and internal regulatory signals converge to determine the fate of the cell: division,

differentiation, quiescence, death. In G2 the cell pauses before entering mitosis as levels of mitotic inducers increase (Pardee, 1989; Sherr, 1996; Baserga, 1999).

Cell cycle progression is precisely regulated. Transmission of genetic information from the parent to the daughter cells requires accurate replication and segregation of the genome. DNA synthesis and mitosis are in fact precisely monitored events and the integrity of the processes that occur during these phases in assured by checkpoints present throughout the cell cycle. These mechanisms assure not only normal cell division but are also required for growth arrest under conditions of environmental stress or after DNA damage (Hartwell and Kastan, 1994; Nasmyth, 1996; Elledge, 1996; Nurse, 1997; Clarke and Gimenez-Abian, 2000). The molecular machinery that drives cell cycle progression is acted upon by the regulatory mechanisms of these checkpoints which halt the cell cycle if damage or mistakes are detected.

The core components of the molecular machinery that drives progress through the cell cycle are the cyclin dependent serine-threonine kinases (cdks), which exert their influence by phosphorylation of other cell cycle regulatory proteins (Morgan, 1995, 1997; Sherr, 1996; Puri et al., 1999). Cdk activation requires binding to a partner cyclin, but it is also regulated by several other mechanisms which will be discussed below. The cyclins and cdks are highly conserved from yeast to mammals. In yeast, there is only one cdk and the different cyclins that are present in the different phases of the cell cycle contribute to the substrate specificity of the cdk during theses phases. In mammalian cells there are at least 9 cdks and 11 cyclins (Sherr, 1996; Puri et al., 1999). The substrate specificity of each cyclin-cdk complex is different and the sequential and transient appearance of different cyclin cdk complexes drives the critical cell cycle events of each phase of the cell cycle in an orderly progression. The principal cdks involved in regulating cell cycle progression are cdk1/cdc2, cdk2, cdk4, and cdk6. Their levels remain relatively constant throughout the cycle, but the levels of their cyclin binding partners vary. In fact, the term cyclin derives from the oscillating levels of these proteins during the cell cycle. Cyclin availability is the first level of cyclin-cdk

regulation. The D type cyclins bind to cdk4/6 and are present in early to mid G1. The E cyclins regulate the transition from G1 to S, and in S phase, the activity of cyclin A-cdk2 is important; initiation of mitosis is triggered by abrupt activation of cyclin B-cdc2 (Morgan, 1995; Sherr, 1996; Morgan, 1997; Puri et al., 1999).

The structure of cyclins and cdks reveals why formation of the complex is required for kinase activity. Cyclins share homology in a 100 amino acid region termed the cyclin box through which they bind to the cdks. All cdks share a sequence in domain III related to EGV**PSTAIR**ISLLKE which is found in the yeast cdk homologues, and a region in domain VII that includes a threonine residue (Thr-160 in cdk2, Thr161 in cdc2) which is important for binding of specific partner cyclins (Pines, 1996). Crystallographic studies (Jaffrey et al., 1995) have shown that in monomeric cdks, the ATP is bound to the kinase in a conformation that precludes its nucleophilic attack on the substrate hydroxy group; this inhibits the scission of the phosphate. Part of the C-terminus of the kinase, the T-loop domain, binds the catalytic cleft. Following interaction of the PSTAIRE region with the cyclin box, the PSTAIRE helix is reorientated thus allowing the ATP phosphate bond to become susceptible to attack by a bound substrate. In addition, interaction of the cyclin with the N-terminus of the T-loop displaces it and exposes the substrate binding site, at the same time exposing the Thr160 in the T-loop which becomes accessible for phosphorylation.

Phosphorylation of cdks is important for regulation of cyclin-cdk activity and can either activate or inhibit cdk activity (Lew and Kornbluth, 1996; Puri et al., 1999). Phosphorylation of Thr160 residue is required for full activation and stabilisation of the cyclin-cdk complex. The kinase responsible for this phosphorylation is CAK kinase, a complex formed between cyclin H and cdk7 (Desai et al., 1992; Fisher and Morgan, 1994; Devault et al., 1995; Fisher et al., 1995; Morgan, 1997). Cyclin H expression is stable throughout the cell cycle, and CAK activity does not appear to be tightly regulated; its phosphorylation of cdks is therefore controlled by substrate availability (Fischer and Morgan, 1994; Morgan, 1995). CAK is a component of TFIIH, and consequently of the basal transcriptional machinery

(Serizawa et al., 1995); it may therefore provide a link between this and the cell cycle machinery. Phosphorylation of cdks within the conserved residues Thr-14 and Tyr-15, which are located in the catalytic cleft, inhibits cyclin-cdk function, and dephosphorylation of these residues is required for activation of the complexes (Lew and Kornbluth, 1996). Dephosphorylation is accomplished by the CDC25 phosphatases. There are three CDC25 proteins in mammalian cells (CDC25A-C; Galaktionov and Beach, 1991). CDC25 A is expressed in early G1, CDC25B is expressed in the G1/S transition, and CDC25C in G2/M (Galaktionov et al., 1995).

Additional regulation of cyclin-cdk activity occurs by binding to CDIs (cyclin dependent kinase inhibitors). There are two families of CDIs which are distinguished on the basis of their sequence similarity and substrate specificity (Harper and Elledge, 1996; Sherr and Roberts, 1999). The Cip-Kip family inhibits all of the G1 cyclin-kinase complexes (cdk2, cdk4, cdk6) and includes p21, p27, and p57 . The INK4 family inhibits cdk4/cdk6 specifically, and includes p15, p16, p18 and p19. The INK4 family members, p15 and p16, compete with D cyclins for binding to cdk4/cdk6 (Hannon and Beach, 1994), and inhibit proliferation only in cells possessing functional *Rb* (Guan et al., 1994; Medema et al., 1995; Lukas et al., 1995). Overexpression of p27, on the other hand, induces G1 arrest independent of the presence of *Rb* (Polyak et al., 1994; Toyoshima and Hunter, 1994) and this may be related to the requirement for cdk2 activity for S-phase entry. The p21 protein may also have a more direct role in inhibiting replication as it is generally found in quaternary complexes with cyclin-cdk and PCNA (Xiong et al., 1993; Zhang et al., 1993).

It was first proposed that CDI regulation of cyclin-cdk activity is stoichiometric (Zhang et al., 1994, Polyak et al., 1994). For example, p21 and p27 co-precipitate with active cyclin-cdk complexes (Xiong et al., 1993a,b; Zhang et al., 1994), and a further increase in p21/p27 or decrease in cyclin-cdk levels are required to inhibit complex activity. More recently, it has been proposed that p21 and other CIP family members can promote cell division by promoting nuclear localisation of

cyclinD-cdk4 as well as cyclin B-cdc2 (LaBaer et al., 1997; Depoortere et al., 2000). Export and cytoplasmic degradation of the CDIs then occurs for cyclin-cdk activation (Tomoda et al., 1999). Indeed, subcellular compartmentalisation is another effective mechanism of cyclin-cdk activity, since most of their substrates are nuclear (Moore et al., 1999; Pines, 1999).

The transitions between the different cell cycle phases will now be examined in more detail, particularly entry to S-phase.

1.4.2 The transition from G1 to S phase - The role of *Rb*

There is a point in G1, termed the restriction point, after which cells become committed to enter S phase and do not require further mitogenic stimulation to do so (Pardee, 1989). Phosphorylation of pRB is thought to be the molecular switch that regulates passage through the restriction point, as pRb inactivation by phosphorylation is required for entry to S (Weinberg, 1995; Mulligan and Jacks, 1998; Kaelin, 1999). The Rb protein is one of three members of the pocket protein family which also includes p107 and p130 (Mulligan and Jacks, 1998). These proteins have a bipartite protein binding domain, the pocket, that is the most conserved region among the three; sequences outside this domain are conserved between p107 and p130 but not pRb.

The hypophosphorylated or active form of pRb is only present in G1. The main activity of pRb in regulating S phase entry is by inhibiting transcriptional activity of the E2F/DP family of transcription factors. These factors bind to the pocket domain and this results in masking of the E2F activation domain (Dyson, 1998). Viral oncoproteins that activate cell cycle re-entry also bind to this region resulting in the release of E2Fs (Whyte et al., 1988; Dyson et al., 1989; Ludlow et al., 1989; reviewed in Harbour and Dean, 2000). Both E2Fs and viral oncoproteins bind preferentially to hypophosphorylated pRb. Phosphorylation results in release of E2Fs and activation of a host of genes whose transcription is important for S-phase entry such as c-myc, B-myb, cdc2, cyclin A, cyclin E, cdk2, thymidine synthetase, thymidine kinase, and DNA polymerase α (reviewed in Sherr, 1996

and Dyson, 1998; Chellapan et al., 1991; ; Dou et al., 1992; Ogris et al., 1993; LaThangue, 1994; Geng et al., 1996). More recently E2F has been shown to also regulate the transcription of genes involved in replication origins such as cdc6 and MCMs (minichromosome maintenance helicases; Leone et al., 1998; Yan et al., 1998). E2F overexpression induces quiescent cells to enter S-phase, and this activity is suppressed by the co-expression of pRb family members. Overexpression of pRb, on the other hand, induces G1 arrest and this arrest can be overcome by overexpressing E2F (Johnson et al., 1993; Qin et al., 1994, 1995; Lukas et al., 1996).

In mammalian cells, pRb is hyperphosphorylated from late G1 to the end of M phase (Weinberg, 1995; Kaelin, 1999). Phosphopeptide analysis suggests that there are more than sixteen distinct sites of phosphorylation on pRb and these are typical of sites modified by the cdks (Knudsen and Wang, 1996; Kaelin, 1999). In mammalian cells, pRb is a substrate of the two G1 cyclin-cdk complexes, cylinD-cdk4/6 and cyclin E-cdk-2 (Ohtsubo and Roberts, 1993; Resniztky et al., 1994). Functional inactivation by both cyclinD-cdk4/6 and cyclin E-cdk2 are required for S-phase entry (Lundberg and Weinberg, 1998; Harbour et al., 1999), and this inactivation is sequential in that cyclin D-cdk4/6 activity is maximal during mid to late G1, whereas cdk2 activity is maximal at the end of G1 and during the G1/S phase transition. The Rb protein is a nuclear protein, and cyclin complexes must be active in the nucleus to exert their pRb regulatory functions. Cyclin D-cdk4/6 complexes are actively imported into the nucleus during G1 and this translocation may involve the p42/p44 MAP kinase pathway which is activated by growth factor stimulation. In unstimulated cells, MAP kinase and its activating kinase MAPKK form a complex that is actively exported from the nucleus because of a nuclear export signal on MAPKK. Growth factor stimulation leads to phosphorylation and release of MAPK, and it translocates to the nucleus (Fukuda et al., 1997; Brunet et al., 1999). The D cyclins are perceived as the target of mitogen induced signalling, as they are the first to be upregulated upon mitogen stimulation of quiescent cells; in the absence of mitogen stimulation, D cyclins are rapidly degraded ($t_{1/2}$=30 minutes; Diehl et al., 1997). During S-phase, the

complexes are excluded from the nucleus and the D cyclin is targeted for degradation in the cytoplasm (Diehl et al., 1997, 1998).

In proliferating cells, cdk2 and cyclin E form complexes at the G1/S boundary and act at the origins of replication to induce initiation of DNA synthesis (Ohtsubo et al., 1995). Cyclin E is a relatively short lived protein and its expression must be maintained for S-phase entry. Cyclin E expression is not regulated directly by mitogen stimulation and its sustained expression may be the molecular basis for passage trough the restriction point. Cyclin E and E2F are both transcriptional targets of E2F and are upregulated as a result of pRb inactivation; release of E2F establishes a positive feedback loop that drives cells into S (Johnson et al., 1994; Leone et al., 1998). In human cells, cyclin E/cdk2 is concentrated in the nucleus (Ohtsubo et al., 1995; Moore et al., 1999). In addition to activation of cyclinD-cdk4/6 and cyclinE-cdk2, phosphatase activity is essential for S-phase entry. The phosphatase cdc25 A is induced by serum in quiescent cells, and cdc25B is expressed at G1/S transition. (Galaktionov et al., 1996).

Although the CDIs also have a role in regulating progression through G1, their function has been examined principally in quiescent, senescent, or differentiating cells and in overexpression studies where they cause G1 arrest. At the end of G1 their ubiquitin-mediated proteolysis is essential for the onset of DNA synthesis (Roberts and Sherr, 1999). The case of p27 regulation is interesting and suggestive of CDI function. In quiescent cells p27 is highly expressed but is degraded following mitogen stimulation (Tomoda et al., 1999). This degradation requires nuclear export and possibly previous nuclear localisation, since a p27 that lacks a nuclear localisation signal cannot be degraded. In proliferating cells p27 is sequestered by cyclin D/cdk4 complexes (Toyoshima and Hunter, 1994). Upon cell cycle inhibition by TGFβ, p15 protein is expressed in the cytoplasm where it binds to newly synthesised cdk4; the p27 remaining in the nucleus is then free to bind to cdk2 and inhibit S-phase entry (Reynisdottir and Massague, 1997). The Cip-Kip and the INK4 families cooperate to induce cell cycle arrest, but only the Cip-Kip family seems to regulate cdk activity levels in cycling cells.

There is an important checkpoint in G1 that inhibits entry to S phase. The function of p21 is important in the G1 DNA damage checkpoint. DNA damage by various treatments blocks pRb phosphorylation through induction of p21 (El-Deiry et al., 1994; Dulic et al., 1994; Deng et al., 1995; reviewed in El-Deiry, 1998), presumably to allow time for the cell to repair DNA and restore genomic integrity before initiating replication.

1.4.3 Progress through S-phase, entry to G2

As mentioned above, cyclin D-cdk4/6 and cyclin E-cdk2 are required for inactivation of pRb and S phase entry. The sequential activation of cyclinE-cdk2 and cyclinA-cdk2 complexes at late G1 early S, on the other hand, is required for initiation of replication (Krude et al., 1997). Cyclin A synthesis is initiated as a consequence of pRb phosphorylation and release of E2F, and the nuclear localisation of cyclin A is an early marker of S phase (Girard et al., 1991, cited in Yoshida et al., 1998). Exit from S-phase is thought to involve the inhibition of E2F. CyclinA-cdk2 phosphorylates E2F-DP1 heterodimers on DP1 and inhibits their binding to DNA (Krek et al., 1994). This inactivates the positive feedback loop set up between cyclin E and E2F at the beginning of S-phase, allowing the cells to exit S-phase (Puri et al., 1999). CyclinA-cdk2 also bind to complexes at origins of replication and may be involved in phosphorylating a variety of substrates at this site, including cdc6 (Leather wood, 1998; Jallepalli and et al., 1997; Lopez-Girona et al., 1998).

Cyclin A-cdk2 may also be involved in preventing rereplication because it phosphorylates cdc6, thereby targeting it for nuclear export and degradation by ubiquitin-mediated proteolysis (Hua et al., 1998; Petersen et al., 1999). The cdc6 protein is a component of DNA replication origins and is imported into the nucleus during G1 phase. Another possibility is that cyclin-cdk phosphorylation of MCM proteins prevents their reloading onto pre-initiation complexes after replication is triggered (Lopez-Girona et al., 1998). Cyclin A is implicated both in the control of S-phase and in the regulation of mitosis (Minshull et al., 1989;

Lehner and O'Farrel, 1989; Furuno et al., 1999; Karlsson et al., 1999). Cyclin A levels continuously increase during S and G2 (Pines and Hunter, 1991; Petersen et al. 1999); it has been suggested that low levels of cyclin A promote passage through S-phase whereas higher levels are required for S phase exit and for its mitotic functions (Strausfeld et al., 1996).

1.4.4 Entry to M

The first irreversible event in mitosis is nuclear envelope breakdown (NEB). It is triggered by hyperphosphorylation of nuclear lamins by cyclin B-cdc2 activity which leads to their depolymerisation (Heald et al., 1993; Fields and Thompson, 1995). Cdc2 is present throughout the cell cycle, but cyclin B is synthesised from late S phase to early metaphase (prophase), and accumulates in the cytoplasm where it forms cyclin B-cdc2 complexes (reviewed in Lew and Kornbluth, 1996; Morgan, 1997; Pines, 1999; Ohi and Gould, 1999; Puri et al., 1999; Sluder et al., 1999). These complexes are inactive and do not promote entry into mitosis until S phase is complete. Dephosphorylation of inhibitory sites in cdc2 is a key determinant of initiation of mitosis in *S. pombe* (Gould and Nurse, 1989; Nurse, 1997), but in mammalian cells the regulation of entry to mitotis is more complex. The dephosphorylation of cdc2 alone is insufficient to trigger mitosis, as demonstrated by the limited premature mitotic events that are induced by the overexpression of cdc2AF, a dominant mutant that cannot be inhibited by phosphorylation (Heald et al., 1993; Jin et al., 1996, 1998). Cyclin B1 constantly shuttles between the nucleus and the cytoplasm during G2, but is predominantly cytoplasmic during this phase (Hagting et al., 1998; Yang et al., 1998). Constitutively nuclear cyclin B1-cdc2 is not fully active in initiating mitotic events (Hagting et al., 1998; Yang et al., 1998; Jin et al., 1999). In the cytoplasm, the complexes are kept inactive by Tyr15 phosphorylation by Myt1 (Mueller et al., 1995; Booher et al., 1997). In the nucleus, the complex is maintained inactive by Thr-14 phosphorylation by Wee1 (Heald et al., 1993; Coleman and Dunphy 1994). Wee1 proteolysis is required for mitotic entry in *Xenopus* extracts and inhibition of replication inhibits wee1 proteolysis, suggesting it is one mechanism of co-ordinating mitotic entry with the termination of replication (Michael and

Newport, 1998).

In prophase, cyclin B translocates to the nucleus before NEB (Pines and Hunter, 1991; Hagting et al., 1998; Pines 1999), possibly as the result of the phosphorylation and masking of a cytoplasmic retention signal in cyclin B (Li et al., 1997). Entry into M phase is triggered when the cdc25C phosphatase is phosphorylated and translocates to the nucleus (Kumagai and Dunphy, 1997; Lopez-Girona et al., 1999). The cdc25C protein is also phosphorylated by cyclinB-cdc2, and once the cdc2-cdc25 positive feedback loop has been activated it drives cells irreversibly to mitosis (Lew and Kornbluth, 1996; Pines, 1999). It has also been suggested that cdc25 may play a role in nuclear localisation of cyclinB by a "piggyback" mechanism (Moore et al., 1999). Other kinases have been identified that act upstream of cdc25 to activate it, but it is still unclear what mechanisms act upstream of these kinases to regulate entry to mitosis. One kinase that has been implicated in activation of cdc25C is polo kinase (Kumagai and Dunphy, 1996; Abrieu et al., 1998; Karaiskou et al., 1999) which in turn is phosphorylated by Plkk1 (polo kinase kinase, Qian et al., 1998). There are also kinases involved in cdc25 inhibition, in particular following the activation of a G2 checkpoint. In mammalian cells the Chk1 and Chk2 nuclear protein kinases inactivate cdc25 by phosphorylation on ser216, following DNA damage. This creates a binding site for 14-3-3 proteins which are also induced after DNA damage, and facilitate cdc25 inactivation by nuclear export (Peng et al., 1997; Sanchez et al., 1997;Blasina et al., 1999; Furnari et al., 1999; Lopez-Girona et al., 1999).

1.4.5 Exit from Mitosis and Entry to G1

At metaphase, the chromosomes condense, align along the midline of the mitotic spindle, and the kinetochores of the sister chromatids attach to spindle microtubules. There are several mechanisms that regulate spindle formation and sister chromatid attachment to microtubules (reviewed in Rieder et al., 1994; Nasmyth, 1999; Sluder et al., 1999), but once everything is in place, the separation of sister chromatids is triggered by the anaphase promoting complex (APC) and anaphase begins. Cyclin B1-cdc2 activity is required for this transition as it

activates the APC (King et al., 1995). This complex is a ubiquitin protein ligase and targets proteins that hold sister chromatids together for proteolysis. It also targets cyclin B for proteolysis, eventually leading to complete inactivation of the cyclin B-cdc2 complex as the cells reach telophase (King et al., 1995; Clute and Pines, 1999; Zachariae and Nasmyth, 1999). Following the expression of non degradable forms of cyclin B, the chromosomes complete anaphase normally, but do not decondense, the nuclear envelope does not reform, and cytokinesis does not occur (Wheatlye et al., 1997; Hinchcliffe et al., 1998). The rapid decrease in cyclinB-cdc2 activity results in nuclear envelope reformation around the separated chromosome masses and cytokinesis. At this point there is very little cyclin-cdk activity and the pRb protein is again hypophosphorylated and active (Weinberg, 1995; Kaelin, 1999; Puri et al, 1999). Cyclin E and A levels are low and the cell is now ready to receive the mitogenic stimuli that will initiate progression through another G1, or to adopt other fates such as differentiation or quiescence depending on the environmental stimuli that it receives.

1.4.6 Cell cycle arrest during myogenesis

The initiation of muscle differentiation in cultured cells seems to be controlled by regulatory cascades involving three players: MyoD, pRb, and the cdk inhibitor p21. Cell cycle arrest during myogenesis occurs in the G1 phase (Nadal-Ginard, 1978; Clegg et al., 1987; Lassar et al., 1994). Muscle specific gene transcription is initiated only when myoblasts are growth arrested in G0/G1, suggesting that the initiation of the myogenic program is dependent on gene products that are expressed or functional in this phase (Olson, 1992). The Rb protein is one such gene product, as it is hypophosphorylated and active only in G1 (Weinberg, 1995; Kaelin, 1999). MyoD is expressed in proliferating myoblasts during G1, but is downregulated as these cells enter S-phase (Kitzmann et al., 1998). Hypophosphorylated pRb may possibly interact with MyoD (Gu et al., 1993), but this association is not sufficient to initiate myogenesis.

There are inumerous accounts of inhibition of muscle differentiation by positive cell cycle regulators that act in G1, and they may exert their activity by inhibiting

MyoD function. Overexpression of immediate early gene products, namely c-jun, c-myc and c-fos inhibit myogenesis, as does overexpression of cyclin D1, and E2F1 (Skapek et al., 1995, 1996; Wang et al., 1995,1996; Guo and Walsh, 1997). In cycling myoblasts, these genes are all expressed at high levels and the myoblasts do not enter the differentiation pathway as long as their expression is maintained (Skapek et al., 1995, 1996; Wang et al., 1995; Puri et al., 1998). On the other hand, forced expression of cdk inhibitors p16 or p21 in proliferating myoblasts results in the activation of MyoD transcriptional function (Skapek et al., 1995). During differentiation, levels of cyclin and cdk proteins and transcripts go down, as do the levels of E2F1 transcription factors (Jahn et al., 1994; Guo et al., 1997; Wang and Walsh, 1996; Tiainen et al., 1996; Mal et al., 2000). These observations would suggest that down regulation of cdk activity is required for MyoD to exert its differentiation functions.

Other observations suggest that this inhibition is independent of cdk4 kinase activity (Skapek et al., 1995; Zhang et al., 1999a). Overexpression of cyclin D1 can inhibit muscle differentiation independent of pRb hyperphosphorylation (Skapek et al., 1995). Binding to cdk4 seems to be sufficient to inhibit MyoD induced transcription, even in the absence of cdk4 kinase activity (Zhang et al., 1999). The cdk4 kinase is cytoplasmic and localises to the nucleus after binding to cyclin D1, but this cyclin is rapidly degraded after mitogen withdrawal (Diehl et al., 1997), and the cdk cannot inhibit MyoD.

Several MyoD transcriptional targets are important for myogenesis, two of which are pRb and p21. The expression of p21 is the first irreversible step in cell cycle withdrawal during myogenesis. After p21 expression is induced, it is no longer downregulated by mitogen stimulation (Guo et al., 1995; Halevy et al., 1995; Jahn et al., 1994 ; Andrés and Walsh, 1996; Wang and Walsh, 1996; Yoshida et al. 1998). Both myogenin and MyoD, on the other hand, are downregulated by serum and myogenin expressing cells can re-enter the cell cycle (Andrés and Walsh, 1996; Yoshida et al., 1998). The inhibition of cdk2 associated kinase activity is observed at the same time as p21 levels increase after serum withdrawal (Mal et al., 2000).

The induction of p21 prevents the formation of E2F and cyclin E/CDK2 as well as E2F cyclin A/CDK2 complexes which are involved in the initiation of DNA synthesis (Puri et al., 1997). The importance of this activity is underlined by the observation that the continued presence of E2F1 or 4 in the nucleus of undifferentiated myoblasts blocks cell cycle exit and promotes progression to S-phase in the absence of mitogenic signals (Puri et al., 1998). As a result of inhibition of cyclin A transcription, its levels go down; cyclin E levels are also reduced, and any residual cyclin E-cdk2 complexes are maintained in an inactive state by p21 binding (Mal et al., 2000).

In differentiated myotubes, the p21 protein also plays an important role in maintaining cell cycle arrest, as it is expressed at high levels in these cells (Guo et al., 1995; Halevy et al., 1995; Jahn et al., 1995; Parker et al., 1995; Skapek et al., 1995; Andrés and Walsh, 1996; Wang and Walsh, 1996; Yoshida et al. 1998; Pajalunga et al., 1999; Mal et al., 2000). The role of p21 may be to maintain cyclin E-cdk2 inactive, since upregulation of cyclin E expression in differentiated myotubes does not lead to cdk2 activation (Pajalunga et al., 1999). In fact, it has recently been proposed that cell cycle arrest is maintained in differentiated myotubes by both p21 and pRb, since E1A mutants that are unable to bind either protein are ineffective at inducing cell cycle re-entry (Mal et al., 2000). When E1A p21 binding mutant is expressed in differentiated myotubes, cyclin E-cdk2 is activated and phosphorylates pRb, but this phosphorylation does not result in DNA synthesis. It is therefore conceivable that phosphorylation of other sites on pRb is required for DNA synthesis in differentiated myotubes. It has been observed that p21 levels begin to go down at about 5 days after serum withdrawal, a time when the expression of p18, and INK4 family member, is upregulated (Franklin and Xiong, 1996; Phelps, 1998). Other CDIs may also have a role in maintaining post mitotic arrest. High levels of p57 are expressed in adult skeletal muscles (Matsuoka et al., 1995; Lee et al., 1995), and p27 is modestly upregulated during myogenesis *in vitro* (Halevy et al., 1995; Wang and Walsh, 1996). The single knockout mice for p21, p18, p27, or p57 do not present muscle phenotypes (reviewed in Zhang, 1999), but the p21-p57 double knockout presents

a phenotype that is very similar to that of the myogenin knockout mouse (Zhang et al, 1999b). Myogenin expression occurs normally in these animals suggesting that their expression may be coordinately regulated.

The maintenance of stable cell cycle arrest in cultured myotubes absolutely requires pRb. Myotubes that are *Rb* null are able to re-enter the cell cycle after serum stimulation (Schneider et al., 1994; Novitch et al., 1996), as are myotubes in which pRb is inactivated by the expression of viral oncoproteins (Endo and Nadal-Ginard, 1989, 1998; Iujvidin et al., 1990; Gu et al., 1993; Okhubo et al., 1994; Crescenzi et al., 1995; Mal et al; 2000). Ectopic expression of p21 or p16 does not prevent cell cycle re-entry in *Rb-/-* myocytes, a situation where p107 and p130 are both present (Novitch et al., 1996). The inactivation of pRb coincides with reinduction of cyclin and cdk expression, probably as a result of increased E2F activity, and inhibition of p21 expression. The overexpression of E2F1 and 4 at very high levels induces DNA synthesis in multinucleate myotubes and cyclin upregulation (Gill and Hamel, 2000).

During myogenesis pRb expression is upregulated and remains high, whereas that of p107 is normally downregulated (Schneider et al., 1994). In *Rb-/-* myotubes, p107 expression remains high. The expression of p107 and of p130 in *Rb -/-* myotubes is not sufficient to maintain stable cell cycle arrest, and p107 is downregulated after serum stimulation of *Rb-/-* myotubes (Schneider, 1994; Novitch, 1996). The myotubes enter S-phase and arrest in G2 (Novitch et al., 1996), This finding is of particular interest in view of the observations in newt myotubes described below. Unlike pRb, p107 and p130 associate stably with cyclin E-cdk2 and cyclin A-cdk2 to form E2F containing complexes still able to bind E2F sites (Whyte, 1995; Muligan and Jacks, 1998). In *Rb-/-* cells cyclin A and E are not downregulated following serum withdrawal (Novitch et al., 1996), and cyclin D is upregulated following serum stimulation, which does not occur in wild type cells (Schneider et al., 1994). Formation of these cyclin-cdk complexes is inhibited by increased expression of p27 and p21 (Shiyanov et al., 1996; Zerfass-Thome et al., 1997), which may explain why E2F4 expression, which is maintained in

differentiated myotubes, does not induce cell cycle re-entry (Puri et al., 1997, 1998).

The requirement for E2F inactivation for muscle differentiation may be due to the fact that, in addition to inducing cell cycle progression, the expression of E2F regulated genes also inhibits transcription of muscle specific genes. Transcription from myogenic promoters can be inhibited by overexpression of cyclins D1, E, and A (Rao et al., 1994, Guo and Walsh, 1997; Skapek et al., 1996) but normal transcription can be restored by coexpression of p21 and p16 or hyperactive mutants of pRb which ultimately result in E2F inhibition (Skapek et al., 1995, 1996; Guo and Walsh, 1997).

1.4.7 Other roles of *Rb* in muscle differentiation

The dramatic cytoskeletal reorganisation that occurs during mitosis and cytokinesis in proliferating cells could disrupt the highly ordered structure of the myofibre cytoskeleton , and this may be one reason why cell cycle arrest is maintained in differentiated muscle. Cell cycle withdrawal, however, is required for muscle differentiation, before myofibrillar proteins are expressed. In addition to stable cell cycle arrest, pRb is required for expression of late markers of muscle differentiation *in vitro* and *in vivo* (Novitch et al., 1996,1999; Zackenhaus et al., 1996). A critical question is whether the requirement for late muscle gene expression reflects entirely the cell cycle arrest dependent of pRb. In the absence of pRb, MyoD induces MEF2C expression, nuclear localisation, and DNA binding, but MEF2C is not transcriptionally active (Novitch et al., 1999). Expression of an E2F-Rb fusion construct containing only the small pocket domain of pRb induces cell cycle arrest, but restores only part of the transcriptional activity to a MEF2C reporter plasmid that is obtained with full length pRb (Novitch et al., 1996, 1999). This suggests that pRb is implicated in a second pathway for MEF2C activation that is independent of its effects on the cell cycle.

The Rb protein may regulate muscle gene expression by virtue of its interaction with other transcription factor families. The association of Rb family members

with the E2F family has been discussed above. This association inhibits the transcriptional activity of E2Fs by masking the E2F activation domain (Dyson, 1998), and therefore inhibits expression of genes required for cell cycle progression. There are other mechanisms by which pRb may inhibit E2F transcriptional activity. A more general mechanism by which pRb actively represses E2F promoters is by recruiting histone deacetylase, which is thought to facilitate formation of nucleosomes and thereby hinder access to promoters by transcription factors (Brehm et al., 1998; Magnaghi-Jaulin et al., 1998). The Rb protein also recruits HBRM and BRG1, homologues of the budding yeast SWI1/SNF2 proteins. These form part of the complexes that facilitate gene transcription by disruption of nucleosomes (Dunaief et al., 1994). In general, the association of pRb with HBRM and BRG1 accompanies binding to transcription factors that induce differentiation (Zhang, 1999). These interactions suggest a model whereby pRb recruits deacetylases in conjunction with transcription factors, such as E2F, whose activity it represses, whereas it recruits BRG1/HBRM in conjunction with transcription factors that act to induce differentiation (Zhang, 1999).

Rb family members also interact with other transcription factor families and these interactions may have functions in muscle differentiation. For example, pRb and p130 bind to members of the HMG transcription factor family. One member, HBP1 functions as a transcriptional repressor of the c-myc promoter and its over expression leads to cell cycle arrest (Tevosian et al., 1997). It is known that pRb interacts with members of the C/EBP family of transcription factors potentiating their DNA binding and transcriptional activities in adipocytes, which also undergo stable post-mitotic arrest (Chen et al., 1996). Glucocorticoid receptor mediated transactivation of reporter genes is also potentiated by interaction with pRb, (Singh et al., 1995), and this may explain the positive regulation of myogenic differentiation by retinoic acid. The Rb protein also binds to c-jun and potentiates its transcriptional activity (Nead et al., 1998).

Rb may also have other roles in myogenesis, notably inhibition of apoptosis. In culture, widespread apoptosis accompanies myocyte differentiation (Wang et al., 1997; Wang and Walsh, 1996). *Rb-/-* myotubes, and myotubes in which pRb is inactivated by the expression of viral oncoproteins, also undergo cell death. The Rb protein may exert its antiapoptotic function in part by inhibiting E2F gene transcription. Indeed, deregulated expression of E2F1 causes apoptosis in differentiated cardiomyocytes and neurons (Kirshenbaum et al., 1996; Azuma-Hara et al., 1999). E2F overexpression also represses p21 expression; it has been suggested that apoptosis occurs as a result of defective cell cycle withdrawal, since both p21 and p16 overexpression limits the number of cells undergoing apoptotis during myogenesis, but only in the presence of *Rb* (Wang et al., 1996; Wang and Walsh, 1997). E2F1 inactivation rescues the lethal phenotype of the *Rb-/-* mouse, but these mice present many developmental defects highlighting the importance of pRb in other processes besides E2F regulation (Tsai et al., 1998).

Surprisingly, *Rb-/-* mice develop normal muscle (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). It is clear therefore that pRb expression is not required for muscle development *in vivo*, and that p107 and p130 may be sufficient to sustain this process. The differentiation of several other tissues is defective in these mice and they die before birth with defects in the hematopoietic and central nervous systems. Embryonic stem cells that are *Rb-/-* are able to contribute to most tissues of the adult in chimeras with wild type cells (Mulligan and Jacks, 1998). The organisation of the cells in the tissue and the resulting interactions of the myofibres with the extra-cellular matrix and other cells in the tissue may stabilise the post-mitotic phenotype *in vivo*, perhaps by enhancing the compensatory activities of p107 and p130 (Mulligan and Jacks, 1998). In mice that express a weak *Rb* allele in an *Rb* null background, the myofibres contained abundant large nuclei which had undergone several rounds of endoreduplication, and no mitosis was observed (Zacksenhaus et al., 1996), indicating that there are defects in the post mitotic arrest. It would be interesting to investigate the response of this muscle and Rb null muscle to injury and stress, as reactivation of cell cycle in these contexts may reveal a role for Rb.

1.4.8 Cell cycle re-entry by newt myotubes in culture

Newt myotubes in culture show a striking distinction from myotubes of other vertebrates (Tanaka et al., 1997). When the serum concentration in the medium was raised to 10% or 15%, the nuclei within the myotube entered S-phase and labelled with BrdU or [^{3}H]-thymidine (Figure 1.5 A and B). Although this process was somewhat asynchronous, it was possible with appropriate labelling periods to show that more than 80% of the myotubes can respond. The duration of S phase was analysed in a double labelling experiment in which myotubes were stimulated with serum, pulsed with [^{3}H]-thymidine to label nuclei entering S-phase, and then incubated with BrdU at various intervals after this. This analysis showed that the duration of S-phase (48-72 hours) in the myotubes was comparable to that in mononucleate A1 cells, and that myotubes arrested stably at the end of S-phase without entering M, or undergoing any pathological event such as apoptosis (Tanaka et al., 1997). These conclusions were further supported by an analysis of the DNA content of individual nuclei in myotubes by quantitative measurement of fluorescence after propidium iodide staining. This showed that nuclei with 4N DNA content were detectable in myotubes after serum stimulation (Figure 1.5 C).

This response of nuclei in a post-mitotic myotube is evoked by all sources of vertebrate serum which have been investigated, but it appears not to be due to the serum growth factors that act on cycling or quiescent cells. The newt myotubes were completely refractory to growth factors such as PDGF, EGF, or IGF-1, which act to stimulate division of A1 mononucleate cells (Tanaka et al., 1997, 1999). It appears therefore that the post-mitotic arrest in urodele cells is quite comparable to that in avian or mammalian myotubes. The responsiveness of the newt myotubes to serum was exquisitely sensitive to cell density (contact inhibition) – a three fold change in density resulted in a 100 fold change in S-phase re-entry – and this can be used as a control manipulation in certain experiments.

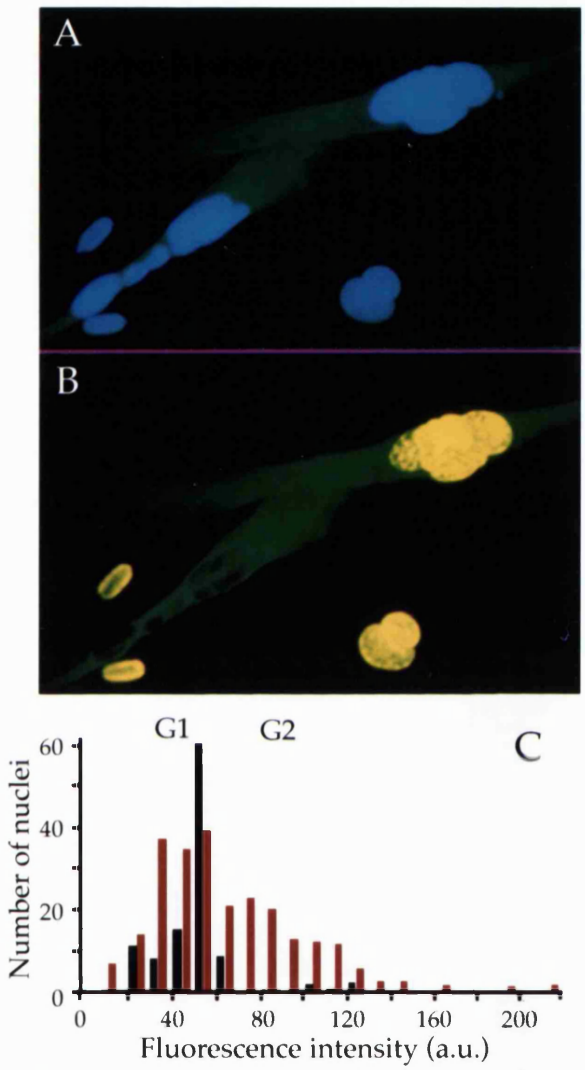


Figure 1.5. Serum-stimulated myotubes re-enter the cell cycle and arrest in G2. (**A**) The two A1 myotubes are stained with muscle-specific myosin heavy chain (MHC, green) and Hoechst to stain DNA (blue). (**B**) The same myotubes showing muscle-specific myosin (green) and BrdU-positive nuclei (yellow). The myotube on the upper right has incorporated BrdU during 24 hour pulse given on day 4 after serum stimulation; the myotube on the lower left is negative for BrdU. (**C**) The DNA content of individual nuclei from MHC-positive myotubes maintained in the medium containing 0.5% serum (black bars) or 10% serum (red bars) for 9 days. Most nuclei maintained in 0.5% serum have a G1 (2N) DNA content consistent with withdrawl from the cell cycle in this phase. A substantial proportion of nuclei maintained in 10% serum have nuclei with a G2 (4N) DNA content, consistent with passage through S-phase and arrest before mitosis (yellow bars). Some serum-stimulated myotubes have DNA content that is higher than 4N. This figure is modified from Tanaka et al., (1997).

The re-entry to S-phase parallels the behaviour of mouse myotubes which are null for both copies of the *Rb* gene (Schneider et al., 1994). The newt Rb gene was cloned and expressed, and affinity purified antibodies prepared to the Rb protein. The newt myotubes are clearly not *Rb* knockout since they express both *Rb* mRNA and protein as detected by immunoprecipitation (Tanaka et al., 1997). Nonetheless, pRb does appear to be central to re-entry because serum stimulated myotubes showed the hyperphosphorylated (inactive) form of pRb after immunoprecipitation. Furthermore, the phosphorylation of pRb is necessary as evidenced by the inhibitory effects of expressing *Rb* constructs (wild type and constitutively active) in the myotubes, and, most important, of expressing the cdk4/6 inhibitor p16. Expression of human p16 in the A1 myotubes completely blocked S-phase entry after serum stimulation, strongly implicating pRb as an endpoint to the pathway (Tanaka et al., 1997). The pivotal role of pRb in the post mitotic arrest is consistent with results in both the newt and mammalian myotubes. Although there are certain parallels between the behaviour of newt and mouse *Rb-/-* myotubes, there are also distinctions. Both appear to be blocked rather stably in G2 (Novitch et al., 1996; Tanaka et al., 1997, this thesis), but in *Rb-/-* myocytes the role of pRb in mediating arrest is apparently played by p107, an Rb family member, and this is the target of serum action (Schneider et al., 1994).

The identity of the extracellular ligand which activates the pathway in newt myotubes is an important question. Although various growth factors and other proteins were inactive, crude preparations of bovine thrombin were of significantly higher specific activity than serum sources. Such preparations showed two peaks of activity on chromatography columns and one peak was further purified and shown to be thrombin (Tanaka et al., 1999). The activity on the A1 myotubes was removed by preincubation with specific thrombin inhibitors such as hirudin. Thrombin required low, subthreshold amounts of serum for its activity and further experiments showed that it acts indirectly by cleaving precursor protein (s), or by

removing an inhibitor to generate a ligand which then acts directly on the A1 myotubes. One other protease which was active when tested in this assay was plasmin, and hence it is two coagulation-related proteases which are able to generate re-entry activity from components in serum.

The second peak of activity in crude preparations of thrombin had the properties expected for the thrombin derived activity. It acted on the newt myotubes in the absence of serum, and interestingly, it did not act on A1 mononucleate cells; the opposite distinction to that made by a growth factor, such as PDGF (Tanaka et al., 1999). It is possible, therefore, that responsiveness to this signal is a property of the differentiated cell rather than the precursor. The activity has been purified significantly from the crude thrombin preparations, but its identity remains unknown.

This mechanism for activating cell cycle re-entry by a post mitotic cell may be relevant for the initiation of regeneration. When frozen sections of an early newt limb regenerate were overlaid with a membrane impregnated with a fluorogenic thrombin substrate, a local maximum of thrombin activity was readily detected in the mesenchyme underlying the WE (Tanaka et al., 1999). It seems plausible that the events of wound healing and coagulation in vertebrates lead to activation of thrombin and plasmin, and hence to the generation of an activity which locally stimulates re-entry of differentiated cells. It is striking that mammalian C2C12 myotubes are completely refractory to this activity, thus providing a possible clue to the restriction of regenerative ability. On this model, the signal may be generated in all vertebrates, but it is only the differentiated cells of urodeles which are responsive. Although the importance of thrombin had not been realised when I began my experimental work, I attempted to examine the nuclear responsiveness of mammalian myotubes to the serum activated pathway by generating A1/C2C12 heterokaryon myotubes (Chapter 3).

1.5. Focus for experiments in this thesis

As discussed earlier in this introduction (sections 1.2.6 and 1.8), two important aspects of cellular plasticity are apparent in newt myotubes: cell cycle re-entry, and reversal of the multinucleate to mononucleate transition. After implantation of newt myotubes into regenerating limbs, DNA synthesis and generation of mononucleate progeny are observed (Lo et al., 1983; Kumar et al., 2000). In culture, serum stimulation leads to phosphorylation of the newt Rb protein and DNA synthesis (Tanaka et al., 1997, 1999). This latter observation in cultured newt myotubes is striking because cell cycle re-entry does not occur in the mammalian system. Mammalian myotubes are stably arrested from the cell cycle in part by virtue of their inability to phosphorylate the Rb protein (Gu et al., 1993; Lassar et al., 1994; Mal et al., 2000); mouse myotubes are able to re-enter the cell cycle following the expression of viral oncoproteins that inactivate pRb (Endo and Nadal Ginard, 1989; 1998; Iujvidin et al., 1993; Crescenzi et al., 1995). Rb-/- mouse myotubes also respond to serum stimulation by synthesising DNA (Schneider et al., 1994; Novitch et al., 1996).

It is possible to analyse whether the serum-activated pathway leading to pRb phosphorylation and DNA synthesis in newt myotubes can operate on mammalian wild type nuclei by forming mouse-newt hybrid myotubes, herein referred to as heterokaryons. In the first year of my PhD, I developed a method to form heterokaryon myotubes from the newt A1 cell line and the mouse C2C12 cell line. In the context of the heterokaryon myotube, mouse nuclei were found to synthesise DNA following serum stimulation. These results are presented in Chapter 3. There is clearly much that can be investigated in the heterokaryon, but the experiments were discontinued because of the difficulties outlined at the end of the chapter.

A major question about plasticity in the newt myotube is the relationship between cell cycle re-entry and the generation of mononucleate progeny, as discussed in

Figure 1.6. Both of these phenomena are observed after implantation of myotubes into a regeneration blastema (Kumar et al., 2000), but only DNA synthesis is observed in culture (Tanaka et al., 1997). It is tempting to speculate that mononucleate cells are produced by fragmentation of the myotube as a result of progression through mitosis and cytokinesis, as illustrated in Figure 1.6 A. In culture, the signals that induce progression through these phases of the cell cycle in the blastema are absent, and the myotube nuclei arrest in G2, explaining why generation of mononucleate cells is not observed (Figure 1.6 B). Although this is an attractive model, the possibility that the production of mononucleate cells occurs independent of cytokinesis also exists. An alternative model for the production of mononucleate cells, involving budding of nuclei from the syncytium, is outlined in Figure 1.6 C and D. Budding of nuclei to form mononucleate cells has been observed in cultured cells of the avian osteoclast lineage, and occurred in the presence of colchicine, a mitotic inhibitor (Solari et al., 1995). In implanted newt myotubes, budding might operate exclusively on nuclei that enter S-phase (Figure 1.6 C), or also on nuclei that are in G1 (Figure 1.6 D).

The experiments described in Chapters 4 and 5 were aimed at distinguishing among these models. First, the "M-phase" entry model (Figure 1.6 A and B) was investigated directly in culture, as outlined in Chapter 4. Attempts to overcome the G2 arrest and induce mitosis and cytokinesis in serum-stimulated newt myotubes were made, particularly by expression of SV40 large T antigen and treatment with caffeine. These experiments underlined the stability of the block, and led me to consider the models of Figure 1.6 C and D. A major focus for my final eighteen months was to test a critical prediction of 4D by rigorously blocking cell cycle re-entry in myotubes and then implanting them into regenerating limbs (Chapter 5).

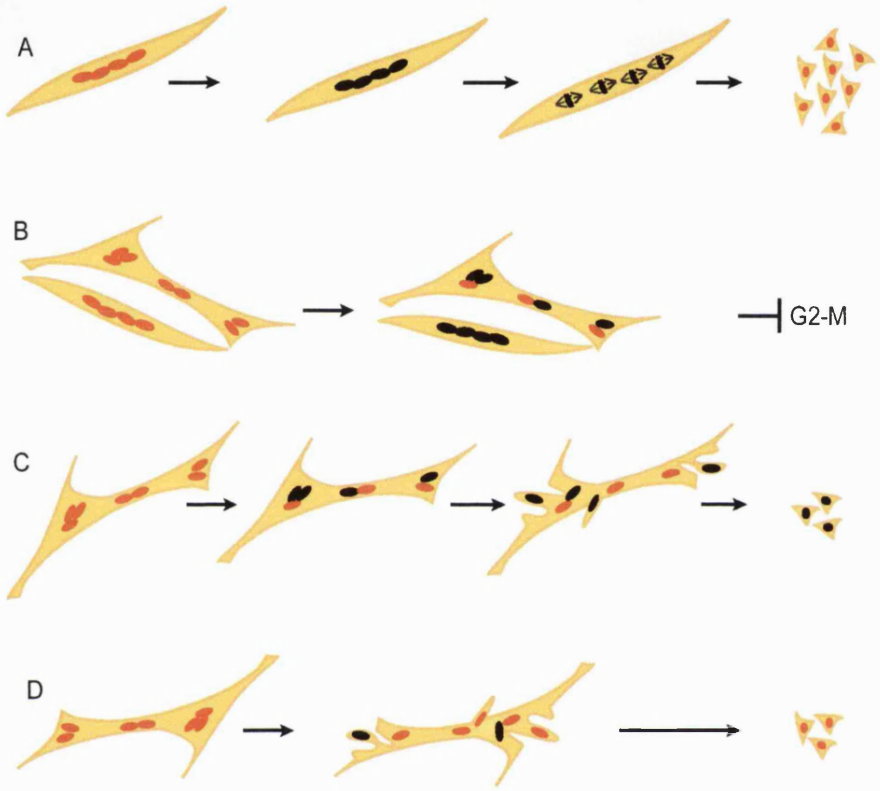


Figure 1.6. Schematic diagram of possible mechanisms for the generation of mononucleate cells after implantation. (**A**) and (**B**). M-phase entry model. (**A**) Nuclei traverse S-phase (black nuclei) and undergo mitosis. The myotube is fragmented by cytokinesis, generating viable mononucleate cells that are in G1 (red nuclei). (**B**) Cultured myotubes traverse S phase and arrest in G2 (Tanaka et al., 1997). Production of mononucleate cells is not observed in culture because the cells do not enter M. (**C**) and (**D**) Budding model. (**C**) Budding operates selectively on nuclei that have entered S-phase. (**D**) Budding operates irrespective of cell cycle re-entry.

Chapter 2

Materials and Methods

2.1 Cell culture

2.1.1 Cell lines and media

All media were supplemented with 50 i.u./ml penicillin , 50 µg/ml streptomycin, 0.29 mg/ml glutamine (Gibco), and 0.28 i.u./ml insulin, (Sigma).

Newt A1 cells, originally isolated from muscle explants (Ferretti and Brockes, 1988), were grown as monolayers in AEMEM (63% Eagle's minimum essential medium, Gibco, 25% H_2O; adjusted to urodele osmolarity) containing 10% heat-inactivated (56°C, 30 minutes), foetal bovine serum (FBS, First Link) on gelatine-coated plastic culture flasks (0.75% w/v porcine skin gelatine in H_2O, Sigma), in a humidified atmosphere of 2% CO_2 at 25°C.

C2C12 clonal mouse myoblast cells (Yaffe and Saxel, 1977) were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 20% FBS, in a humidified atmosphere of 10% CO_2 at 37°C.

2.1.2 Treatment with trypsin

Cells were digested with trypsin/EDTA (Gibco) diluted from a 10X stock (5g Trypsin, 2g, EDTA, 8.5 g NaCl per litre H_20)to 1X using PBS (phosphate buffered saline, Gibco) for C2C12 cells and heterokaryon myotubes, or APBS (75 % PBS, 25% H_2O) for A1 cells. Cells were washed in 2-5 ml PBS (C2C12, heterokaryons) or APBS (A1), and digested with 1-3 ml 1X trypsin/EDTA. The enzyme was inactivated by the addition of 2-5 ml medium containing 10% or 20% FBS. A cell pellet was collected by centrifugation on a table top centrifuge at an average RCF of 45 x g (myotubes) or 129 x g (mononucleate cells) for 3 minutes, and the cells resuspended in 1-5 ml medium with 0.5%, 10%, or 20% FBS.

2.1.3 Cell counting

After treatment with trypsin and resuspension in 1-5 ml of medium, cells were counted on a haemocytomer with 4 chambers of 10^{-4} ml. The number of cells in the four chambers was averaged, and the final cell number was calculated from the number of cells per 10^{-4} ml and the total volume in which the cells were resuspended. For adherent cells, the number of cells present in 10 random fields of view were counted and the average was determined. The area of the field of

view was calculated from its diameter measured with a graticule divided at every 0.01 mm. The final number of cells was expressed as cells per/mm^2.

2.1.4 Passaging

A1 cells were passaged once per week. After treatment with trypsin, cell pellets were resuspended in AEMEM containing 10% FBS and counted. Typically $2x10^5$ A1 cells were plated on a 162 cm^2 tissue culture flask. C2C12 cells were passaged every 2-3 days, in order to maintain the cultures at low density, that is without contact between the cells. After treatment and resuspension in DMEM containing 20% FBS, 0.5 - 1 x 10^5 cells were plated on an 80cm^2 flask with 10 ml of medium.

2.1.5 Myotube formation

A1 or C2C12 cells were plated on gelatine-coated 100 mm or 60 mm tissue culture dishes scored with a sterile scalpel to form a fine mesh pattern, thereby limiting the size of the myotubes by limiting the number of cells that were able to fuse in each square. The cells were grown to high density (A1 at 25°C and C2C12 at 33°C), so that almost no cytoplasm was visible between the cell nuclei, and fusion was induced by lowering the serum concentration in the medium to 0.5% for 5 days.

The final protocol for heterokaryon C2C12-A1 myotube formation is illustrated in Chapter 3, Figure 3.4. A1 cells were grown at 25°C in 10% FBS AEMEM to 90% confluence (cytoplasm was visible between cell nuclei, typically this was a density of 33 cells/mm^2), and placed in 0.5% FBS medium at 25°C (AEMEM) or 33°C (DMEM) for 0 to 2 days. The number of adherent A1 cells per dish was estimated as described in section 2.1.3. C2C12 cells at low density (with no contact between cells) were placed in 0.5% FBS DMEM (DM) at 33°C for 0 to 48 hours, digested with trypsin, resuspended in DM, counted in a haemocytometer, and added to the A1 dishes. The number of C2C12 cells added to each dish of A1 cells was between 4 - 5 x 10^4. Cells were allowed to fuse for 3-5 days in DM at 33°C, until myotube formation was observed.

2.1.6 Myotube purification by sieving

The myotubes were sieved, as described (Lo et al., 1993; Tanaka et al., 1997). Meshes of different pore sizes (100 μm, 35 μm, and 20 μm) were cut into squares measuring 2cm x 2cm. The squares were placed over 20 ml universal vials and depressed in the centre to form a funnel shape. Meshes were attached to the vials at three points by heating with the tip of an incandescent scalpel. The A1, C2C12, and heterokaryon myotubes were digested with trypsin, and resuspended in 5 ml medium containing serum 10-20% serum. The suspension was sieved through a 100 μm pore size nylon mesh (Biodesign, Inc., New York). Cells clumps retained by the mesh were discarded and the flow through fraction was sieved through a 35 μm mesh which does not retain mononucleate cells. Cells retained by the 35 μm mesh were rinsed off the mesh by inverting it onto a 60 mm tissue culture dish with 2 ml of medium containing 0.5% - 20 % serum, and rinsing the mesh with another 2 ml of the same medium. The cells were plated onto 35 or 60 mm dishes coated with gelatine or fibronectin (10-20 μg/ml, 1hr, room temperature). This procedure resulted in cultures in which greater than 85% of the nuclei were in myotubes. The flow through fraction of the 35 μm mesh of heterokaryon and C2C12 myotube preparations was retained because C2C12 myotubes tended to flow through this mesh. The fraction was sieved through a 20 μm mesh, and the cells retained by this mesh were plated onto 35 or 60 mm dishes.

2.1.7 Labelling with BrdU, thymidine, and uridine

Cells were labelled by adding 3-bromo-2-deoxyuridne (BrdU) 10mM or [^{3}H]-thymidine 1 μCi/ml to the medium for six hours to ten days prior to fixation and subsequent processing. Labelling with [5-^{3}H]uridine 2μCi/ml (Amersham) was for 1, 6 or 24 hours.

2.1.8 X-ray and ultraviolet irradiation

For ultraviolet (UV) irradiation cells were rinsed briefly in APBS and placed under the UV source (UV Stratalinker 2400, Strategene) at the same height as the sensor. After irradiation at 5-4500 J/m^2, the cells were placed in AEMEM containing 0.5% or 10% FBS. A 60 J/m^2 dose with this source was effective in

inhibiting DNA synthesis in NIH3T3 fibroblasts after 24 hours, as reported by Cheng et al. (1994).

For X-irradiation, cells were placed for 1-5 minutes in serum free L15 (Gibco, SF-L15) medium under a 250 kilovolt source, emitting a dose of 4.61 Gray (Gy)/minute at 40 cm from the source, for 0.4-4.3 minutes to obtain the doses of 1.2 to 20 Gy. After irradiation, myotubes were maintained in SF-L15 for implantation, whereas myotubes to be cultured were placed in AEMEM without serum for 24 hours. The irradiated mononucleate cells used for the experiment described in Chapter 5, Figure 5.1 were placed in AEMEM with 10% FBS after irradiation.

2.1.9 Transfection

2.9.1.1 Microinjection

Dextran and plasmid DNA microinjection were performed using a Narishige MMO 202 micromanipulator connected to a pneumatic picopump PV820 (World Precision Instruments) mounted on a Zeiss Axiovert 135 microscope. Micropipettes were prepared with a p-97 micropipette puller (Sutter, USA) from glass capillaries (Clark Electromedical Instruments) with an inner filament and diameters of 1.2 mm (external) and 0.69 mm (internal).

Cells or myotubes were placed in serum free L-15. The plasmid concentration for microinjection was 0.3-0.5 mg/ml, and the plasmids were diluted in TE (10mM Tris, 1mM EDTA pH8.0). Microinjection in one nucleus per myotube was sufficient to obtain protein expression in the entire syncytium. Typically, the transfection efficiency by microinjection of myotubes was 10-20%, and by microinjection of mononucleate cells was greater than 80%.

Dextrans (Texas red, TR-conjugated or fluorescein-conjugated dextran 70 kD 15mg/ml in H_2O, Molecular Probes) were microinjected cytoplasmically into myotubes as soon as possible after irradiation or into the parallel controls.

After microinjection cells were placed in the AEMEM with or without serum or left in SF-L15 for implantation.

2.1.9.2 Particle bombardment

For transfection with the DNA particle gun (Helios Gene Gun, Biorad), 10^5 cells or a 10cm dish of myotubes were digested with trypsin, resuspended in 1 ml of medium, placed in a heat-sealed 1 ml plastic micropipette tip, and centrifuged at 600 or 1000 RPM. Following this, the supernatant was aspirated and the pipette tip was cut open with a scalpel. The cell pellet was expelled on to a dry 35 mm tissue culture dish with the aid of a Gilson micropipette. Cells were bombarded by placing the gun as close as possible to the dish holding the pellet while avoiding contact with the dish. DNA-coated 1µm gold microprojectiles were expelled from a cartridge by a 150 psi (pounds per square inch) helium pulse from a cylinder attached to the Gene Gun. After bombardment, the cells were resuspended in AEMEM with or without serum and allowed to recover overnight.

2.10 Treatment with caffeine

A stock solution of 0.5 M caffeine (1,3,7-trimethylxanthine, Sigma) was prepared in H_20, divided into 100 µl aliquots, and stored at –20°C. Caffeine was added directly to the cell culture medium after thawing to final concentrations of 5, 10, and 25 mM.

2.11 Treatment with nocodazole

A stock solution of 1 mg/ml nocodazole (methyl-[5-(2-thienylcarbonyl)-1H-benzimidazol-2-yl]carbamate, Sigma) was prepared in dimethylsulfoxide (DMSO, Sigma). A volume of 0.1 ml of this solution was diluted serially in equal volumes of medium to obtain a final conentration of 10 µg/ml. This concentration was found to be effective in mononucleate A1 cells. Treatment of a cycling population of A1 cells for 24 hours resulted in an eight fold increase in the number of mitotic figures as compared to DMSO-treated controls (1.2% to 8.4% of 1000 cells counted).

2.2 Processing of cultured cells

2.2.1 Fixation

Cultured cells were fixed, after rinsing with PBS, with 100% methanol at –20°C for 5 to 10 minutes. For cells labelled with green fluorescent protein, dextrans, or alkaline phosphatase fixation was in 3% paraformaldehyde in PBS containing 0.2% Triton-X100 pH 7.4, for 5-10 minutes.

2.2.2 Immunocytochemistry

The fixed cells were rinsed in PBS 3 times for 10 minutes to remove the fixative. After a 30 minute blocking step in PBS containing 10% goat serum (PBS-GS), primary antibodies were added and incubated for 1 hour at room temperature or overnight at 4°C (anti-BrdU). After three 10 minute washes in PBS-GS, secondary antibodies were added for 1 hour at RT followed by two 10 minute washes in PBS-GS. For BrdU staining, a 10 minute hydrolysis in 2N HCl was performed prior to the blocking step in PBS-GS. All antibodies were diluted in PBS-GS. Cells were mounted in 90 % glycerol in 200 mM Tris-HCl buffer pH 8.2.

In double and triple labelling experiments antibodies of different subclasses were used. All the primary antibodies were incubated together, followed by washes and incubation with the secondary antibodies simultaneously. For T antigen and MHC staining, the cells were processed first for T antigen staining (nuclear) followed by MHC staining (cytoplasmic). Controls were stained with secondary antibodies only, or with primary antibodies followed by secondary antibodies to the wrong immunoglobulin subclass or species type. The controls were all negative.

Staining of the nuclei with Hoechst 33258 was at a concentration of 1 μg/ml in PBS for 5 minutes.

2.2.3 Autoradiography

Isotopically labelled cells were processed for autoradiography after antibody staining. Cells were air dried and coated with Ilford K5 emulsion diluted 1:1 in H_2O. Development was after 2 or 3 days in Phenisol (Ilford), diluted 1:6 in H_2O

for 6 minutes followed by fixation was in Hypam (Ilford) diluted 1:6 in H_2O for 6 minutes.

2.2.4 Alkaline phosphatase cytochemistry

For alkaline phosphatase (AP) cytochemistry, samples were incubated in PBS at 65°C for 10 minutes to destroy the endogenous AP activity. The cells were washed in 1mM $MgCl_2$/PBS and developed in AP-Orange substrate solution (Zymed, CA) for 4-5 hours at 37°C. After AP staining, the cells were washed in PBS. The nuclei were counterstained in Mayers hematoxylin solution (Sigma) for 10 minutes washed 3-5 times with H_2O, and mounted in 90 % glycerol in 200 mM Tris-HCl buffer pH 8.2.

2.3 Microscopy

Cells were observed under bright field illumination using phase contrast optics or under epi-illumination using standard fluorescein (excitation 450-490 nm, emission between 515-565 nm), rhodamine set (excitation 538-558 nm and emission above 590 nm), and UV (excitation 390- 420 nm and emission above 425 nm) filter sets on a Zeiss Axiophot 2 microscope. Images were recorded on Kodachrome 400 ASA colour slide film or on Kodak Tmax 100 ASA PRO black and white film. Images were also collected on a CV-12 cooled monochrome digital camera (Photonic Sciences, UK) as 12-bit images with Image Pro Plus software (Media Cybernetics, USA). The individual grey scale images from each channel were converted to 8 bit images, and merged to obtain 24 bit colour images. These images were exported to Adobe Photoshop 4.0 (Adobe Systems, USA) for colour correction and contrast enhancement and printed on a Kodak DS8650 PS dye sublimation printer.

2.4 Cytometry

After processing for immunocytochemistry, the cells were incubated in Hoechst 33258 2 μg/ml for 24 hours 4°C, mounted under glass coverslips using PBS and analysed immediately.

Images of Hoechst-stained nuclei were taken on a CV-12 digital camera as 12 bit images. To assign DNA values to phospho-histone H3 and BrdU-positive nuclei and to ensure that nuclei were in myosin heavy chain (MHC)-positive myotubes, images of nuclei (UV filter set) were overlayed with images taken with standard fluorescein (BrdU, MHC) and rhodamine (phospho-H3) filter sets.

2.4.1 Adjustment of images for uneven illumination

The uneven illumination from the fluorescence light source was corrected from the field of view by the following method. The nucleus of a cell labelled with phospho-histone H3 was focused at the centre of the field of view and an image was collected with the UV filter set. The exposure time to acquire the image was set, such that the nucleus had an intensity value near 3000. Following this, concentrated Hoechst solutions (20-50 mg/ml) were placed in the field of view and imaged with the set exposure time. The concentration of the Hoechst solution which gave a value of 300 was selected. Ajustment was performed by dividing the active image (nuclei) by the background image (solution) in the operations menu of the software.

Figure 2.1 shows an example of this procedure. Images of Hoechst-stained nuclei (A) were divided by the background image solution (B) in the operations menu of the software. The resulting image is shown in C. The nuclei were selected and separated from the background using the count/size function in the measure menu of the software. The range of intensity to be measured was set at a value 300 units below the intensity of the faintest nucleus. The average intensity value (dens lum on the menu) and area of each nucleus was determined. The DNA content was calculated as the product of the two parameters.

Area of nucleus x average intensity of nucleus = DNA content

This value was simplified by dividing by 10^5 and was expressed in arbitrary units (a.u.) For each plate of cells analysed the same Hoechst adjustment image and the same cut-off detection values were used.

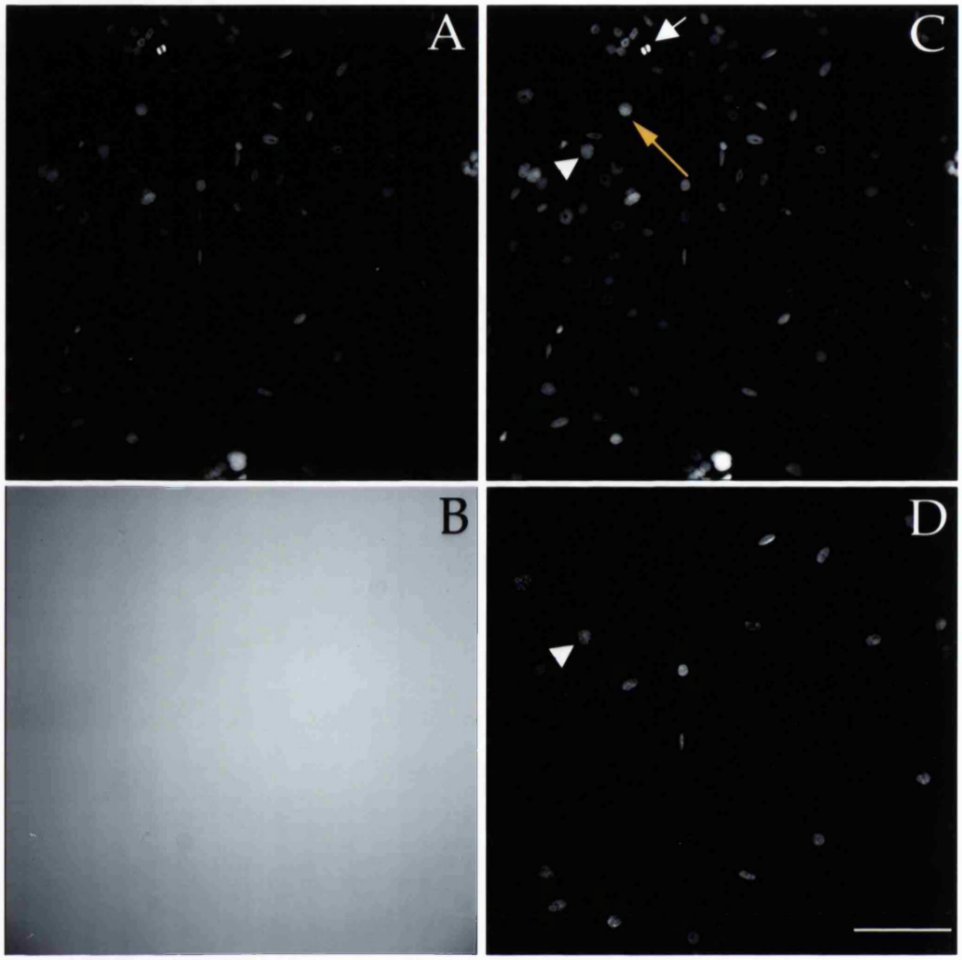


Figure 2.1. Background calibration of images using Hoechst solution for cytometric quantitation of nuclear DNA content. (**A**) The original image of Hoechst-stained nuclei. (**B**) The image of the concentrated (40mg/ml) Hoechst solution. The uneven illumination of the field can be seen; the central portion is bright, whereas the edges of the image, particularly on the bottom and left sides, are darker. (**C**) Image A after adjustment by image B. As a result of the division process, the intensity of the nuclei in the left and bottom portions of the original image increases while the intensity of the nuclei in the central portion of the image do not vary visibly. (**D**) BrdU staining to identify the nuclei that were in S phase. The DNA content value of the mitotic (G2/M) nucleus indicated in image C (arrow) was 17.4 arbitrary units (a.u.); in image A it was 10.6 a.u. The DNA content value of the nucleus indicated with the arrowhead (BrdU labelled, S-phase) in image C was 14.5 a.u., whereas in image A it was 8.84 a.u. The DNA content value of the nucleus indicated by the yellow arrow (BrdU negative, phospho-H3 negative, G1 nucleus) in image C is 10.5 a.u. whereas in the original image it was 11.0 a.u. The G1 values calculated from one chromatin mass of the mitotic nucleus indicated by the white arrow was 7.9 a.u. in image C and 5.8 a.u. in image A. The scale bar is 100 μm.

2.4.2 Setting reference DNA values and validation of the cytometric procedure

Staining with the phospho-histone H3 antibody was taken as a marker for cells with G2 DNA content. Phosphorylation of histone H3 at Ser10 correlates with mitosis in mammalian (Hendzel et al., 1997), *Tetrahymena* (Wei et al., 1999), and yeast (Dang et al., 1999). In A1 cells staining is observed in 0.8-1.5% of a cycling population with condensed chromatin in prophase and metaphase, but not late anaphase/telophase (Figure 2.2).

For each cytometry experiment a population of cycling cells plated on the same dish as the experimental cells and myotubes was used as an internal reference for determining the value of DNA content in G2. The mean value of 10 to 20 phospho-H3 labelled cells in this cycling population was determined and the G2 compartment was calculated as mean $\pm$ s.d. (standard deviation). The G1 cut off value, i.e. the value below which cells were considered to be in G1, was established as one half of the mean G2 [(G2 mean+s.d)/2]. S phase values were between the G1 cut-off and G2 values.

This method was validated by measuring the DNA profile of a cycling population of cells labelled with BrdU for 2 hours immediately prior to fixation to identify cells in S phase (Figure 2.3, black line). The percentage of BrdU-labelled cells relative to the total number of cells in each value category is shown in Figure 2.3 in red. It can be seen that cells with a DNA content of G1 or less are not labelled with BrdU, and that the percentage of labelled cells falls dramatically at values corresponding to G2/M. Furthermore, the percentage of cells with S-phase values that label with BrdU is very high.

2.4.3 Preparation of myotubes for cytometry

Myotubes were purified and plated on a well, constructed in the following manner. A hole, approximately 2 cm in diameter, was cut in the bottom of a 35 mm plastic dish, and the dish was then inverted and stuck with vacuum grease (Dow Corning) on the centre of a 60 mm dish. The purified myotube suspension was introduced through the hole to the area covered by the 35 mm dish. The myotubes were irradiated and placed in AEMEM containing 20% serum 24 hours

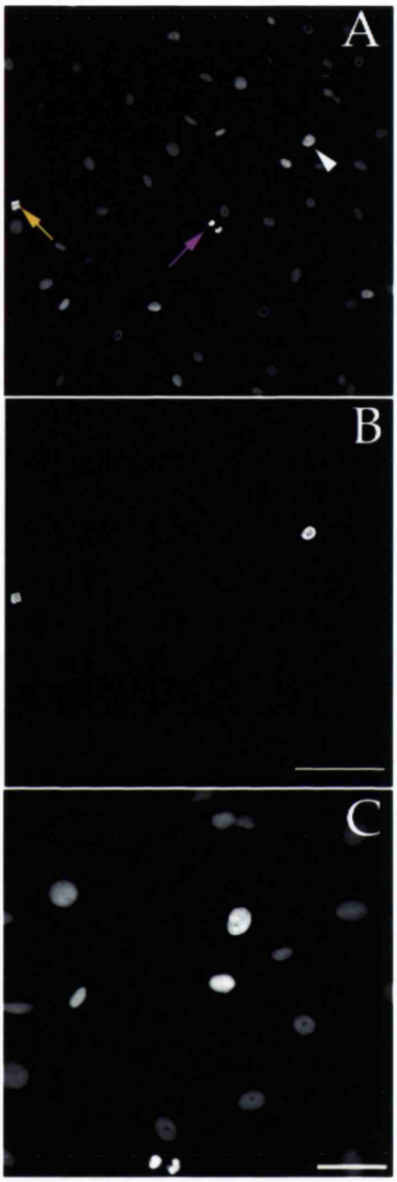


Figure 2.2. The anti phospho-H3 antibody labels A1 cells with visibly condensed chromatin, and cells in metaphase. (**A**) Hoechst-stained nuclei are shown. The arrow in the top left panel indicates a metaphase figure that labels with phospho-H3, as shown in panel B. (**B**). Phospho-H3 labelled cells. The cell in late anaphase/telophase indicated by the pink arrow does not label with the antigen. The interphase nucleus with condensed chromatin indicated by the white arrowhead in A is labelled with phospho-H3, but other nuclei with uncondensed chromatin are not. (**C**) Higher maginfication of A to show the phospho-H3 positive nucleus indicated by the white arrowhead in A. The scale bars are 100 μm for panels A and B, and 50 μm for panel C.

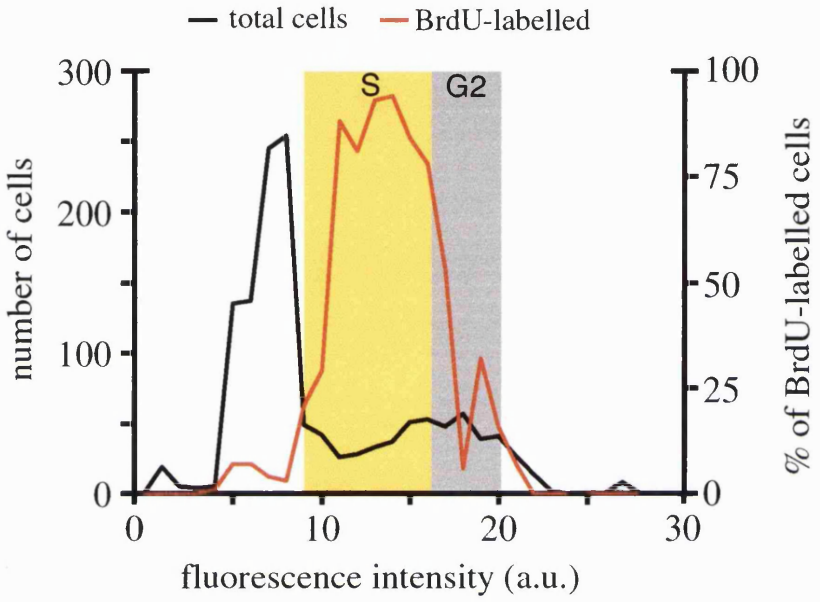


Figure 2.3. DNA content profile of a cycling population of A1 cells shown in Figure 2.1. Cells maintained in 10% serum were incubated with BrdU for 2 hours prior to fixation in order to label cells in S-phase. The cells were processed for BrdU immunocytochemistry, stained with Hoechst 33258 2 mg/ml for 24 hours at 4°C, and analysed immediately as described in section 2.4.1. The cells have a DNA content distributed between S, G2 and M phases as shown by the black line. The G2 DNA content was determined from 17 phospho-histone H3 labelled cells and was 18 ± 2 arbitrary units (a.u.); the G2/M compartment is highlighted in grey. The red line indicates the percentage of BrdU labelled cells for each value category. The theoretical S-phase values, determined as described in section 2.4.2, are highlighted in yellow; most of the BrdU labelled cells are in this category. Some BrdU labelled cells are in the G2 compartment, as might be expected from cells that were at the end of S-phase when the BrdU label was added, and had entered G2 at the time of fixation. In contrast, almost no BrdU-labelled cells are found in the G1 compartment. A total of 1360 cells were measured of which 304 labelled with BrdU.

after irradiation. The medium was changed after 5 days and the myotubes were fixed 10 days after serum stimulation. Three days prior to fixation, cycling mononucleate cells were added to the area outside the inverted 35 mm dish. In this way, each 60 mm dish had a central area in which irradiated myotubes and mononucleate cells were located and which was clearly separated from a ring of cycling mononucleate cells surrounding it. This cycling population served as the standard for determining DNA content values for nuclei in G2/M in each dish. The DNA content of control myotubes was measured on parallel plates of myotubes which were not irradiated.

2.5 Cell implantation and BrdU labelling *in vivo*

2.5.1 Animals and implantation

Adult *Notophthalmus viridescens* were obtained from Charles Sullivan and Co. (Tennessee, USA) and maintained as previously described (Ferretti and Brockes, 1988; Lo et al., 1993). Both forelimbs were amputated from animals anaesthethised by immersion for 10-15 min in 0.1% w/v Tricaine (Sigma), just proximal to the elbow. For experiment involving implantation of fluorescent-labelled myotubes, the humerus was removed to minimise damage to the thick sections (80-100 mm). The humerus was removed by detaching the muscle tissue from the protruding end of the bone with forceps, and dislocating it from the joint by gentle tugging. The humerus could then be pulled free from the limb stump. The stump tissues were trimmed to provide a flat surface and the newts were left to regenerate at 23-24°C.

A cell pellet containing labelled myotubes mixed with 1000-10000 carrier mononucleates, as required, was implanted into left forelimb regenerates 3 days after amputation. Implantations were as previously described (Lo et al., 1993, Kumar et al., 2000) and are illustrated in Figure 2.4. On the day of implantation, the myotubes were quantitated as described in section 2.5.2, digested with trypsin, mixed with unlableled mononucleate cells as carriers, and centrifuged in small-bore siliconized Pasteur pipettes (0.55 mm, Bill Bates Glass, Daventry, UK), plugged with Sylgard 184 elastomer (Dow Corning). Initially, a carrier layer of 1000 cells was seeded above the silicone bed and spun in the centrifuge to avoid

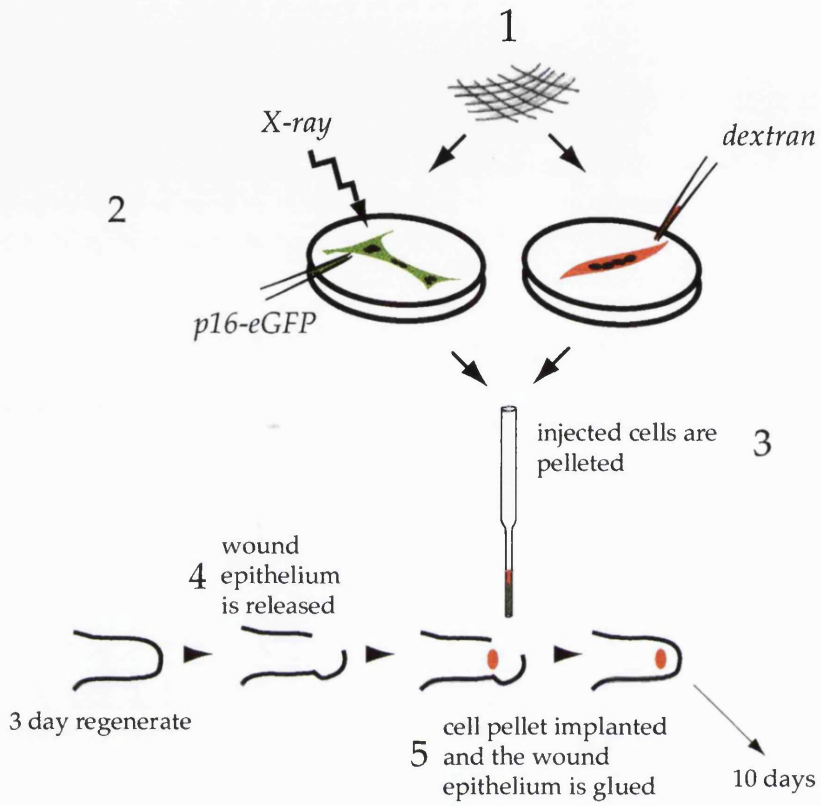


Figure 2.4. Schematic representation of an implantation experiment (modified from Lo et al., 1993). (1) Myotubes are purified by sequential sieving through 100 µm and 35 µm nylon meshes. (2) Myotubes are irradiated and injected with dextran lineage tracer or with plasmid DNA expressing p16 and eGFP. Control myotubes are injected with dextran lineage tracer. (3) Experimental and control myotubes are mixed and pelleted in a sealed Pasteur pipette. (4) The wound epithelium of a 3-day blastema is released from the underlying tissue using iridectomy scissors. (5) The pellet is implanted after scoring and breaking the Pasteur pipette, and the wound epithelium is sealed over the implanted cell pellet with tissue adhesive. The limb is left to regenerate for 10 days.

any myotube loss during the release of the cell pellet. The cell pellets were released from the pipette by scoring and breaking the glass. The cell pellet was implanted by first releasing the wound epithelium from underlying tissue using iridectormy scissors, and trimming the musculature to form a pocket. The cell pellet was placed into this pocket and the wound epithelium was then reattached by using a cyanoacrylate ester adhesive (Histoacryl Blue). The animals were allowed to regenerate for 10 days.

2.5.2 Quantitation of myotubes and nuclei for implantation

The number of fluorescent - labelled myotubes and the number of nuclei in each labelled myotube was determined for irradiated and control populations, on the day of the implantation and 24 hours after irradiation and microinjection of dextrans. For myotubes injected with plasmid DNA, the number of eGFP-expressing cells was counted 2 days after microinjection, and an equivalent number of non-expressing myotubes in parallel plates were microinjected with TR-dextran. If necessary, cells were selectively eliminated from one or the other of the labelled myotube populations with a microinjection pipette in order to equalise the number of implanted nuclei. The number of nuclei in labelled myotubes was then counted. For double implantation experiments, myotubes of different populations were mixed before addition of carrier mononucleates and pelleting.

For myotubes transfected with alkaline phosphatase, the number of myotubes implanted was counted on the day of implantation. To estimate the number of alkaline-phosphatase expressing myotubes implanted, a parallel sample was fixed at the time of implantation and processed for alkaline phosphatase staining. The percentage of stained myotubes and the total number of myotubes implanted, were used to calculate the alkaline phosphatase expressing myotubes implanted. For myotubes transfected by particle bombardment, the mononucleate cells in the culture were mechanically removed with the micromanipulator 24 hours after transfection and again on the day of implantation. A glass needle was used to tear through the mononucleate cell and pull it away from the surface of the dish. This procedure was repeated 24 hours later, on the day of implantation, and the

number of myotubes was counted. Myotubes transfected by microinjection were implanted 24-48 hours after microinjection.

2.5.3 BrdU labelling

For 3-bromo-2-deoxyuridne (BrdU) labelling experiments, animals were injected intraperitoneally with BrdU (0.25 mg/g body weight) at 9 days post implantation (Kumar et al., 2000). The regenerates were harvested after 24 hours of BrdU incorporation and processed for immunohistochemistry.

2.6 Histology

2.6.1 Harvesting of blastemas

Animals were anaesthetised with 0.1% tricaine (Sigma) and perfused with 0.6% saline 30-35°C followed by 2% paraformaldehyde, 0.2% Triton X-100 (PFA-TX) room temperature, pH 7.4. Regenerates were collected and fixed further in PFA-TX at 4°C for 3 hours, washed in PBS 3 times for 10 minutes, and infused with 10% sucrose in PBS overnight at 4°C . The tissues were washed in PBS, embedded in Tissue-Tek (Sakura Finetek), and frozen over dry ice .

2.6.2 Sectioning

Blastemas implanted with fluorescent labelled cells were sectioned longitudinally at 80-100 µm (thick sections) on a sledge microtome (Leitz 1400) and floated in PBS to remove the mounting medium. For alkaline phosphatase - labelled myotubes, 15 to 20 µm sections were cut on a cryostat microtome (Leica) and adhered to glass slides coated with 2% silane (3-aminopropyl triethoxy-silane, Sigma). Coating of slides was performed by dipping the slides first in 100% acetone, followed by acetone containing 2% silane for 10 minues. Slides were then dipped in 100% acetone, in H_2O, and air dried.

2.6.3 Histochemistry and immunohistochemistry

For direct observation of thick sections implanted with fluorescent-labelled myotubes, the sections were floated in PBS containing 1 µg/ml Hoechst 33258 for counterstaining nuclei, and mounted in 90 % glycerol in 200 mM Tris-HCl buffer

pH 8.2. Sections were sandwiched between two coverslips that were subsequently sealed with clear nail varnish and slides stored at –20°C.

For detection of BrdU label, sections were floated in 2 M HCl for 4 hours at 37°C , neutralised in 100mM borate buffer (pH 8.4), followed by three 10 minute washes in PBS, and incubation in PBS-Triton X100 0.2% + 10% goat serum (PBS-T-GS) for 2 hours. A mouse monoclonal anti-BrdU antibody (was added to the culture dishes diluted in PBS-T-GS (1:500) and placed on a rocker at 4°C for 48-72 hours. Sections were washed three times in PBS-T-GS for 2 hours and fluorescein-conjugated goat anti-mouse secondary antibody was added for 24 hours. Sections were mounted in 90 % glycerol in 200 mM Tris-HCl buffer pH 8.2.

For alkaline phosphatase labelled myotubes, the sections were thawed at room temperature, air dried and rehydrated in PBS. Sections were processed for AP-orange substrate as described in section 2.2.4 except that that colour development was for 30 to 60 minutes. Sections were washed in PBS, the nuclei were counterstained with Mayers hematoxylin, and mounted in glycerol.

2.6.4 Analysis of sections and photography

Sections containing fluorescent-labelled cells were observed under epi-illumination and images were collected as described in section 2.3. Sections containing alkaline phosphatase-labelled cells were observed under bright field illumination and recorded on photographic film (Fuji 64 T).

All the sections were analysed for BrdU, eGFP, dextran, or alkaline phosphatase-labelled cells and the number of nuclei per cell was determined. To eliminate scoring of false positive fluorescent cells due to background fluorescence, cells fluoresceing only under the appropriate excitation and emission wavelengths were considered. Cells which were fluorescent when observed with other filter sets were considered background. The eGFP label, however, could be detected both with the fluorescein filter set and with the UV filter set. The eGFP labelled cells were visible as green in the blue background fluorescence from the UV filter set. In addition, eGFP-positive cells were negative when examined with the

rhodamine filter set. The eGFP protein has an excitation maximum of 488 nm and emission maximum at 507 nm. True positive cells, identified by the above criteria, were not found in the unoperated right arms that served as controls. For alkaline phosphatase implants, samples in which the activity of the endogenous alkaline phosphatase was high, as assessed by staining of the right limb, were discarded.

2.7 Reagents

2.7.1 Antibodies

Mouse monoclonal antibodies were used against BrdU (IGg1, BU-20, Amersham), muscle specific myosin heavy chain (IgG2a, A4.1025 Dr. Simon Hughes, Randall Institue, King's College, London), p16 (IgG1 DCS-50.2, Gordon Peters, Imperial Cancer Research Fund, London), wild type T antigen (IGg2a, pAb101, Dr. Parmjit Jat, Ludwig Institute for Cancer Research, London), and mouse lamin B (IgM, XB10, Brian Burke, University of Calgary, Canada). Other antibodies were polyclonal rabbit anti mouse: anti-phospho histone H3 (Upstate Biotechnology, New York, USA), and anti-human placental alkaline phosphatase (Dako). Primary antibodies were used at a dilution of 1:100 to 1:500. XB10 and Pab101 were hybridoma culture supernants and the other antibodies were affinity purified at the source.

Secondary antibodies were FITC or TRITC conjugated goat anti-mouse, rabbit anti-mouse, or swine anti-rabbit (DAKO) or subclass specific FITC, TRITC, or biotin conjugated goat anti mouse (Jackson). Streptavidin conjugated to cascade blue was from Molecular Probes.

2.7.2 Plasmids

The pSE plasmid, encoding wild type T antigen under control of the SV-40 promoter, and pMTSVtsA58, encoding a temperature sensitive mutant with wild type activity at a temperature below 37°C under the control of metallothionein promoter, were a gift of Dr. Parmjit Jat (LICR, London, UK). The pMTV plasmid encoding v-myb under the control of the metallothinein promoter was a gift of Dr. Kathy Weston (Institute for Cancer Research, London, UK). Expression from the

metallothinein promoter was induced by addition of $ZnCl_2$ 100mM to the medium immediately after microinjection, unless otherwise stated.

PTL1-p16 was constructed by inserting an EcoRI-BamHI fragment of pcDNA3WTp16 (a gift of Drs. David Parry and Gordon Peters, Imperial Cancer Research Fund, London, UK) into the BamHI-NotI site of PTL1 (Ragsdale et al., 1989) which contains an SV40-promoter. pSG5-eGFP was constructed in this laboratory by Dr. Sara Morais da Silva by blunt ligating the SalI-NotI fragment of pEGFP (Clontech) into a BamHI site of pSG5 (Strategene). The plasmids pCAP, in which the expression of the human placental alkaline phosphatase gene is under control of the SV-40 promoter (Schiltius et al., 1993), and pCAPp16, in which the SalI expression cassette of p16 PTLI was blunt ligated into the BamHI site of pCAP, were engineered in this laboratory by Mr. Phillip Gates.

2.8 Preparation of DNA for transfection

2.8.1 CsCl purification of plasmid DNA

Plasmid DNA was used to transform competent B1H1 cells (Stratagene) according to the protocol of Sambrook et al. (1989). The bacteria were spread on 1.5% bacto-Agar (Difco)-NZCYM (Gibco) containing 100 µg/ml ampicillin and incubated overnight at 37°C. A single colony was used to innoculate 5 mL of NZCYM containing 100 µg/ml ampicillin, and the culture was incubated with shaking at 37°C for 10 hours, and inoculated into 250 ml NZCYM 100 µg/ml ampicillin with shaking overnight. The following morning another 250 ml of medium with fresh ampicillin was added for a further 5 hours. Preparation and purification of plasmid DNA were by alkali lysis and equilibrium centrifugation on a CsCl gradient (Sambrook et al., 1989). The solution containing plasmid DNA and CsCl 1.55g/ml was introduced into Beckman pollyallomer centrifuge tubes, heat sealed, and equilibrated overnight at 80,000 RPM room temperature in a Beckman TLN100 rotor. The bottom band containing supercoiled plasmid DNA was collected, and a second equilibration was performed at 100,000 RPM for 4 hours at room temperature. The ethidium bromide was removed by water saturated butanol extraction and the DNA was precipitated and resuspended in TE. An

was determined spectroscopically by measuring the A_{260}/A_{280} ratio (Sambrook et al. 1989).

2.8.2 Cartridge preparation

Cartridges for the gene gun were prepared according to the manufacturer's protocol. 100 µg plasmid DNA was precipitated onto 25 µg of gold particles with $CaCl_2$. First the gold was measured in a 1.5 ml microfuge tube to which 100 µl of spermidine 0.05 M was added. The gold and spermidine were vortexed for 5 seconds at high speed and sonicated for 5 seconds in a sonicating waterbath. The plasmid DNA (100µl, 1mg/ml) was added to the gold and spermidine mixture. The mixture was vortexed again for 5 seconds at high speed. The speed of the vortexing was reduced and 100 µl of freshly prepared 1M $CaCl_2$ was added dropwise to the mixture. The mixture was allowed to precipitate at room temperature for 10 minutes, and was then spun in a microfuge for 15 seconds to pellet the gold. Most of the supernatant was removed, and the gold was resuspended in the remaining 5-10 µl supernatant by vortexing. The pellet was washed three times in 1 ml of fresh 100% ethanol by spinning the solution to pellet the gold and discarding the supernatant each time. After the final ethanol wash, the pellet was resuspended in 3.5 ml of an ethanol solution containing 0.1 mg/ml of polyvinylpyrrolidone (PVP), which serves as an adhesive during the cartridge preparation procedure.

A 50 cm length of tubing (Biorad) was coated with the DNA coated particles according to the manufacturer's protocol. The tubing preparation station (Biorad) was connected to a nitrogen tank, the tubing was inserted in the tubing support cylinder, and the nitrogen was passed through the tubing for 15 minutes to ensure that it was completely dry. The tubing was removed from the preparation station, and the microparticle/ethanol/PVP suspension was drawn into the tubing with a syringe; the tubing was pushed back into the tubing support cylinder. The microparticles were allowed to settle for 5 minutes, and the ethanol was removed with a syringe. The tubing station was turned on to start rotating the tubing and enable the gold to smear in the tube. After 30 seconds the nitrogen flow was turned on and the tubing allowed to spin as it dried. The tubing was removed

from the preparation station and cut into 42-55 0.5 cm cartridges, such that 1.8-2.5 μg DNA were used for each transfection. Cartridges were stored at 4°C in vials containing silica gel and were stable for 2 months. The variation in protein expression efficiency of mononucleate cells transfected with cartridges from the same preparation was not significant.

Chapter 3

Cell cycle re-entry in newt-mouse heterokaryon myotubes

3.1 Introduction

Mammalian myotubes are in a state of stable post mitotic arrest and are refractory to serum stimulation. This is due in part to their inability to phosphorylate and thereby inactivate the retinoblastoma protein (pRb; Olson, 1992; Gu et al., 1993; Lassar et al., 1994; Schneider et al., 1994; Mal et al., 1999). In contrast, newt myotubes are able to phosphorylate pRb in response to serum stimulation and re-enter the cell cycle (Tanaka et al., 1997, 1999). This difference in pRb regulation between mammalian and newt myotubes is striking, and suggests that a pathway leading to pRb phosphorylation is present in newt myotubes and is absent in their mammalian counterparts (Tanaka et al., 1997,1999). The stimulus for this pathway is generated by the activity of plasmin or thrombin on serum from a variety of sources, including mammalian sera (Tanaka et al., 1999). These observations emphasise that the responsiveness to serum stimulation is intrinsic to the differentiated newt myotube.

There are several mechanisms that could account for this observation; the most likely is that newt myotubes express a unique receptor that confers responsiveness to the thrombin/plasmin-generated activity. It is possible that expression of this putative receptor in mammalian myotubes would lead to cell cycle re-entry following serum stimulation. A critical question, therefore, is whether the mechanisms that ensure post-mitotic arrest in mammalian myotubes can be subverted by the cell cycle re-entry pathway that is active in their newt counterparts. One way to investigate this question and establish the dominance relationship between the mouse and newt responses is by the formation of mouse-newt heterokaryon myotubes.

A heterokaryon is a cell that contains nuclei from two different cell types within a common cytoplasm and plasma membrane. They can be formed from a variety of different cell types of the same or different species. Heterokaryons have been used extensively to study processes that are regulated by the activity of diffusible cytoplasmic factors such as cell cycle progression, differentiation, gene transcription, and chromatin remodelling (Johnson and Rao., 1970; Rao and

Johnson, 1970; Blau et al., 1983, 1985; Wright, 1984, 1990; Ringertz et al., 1985; Schafer et al., 1990; Tapscott et al., 1993; Mook-Jung and Gordon, 1996; Komarova et al., 1997; Hache et al., 1999). Studies in muscle heterokaryons have generated significant insights into the mechanism of muscle determination (Schafer et al, 1990; Tapscott et al, 1993) and the establishment of the post-mitotic state (Schwab and Luger, 1980; Clegg and Hauschka, 1987). Examples of cross species heterokaryon myotubes are those formed by Duchenne human and normal mouse myoblasts (Denetclaw et al., 1993), and the chick myocyte-rat myoblast hybrid (Wright and Lin, 1990). Myoblasts naturally fuse during the process of differentiation to form multinucleate myotubes, and therefore the formation of muscle heterokaryons does not require the use of a fusogen, such as polyethylene glycol (PEG), which is normally employed for fusing other cell types.

In this chapter the formation of mouse-newt heterokaryons, and an analysis of their ability to re-enter the cell cycle was undertaken in order to investigate the dominance relationship of the newt and mammalian myotube responses to serum stimulation.

Results

3.2 Culture condition of parental cell lines are unsuitable for heterokaryon formation.

Culture conditions for newt (A1) cells and mammalian (C2C12) myoblasts are very different; whereas A1 cells grow optimally at 25°C in 75% EMEM (AEMEM, adjusted to urodele osmolarity), C2C12 cells grow optimally at 37°C in DMEM. Therefore, finding culture conditions compatible with the growth and differentiation of both cell types, as well as with the cell cycle re-entry of A1 myotubes, was the first requirement for heterokaryon myotube formation. To this end, A1 cells were placed at 37°C in DMEM and C2C12 cells at 25°C in AEMEM. After one week, the A1 myoblasts began to die but the C2C12 myoblasts appeared healthy. Nevertheless, proliferation of C2C12 myoblasts was low at 25°C (Figure 3.1), and only a small number incorporated BrdU one week after the temperature shift (2.7% at 25°C compared to 43.3% at 37°C following a 6-hour BrdU pulse).

Furthermore, the transcriptional activity of these cells was reduced as ascertained by the very sparse labelling obtained with tritiated uridine after a 24 hour exposure. In contrast, a strong signal was obtained at 37°C after an exposure of only 1 hour (data not shown). Therefore, the culture conditions for either of the parental cell lines were inadequate for formation of heterokaryon myotubes.

3.3 A1 myotubes respond to serum stimulation at 33°C.

The growth parameters of both cell lines were investigated next at 33°C, as many studies have shown that this temperature is appropriate for mammalian cell culture. As expected, C2C12 cells which had been at 33°C for 1 week were proliferating (38.4% were labelled with BrdU after a 6 hour pulse) and underwent myogenesis. In addition, the post-mitotic arrest was clearly intact in the 507 myotubes which were analysed after purification by sieving. Following a 24 hour BrdU pulse at 1,3, and 5 days after serum stimulation, no BrdU labelled C2C12 myotubes were observed.

Mononucleate A1 cells maintained at 33°C for two weeks incorporated BrdU following a 6 hour pulse (14.1% at 33°C compared to 23.4% at 25°C) and underwent almost 1.7 population doublings during the two weeks (Figure 3.1 B). A1 cells placed at 33°C also fuse to form myotubes, though less efficiently than at 25°C (not shown). These myotubes synthesised DNA following serum stimulation (Figure 3.2 A), but at a level two to five fold lower than at 25°C. A change in the culture medium to EMEM or AEMEM did not result in an increase in the percentage of myotubes that entered S-phase (Figure 3.2 B). Despite the lower level of cell cycle re-entry observed in A1 myotubes at 33°C, I decided to proceed with the experiments because the timing of S-phase entry was similar at the two temperatures.

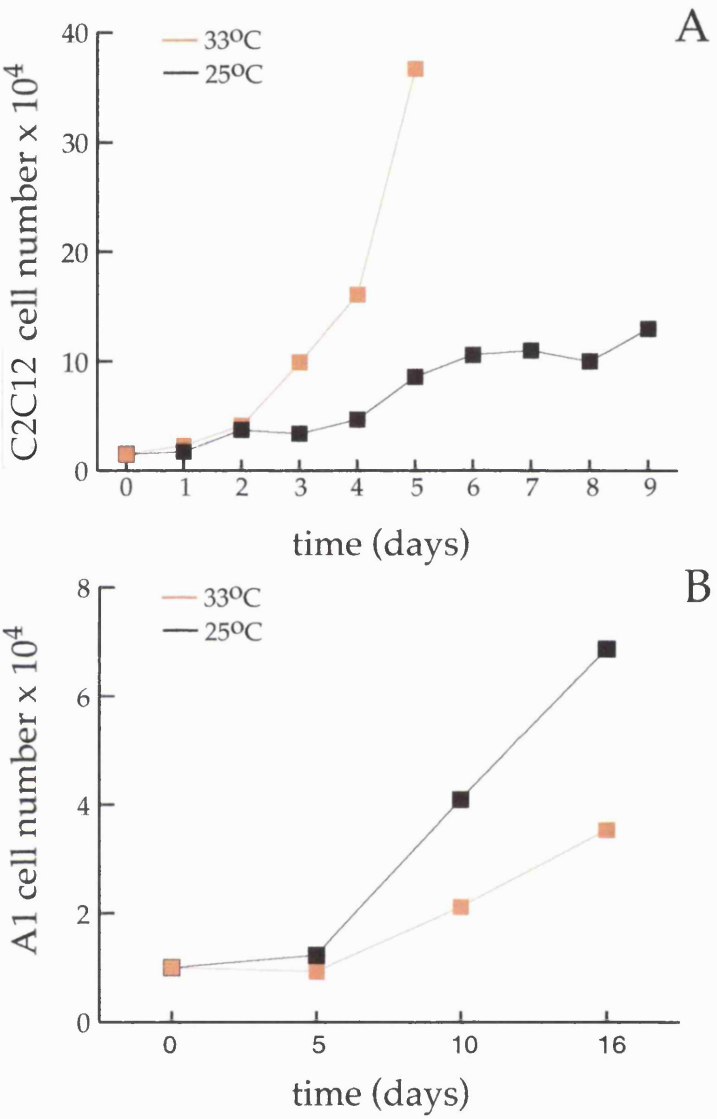


Figure 3.1. Growth of C2C12 (**A**) and A1 (**B**) cells at different temperatures. Equal numbers of cells were plated and placed at 25°C or 33°C. At the indicated times, the cells were trypsinised and counted as explained in the Material and methods section. The experiments were repeated once, with a different population starting size, and gave comparable results.

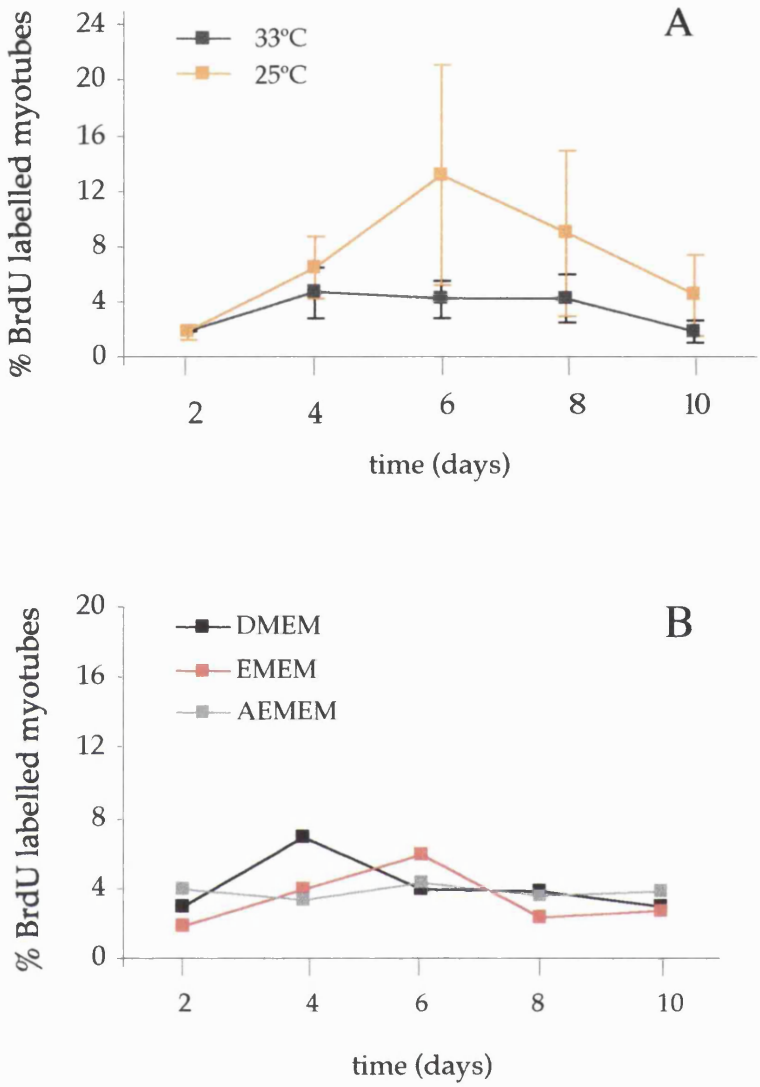


Figure 3.2. A1 myotubes respond to serum stimulation at 33°C in DMEM. Confluent plates of A1 cells were placed at 33°C, allowed to fuse, purified as described in the Material and methods section, and immediately placed in medium containing 20% serum. Myotubes were given a 24-hour BrdU pulse prior to fixation at the indicated times, and processed for BrdU and MHC immunocytochemistry. (**A**) Myotubes incorporated BrdU after serum stimulation in DMEM at 33°C. Controls were fused and stimulated in AEMEM at 25°C. The response of myotubes at 33°C is two to five fold lower than the response at 25°C, but follows the same time course. Graphs show the average of three separate experiments $\pm$ the standard deviation. (**B**) The response of myotubes fused and stimulated in different media at 33°C is compared. Culture in medium of appropriate newt osmolarity (AEMEM) does not increase BrdU incorporation by the myotubes. At least 120 myotubes were scored for each time point.

3.4 A1 and C2C12 myoblasts fuse to form heterokaryon myotubes.

Cultured myoblasts naturally fuse to form myotubes, and I took advantage of this property for the formation of muscle heterokaryons. The fusion times of A1 and C2C12 cells are compatible at 33°C; fusion of A1 myoblasts into myotubes takes five days and fusion of C2C12 myoblasts takes four days. C2C12 cells were added at various ratios to a 90% confluent dish of A1 cells and, concomitantly, the A1 cells were transferred from 25°C to 33°C. C2C12 cells were allowed to adhere for 36 hours, and the cells were then placed in low serum (0.5% FBS). After three days, the myoblasts had assumed a spindle shape, and after four days myotubes began to appear; on the fifth day the cells were treated with trypsin and sieved through nylon meshes to remove unfused myoblasts. The purified myotubes were left to adhere overnight, fixed, and stained with monoclonal antibodies to muscle-specific myosin heavy chain (MHC) and mouse lamin B. An example of a heterokaryon with a single mouse nucleus identified by lamin staining is shown in Figure 3.3. (The lamin B antibody failed to stain A1 nuclei in myotubes and mononucleate cells, confirming its specificity for mouse lamin. See Figure 3.6).

As shown in Table 3.1, reducing the relative contribution of C2C12 myoblasts by as much as eight-fold did not effect the efficiency of heterokaryon myotube formation or the ratio of C2C12 to A1 nuclei within the heterokaryons. In all cases the sieved myotube preparation contained a significant number of C2C12 mononucleate cells.

Fusion among different cell types after mixing was expected to be random and result in the formation of A1 and C2C12 homokaryon as well as heterokaryon myotubes. C2C12 homokaryons, however, were rarely observed in the final preparation, probably because procedure of plating C2C12 cells on confluent A1 cells resulted in few C2C12 cells contacting one another. Furthermore, those few C2C12 homokaryons that did form would tend to flow through the 35 μm mesh.

Although the protocol involving the mixture of the two cell types immediately prior to lowering the serum concentration resulted in the formation of

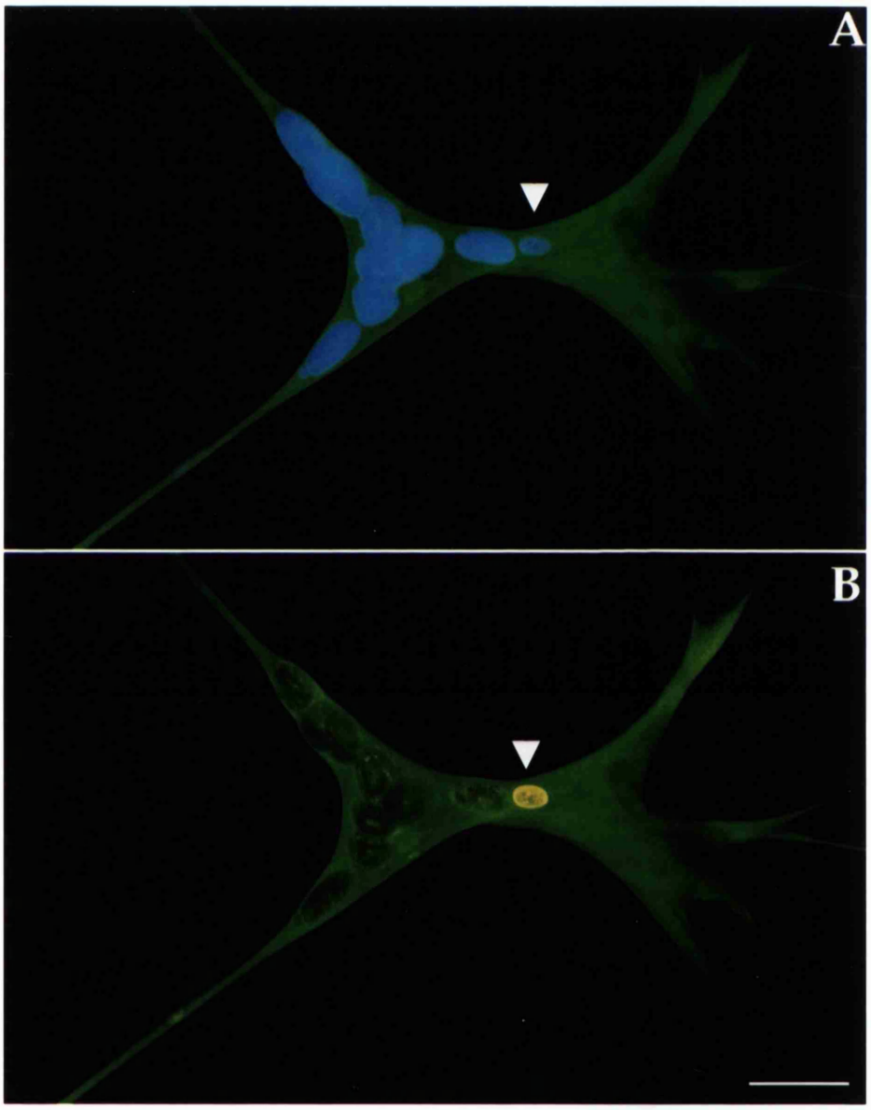


Figure 3.3. C2C12 and A1 cells fuse to form heterokaryon myotubes. Myotubes were prepared as explained in Figure 3.4, by prefusion at 33°C. Immunocytochemistry for MHC and lamin B was performed, and the nuclei were stained with Hoechst 33258. (**A**) An MHC-positive myotube (green) containing 10 nuclei (blue). The arrowhead indicates a C2C12 nucleus. (**B**) Same myotube as in A, showing lamin B staining of the C2C12 nucleus (arrowed). Note the difference in size between the mouse and the newt nuclei. The haploid DNA content of newt nuclei is 13 fold the content of mouse cells. The scale bar is 50 μm.

Table 3.1. Varying the ratio of C2C12 to A1 mononucleate cells mixed does not result in a corresponding variation in the nuclear ratio of the heterokaryons.

ratio of nuclei mixed	average number of nuclei in heterokaryon myotubes		Number of myotubes obtained		
C2C12:A1	C2C12	A1	heterokaryons	total	% heterokaryons
1:8	1.4	2.5	43	141	30
1:4	1.8	2.2	24	94	26
1:2	1.8	1.8	68	152	45
1:1	1.3	1.7	8	32	32

A1 mononucleate cells were grown to 90% confluence at 33°C and counted. C2C12 mononucleate cells were trypsinised, counted and added to the A1 plates in the appropriate number to give the ratios indicated. Cells were allowed to fuse for 5 days in medium containing 0.5% FBS, purified, and left to adhere overnight. Cells were fixed, processed for MHC and lamin B immunocytochemistry, and stained with Hoechst. The preparations were analysed for multinucleate MHC positive cells containing nuclei labelled with lamin B. The number of A1 and C2C12 nuclei in each of heterokaryon myotubes was counted, and the average number is presented. Note that an 8 fold variation in the C2C12 to A1 ratio lead to little or no variation in the nuclear ratio in the heterokaryons.

heterokaryon myotubes, several other parameters were altered with the objective of optimising heterokaryon yield, and obtaining myotubes with different ratios of A1 to C2C12 nuclei. Increasing the time of fusion from five to either seven or nine days had no effect, and the myotubes formed after 5 days appeared healthier. A procedure termed prefusion also seemed to have little effect on heterokaryon myotube yield, or on the final nuclear ratio obtained. During prefusion, subconfluent dishes of A1 or C2C12 cells were maintained in low serum medium for one or two days prior to mixing of the two cell types. One advantage of prefusion was that it resulted in a significantly reduced presence of C2C12 mononucleate cells in the final myotube preparation. Therefore, prefusion was employed in the protocol for the S-phase entry assays, as illustrated in Figure 3.4.

3.5 C2C12 nuclei in heterokaryon myotubes enter S-phase.

Two different prefusion conditions were used in the cell cycle re-entry assay. A1 cells were either prefused at 25°C (experiments I-IV, Table 3.2) or 33°C (experiments V-IX, Table 3.2), to minimise the time A1 cells were maintained at 33°C. C2C12 myotubes were also prepared in dishes containing only C2C12 myoblasts and observed in parallel to the heterokaryon preparation. After sieving, the myotubes were stimulated with serum for 5 days and given a 32-hour BrdU pulse from the fourth to the fifth day. Nine experiments were performed entailing an analysis of a total of 3356 A1 nuclei and 493 C2C12 nuclei in 440 heterokaryon myotubes. The efficiency of heterokaryon formation was not comparable in each experiment and in the vast majority of heterokaryons, the A1 nuclei were present in an excess of three to four fold.

The response of the parental homokaryon myotubes to serum stimulation at 33°C was very distinctive; A1 homokaryon myotubes incorporated BrdU (Table 3.2), but C2C12 homokaryons did so only very rarely (4 nuclei in 3 myotubes of 3041 myotubes analysed), and these are not tabulated in Table 3.2. In the heterokaryon myotubes a total of 31 (6.3%) C2C12 nuclei in 29 myotubes incorporated BrdU (Table 3.2, examples are shown in Figures 3.5 and 3.6). This response was

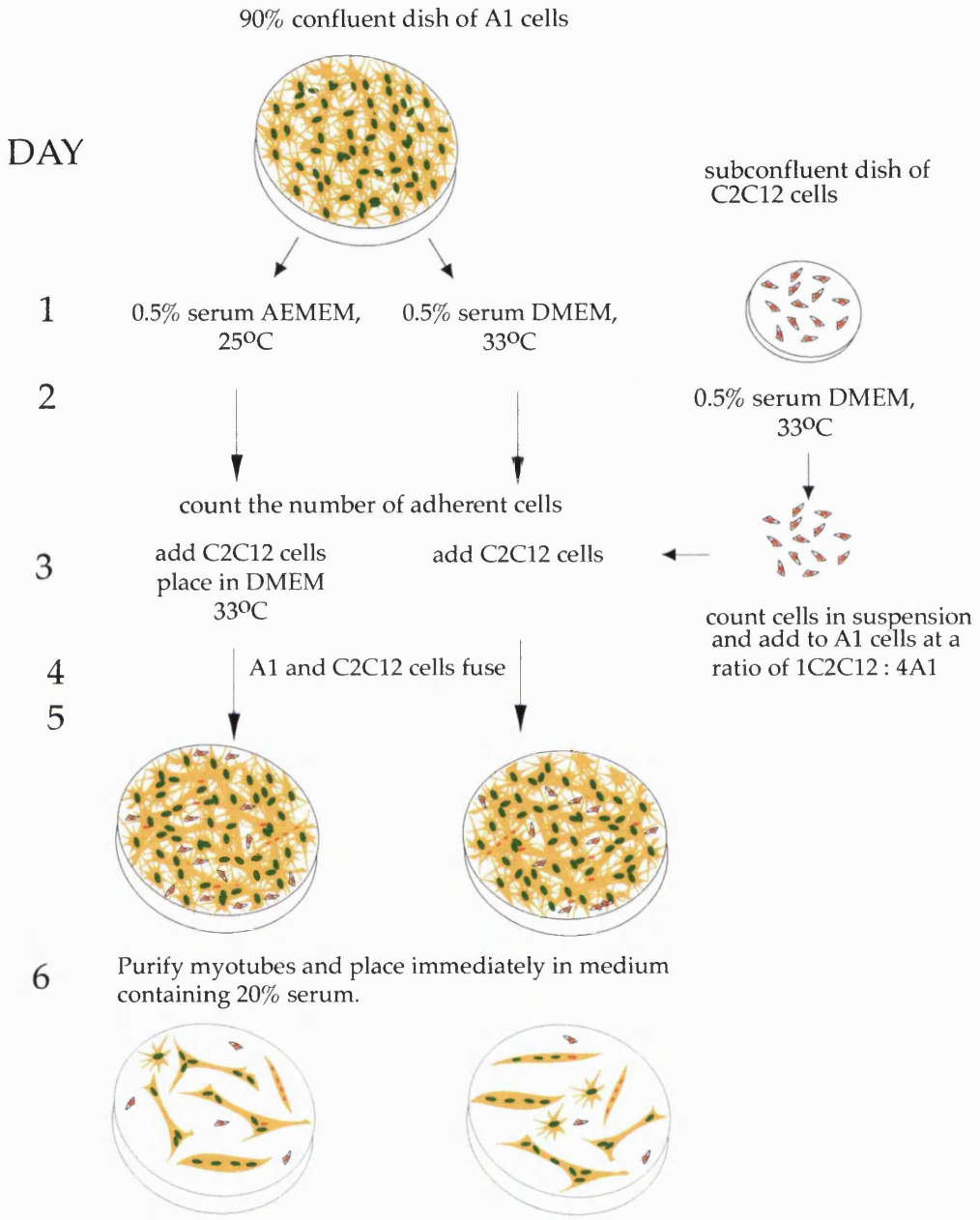


Figure 3.4. Final protocol for obtaining heterokaryon myotubes for S-phase entry assay. On the left is the day on which each step was carried out. On day 1, 90% confluent dishes of A1 cells (containing from 1.8 to 2.5 x 10^5 cells) were placed in low serum (0.5%) medium at 25°C or 33°C (prefusion). On the following day (day 2), sub-confluent C2C12 cells were prefused at 33°C for 24 hours. On day 3, the number of A1 cells in the plates was determined, and C2C12 cells were treated with trypsin, resuspended in low serum medium, counted and added to the A1 cells at a ratio of 1 C2C12 to 4 A1 cells. At this time the A1 cells that had been pre-fused at 25°C were placed at 33°C and the medium changed to DMEM. The cells were allowed to fuse for a further 70-80 hours (days 3 to 6). On day 6, the myotubes were purified, placed immediately in DMEM containing 20% serum and left to adhere overnight. For the S phase entry experiments, the cells were incubated with BrdU for 32 hours and fixed on day 11.

Table 3.2.. C2C12 nuclei in heterokaryon myotubes synthesise DNA in response to serum stimulation.

	A1 homokaryon myotubes			Heterokaryon myotubes			
	number labelled with BrdU	**total number analysed**	**% BrdU labelled**	number of myotubes containing BrdU labelled nuclei			total number analysed
Experiment				C2 only	A1 only	C2 and A1	
I	**57**	**130**	**43.8**	3	0	1	16
II	**5**	**346**	**1.4**	1	0	0	4
III	**11**	**366**	**3.0**	0	2	0	80
IV	**37**	**571**	**6.5**	1	0	0	55
Total I-IV	**110**	**1413**	**7.8**	5 (3.2%)	2 (1.3%)	1 (0.6%)	155
V	**23**	**144**	**16.0**	4	0	4	35
VI	**6**	**406**	**1.5**	2	0	0	8
VII	**24**	**372**	**6.5**	5	0	1	47
VIII	**12**	**145**	**8.3**	0	0	1	22
IX	**21**	**960**	**2.2**	4	0	2	173
Total V-IX	**86**	**2027**	**4.2**	15 (5.3%)	0	8 (2.8%)	285
Total I-IX	**196**	**3440**	**5.7**	20 (4.5%)	2 (0.5%)	9 (2%)	440

Heterokaryon myotubes were prepared as explained in figure 3.4. In experiments I-IV, the A1 cells were prefused at 25°C, and in experiments V-IX prefusion was at 33°C. The myotubes were labelled with BrdU for 32 hours prior to fixation 5 days after serum stimulation (on day 11 according to the time table in figure 4). In a total of 3041 C2C12 homokaryon myotubes analysed in the same experiments, only 3 (0.1%)had BrdU-labelled nuclei. Note that the C2C12 nuclei in 6.6% of heterokaryon myotubes synthesise DNA and that cell cycle re-entry by the mouse nuclei may occur independently of re-entry by A1 nuclei.

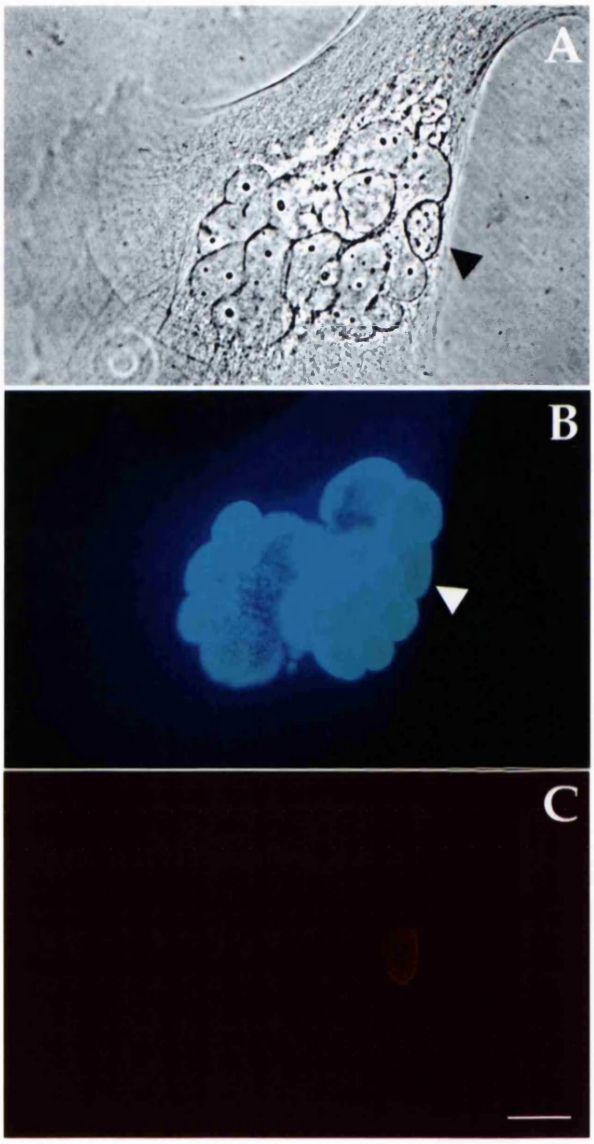


Figure 3.5. Mouse and newt nuclei synthesise DNA in heterokaryon myotubes. Myotubes were prepared as explained in figure 3.4, with prefusion of A1 cells at 33°C. The myotubes were left in high serum for 4 days (96 hours) before addition of BrdU for 24 hours prior to fixation (on day 11 according to the time table in Figure 3.4). Myotubes were processed for BrdU, MHC, and lamin B immunocytochemistry. (**A**) A phase contrast image of a multinucleate myotube. The arrowhead indicates the C2C12 nucleus. (**B**) The cytoplasm shows MHC staining (cascade-blue), and all nuclei, including the C2C12 nucleus (arrow) are positive for BrdU staining (green-blue). (**C**) The nucleus indicated in A and B is the only nucleus that stains for lamin B (red). The scale bar is 25 μm.

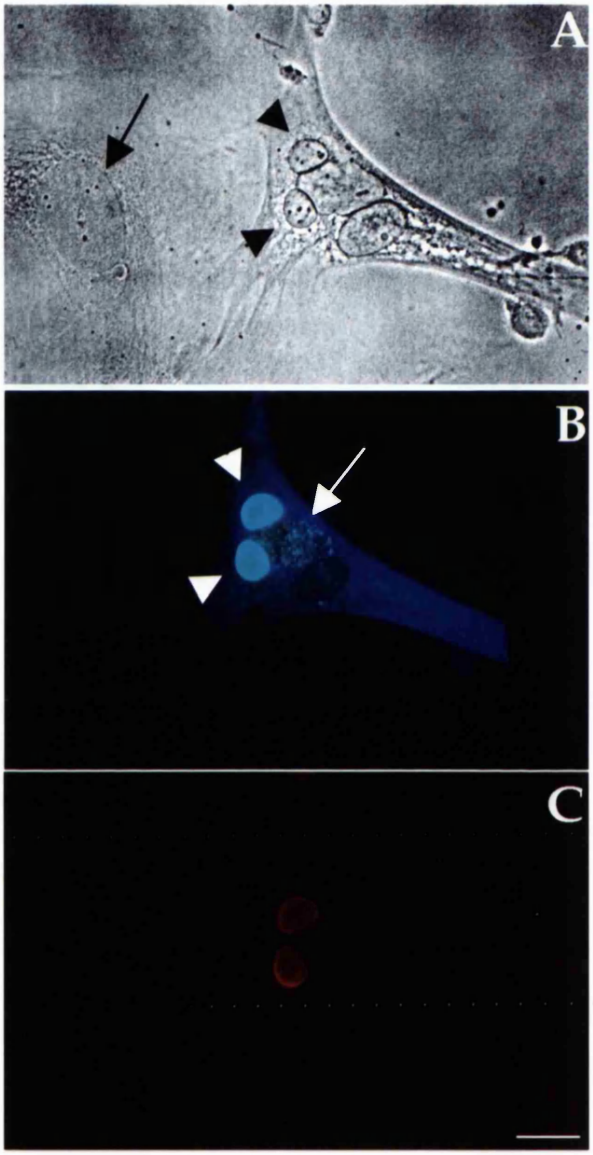


Figure 3.6. Mouse and newt nuclei in heterokaryon myotubes label with BrdU. Myotubes were prepared and processed as in the legends to figures 3.4 and 3.5. (**A**) A phase contrast image of a multinucleate myotube containing two mouse nuclei (arrowheads). The arrow indicates a mononucleate A1 cell in the vicinity of the myotube. (**B**) The C2C12 nuclei incorporated BrdU (arrowheads), as did the A1 nuclei (arrow). The cytoplasm is positive for MHC (blue). (**C**) The mouse nuclei indicated in A and B are positive for lamin B (red). Neither the A1 nuclei in the myotube, nor the A1 nucleus in the mononucleate cell indicated by the arrow in A stains for lamin B. The scale bar is 25 μm.

independent of the ratio of A1 to C2C12 nuclei in the heterokaryons (Figure 3.7), although the sample size is limited.

In the homokaryon A1 myotubes analysed in these experiments, 2.8% of the nuclei incorporated BrdU at 33°C (689 out of 24410 total nuclei). In the heterokaryon myotubes only 1.9% (40 nuclei out of 3356) did so, suggesting that the A1 response in heterokaryons myotubes may be inhibited (Table 3.2 shows the figures for myotubes).

Interestingly, BrdU labelling of C2C12 nuclei within the heterokaryon myotubes was not always accompanied by labelling of the A1 nuclei present in the same heterokaryon (Figure 3.8; Table 3.2, column labelled C2 only). The reverse labelling pattern in which only A1 nuclei incorporated BrdU was also observed, but only rarely (Table 3.2, column labelled A1 only; an example is shown in Figure 3.9). This suggests that cell cycle re-entry in each nucleus can occur independent of events in neighbouring nuclei.

3.6 Discussion

The work presented in this chapter establishes for the first time that mouse and newt myogenic cells are able to form viable heterokaryon myotubes that express MHC, a late marker of muscle differentiation, and that the post-mitotic arrest of the mouse nuclei in the syncytium can be undermined. DNA synthesis was observed in the mouse nuclei within the heterokaryon myotubes up to 8 days after fusion. In contrast, and as expected, cell cycle re-entry almost never occurred in mouse homokaryon myotubes (0.1% of C2C12 myotubes contained nuclei that incorporated BrdU).

One interesting aspect of the cell cycle re-entry response of the heterokaryon myotubes was that a high frequency of BrdU labelling occurred in the mouse C2C12 nuclei independent of labelling in the newt A1 nuclei. It is possible that S-phase entry in mouse and newt nuclei was asynchronous, occurring faster in the mouse nuclei because they are closer to their physiological temperature. The

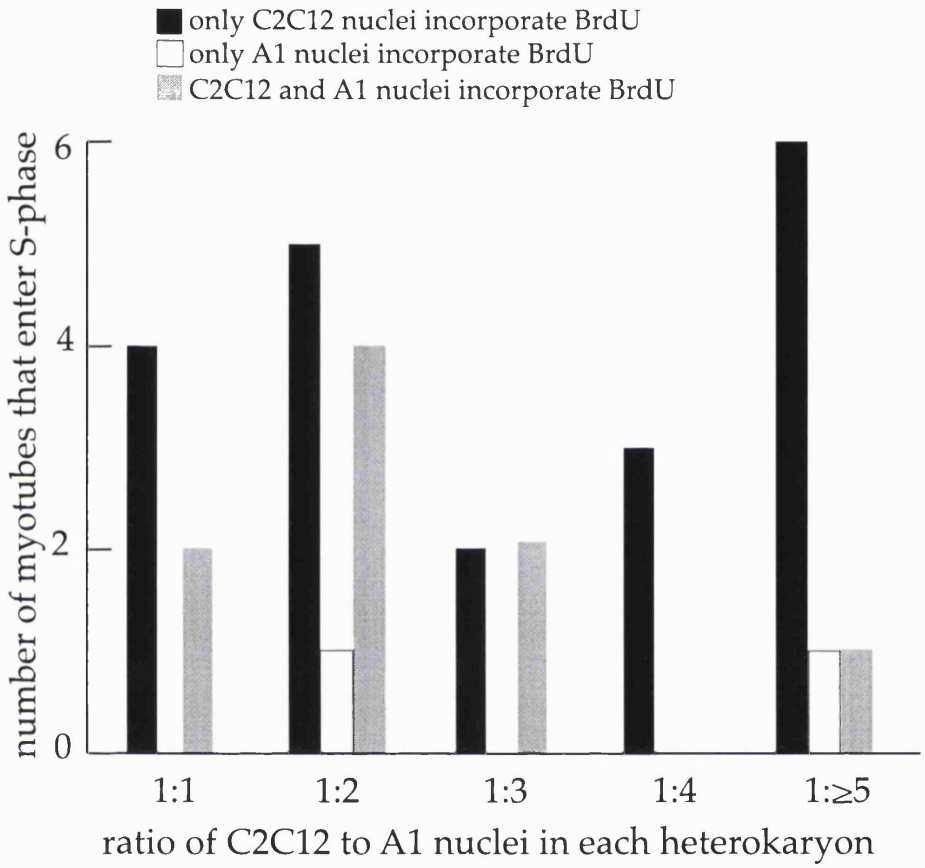


Figure 3.7. DNA synthesis in C2C12 nuclei within heterokaryon myotubes appears to be independent of nuclear dosage. The ratio of nuclei in the 31 heterokaryon myotubes with BrdU labelled nuclei shown in Table 3.2 was determined. The number of myotubes in which labelling occurred only in the C2C12 nuclei (black bars), only in the A1 nuclei (white bars), or in both types of nuclei (grey bars) were counted for each category. Note that, althought the sample size is small, C2C12 nuclei incorporate BrdU at all nuclear ratios (black and grey bars).

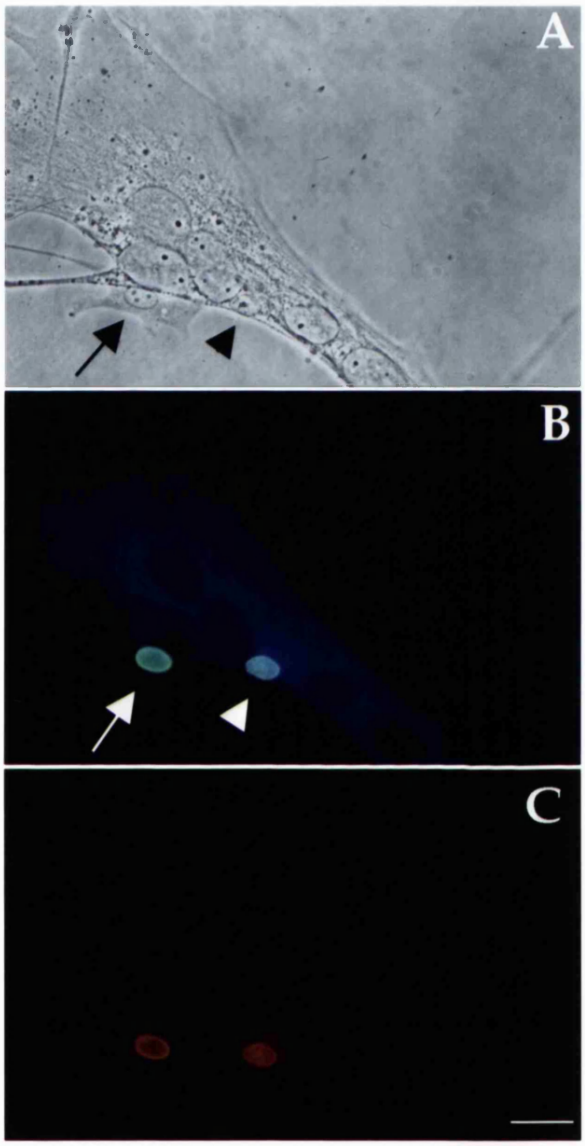


Figure 3.8. BrdU incorporation in mouse nuclei can occur independent of detectable DNA synthesis in the newt nuclei. Myotubes were prepared and processed as in the legends to figures 3.4 and 3.5. (**A**) A phase contrast image of a multinucleate myotube containing a mouse nucleus (arrowhead). The arrow indicates a mononucleate C2C12 cell lying underneath the myotube. Note that it is readily distinguishable from the myotube. (**B**) Only the C2C12 nucleus in the myotube incorporated BrdU (arrowhead), as did the nucleus in the mononculeate cell indicated in A (arrow). The cytoplasm is positive for MHC (blue). (**C**) Only the nuclei indicated in A and B are positive for lamin B (red). The scale bar is 25 μm.

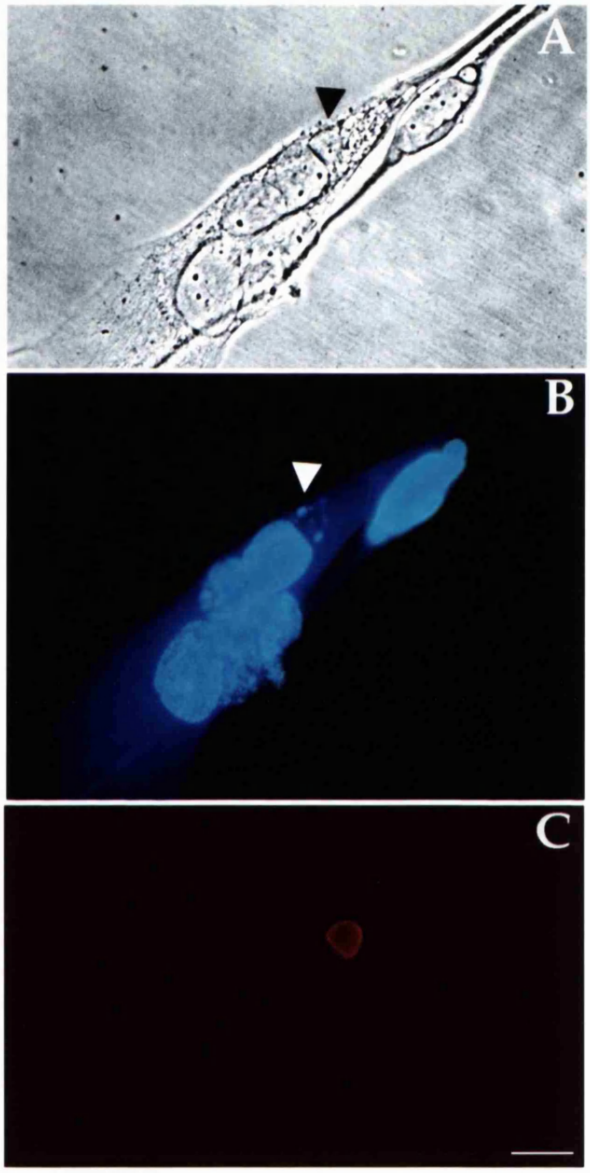


Figure 3.9. BrdU incorporation in newt nuclei can occur independent of labelling in the mouse nucleus. Myotubes were prepared and processed as in the legends to figures 3.4 and 3.5. (**A**) A phase contrast image of a multinucleate myotube containing a mouse nucleus (arrowhead). (**B**) Only the A1 nuclei in the myotubes incorporated BrdU. The mouse nucleus (arrowhead) is negative for BrdU. The cytoplasm is positive for MHC (blue). (**C**) Only the nucleus indicated in A and B is positive for lamin B (red). The scale bar is 25 μm.

pattern of BrdU labelling observed in Figure 3.6, dense in C2C12 nuclei and sparse in A1 nuclei, could be considered indicative of asynchronous S-phase entry, although there are other possibilities. Asynchrony of DNA synthesis among A1 myotube nuclei was observed after implantation of these myotubes into limb blastemas (Kumar et al., 2000).

The available data offer no support to the idea of an effect of nuclear dosage on cell cycle re-entry (Figures 3.5, 3.6, and 3.7) but it cannot be ruled out, since heterokaryon myotubes with an excess of C2C12 nuclei were never observed. It is possible that the larger volume of the A1 cells compared to the C2C12 cells would result in the dilution of cell cycle inhibitors produced by the mouse nuclei (Figure 3.3). One way to address the issue of inhibitor dilution would be to culture the heterokaryons at 25°C in AEMEM, where the cell cycle re-entry response of newt nuclei should be maximal and equivalent to the response of A1 homokaryon myotubes. The continued presence of an inhibitory activity would indicate that nuclear dosage is an important factor effecting the experimental outcome. Additionally, alternative strategies for the production of heterokaryons with an excess of C2C12 nuclei, such as PEG-mediated fusion (Chiu and Blau, 1984; Clegg and Hauschka, 1987), remain to be explored.

The most immediate interpretation of the results presented in this Chapter is that the post-mitotic arrest in mammalian nuclei can be subverted by the pathway leading to pRb phosphorylation in newt myotubes. Two observations suggest that the response of heterokaryons to serum stimulation is due to activation of DNA synthesis by serum and is not an aberrant response: 1) The peak in DNA synthesis in A1 myotubes is observed at the same time at both 25°C and 33°C; 2) A1 cells are able to grow and differentiate at the higher temperature. There were probably no differences in the DNA synthetic activity or in the cell cycle stage of the mouse and newt nuclei that fused to form heterokaryon myotubes. It is well established in the mammalian system and strongly suggested in the newt that myoblast fusion is preceded by cell cycle withdrawal (Olsen, 1992; Lassar et al., 1994; Andrés and Walsh, 1996; Novitch et al., 1996; Tanaka et al., 1997; Yoshida et al.,

1998). It is noteworthy that in mouse myocyte-fibroblast heterokaryons, DNA synthesis in the muscle nuclei is only observed in the 24 hours subsequent to fusion (Schwab and Luger, 1980; Clegg and Hauschka, 1987). Thereafter, the mechanism that ensures post-mitotic arrest in the muscle nuclei becomes dominant and stable, and the heterokaryon cells are refractory to serum stimulation. In the experiments reported here, the C2C12 and the A1 cells were maintained in low serum medium for five days prior to serum stimulation, a time during which the post mitotic state is established irreversibly in C2C12 myotubes.

These experiments were performed during the first year of my PhD. Continuing the experiments with a view to clarifying the question of nuclear dosage or to increase the numbers of heterokaryon myotubes for a more accurate statistical analysis was considered unjustified in light of problems with heterokaryon yield and the low response of the A1 nuclei in the heterokaryon myotubes. At the same time, an experiment that would conclusively establish the link between cell cycle re-entry in C2C12 nuclei and the activation of a specific newt myotube signalling pathway was not apparent. Subsequent investigations revealed that the activity responsible for inducing cell cycle re-entry in newt myotubes is specifically generated by thrombin on an unidentified serum component (Tanaka et al., 1999). The possibility of attributing DNA synthesis in C2C12 nuclei to the thrombin-generated activity now exists. This is an important opportunity that can be explored with the basis of the experiments in this chapter. It would also be desirable to show that the heterokaryons are arrested in respect of their response to a defined growth factor, for example PDGF or EGF, as these growth factors do not induce DNA synthesis in newt myotubes (Tanaka et al., 1997, 1999).

Chapter 4

Viral oncogene expression, caffeine treatment, and ultraviolet irradiation of cultured newt myotubes

4.1 Introduction

Newt myotubes implanted into regenerating limbs enter S-phase and give rise to mononucleate cells (Lo et al., 1993; Kumar et al., 2000). In culture, the myotubes re-enter the cell cycle in response to serum stimulation, and arrest with a DNA content of the G2 phase (Tanaka et al., 1997). The multinucleate to mononucleate transition has not been observed *in vitro*. One hypothesis that I put forward in the Introduction is that mononucleate progeny may be generated after implantation as a result of progression through mitosis followed by cytokinesis (Figure 1.6 A). Some evidence to support this hypothesis comes from experiments in mammalian myotubes. Expression of viral oncoproteins, such as SV40 large T antigen and adenovirus E1A, in mouse myotubes induces mitosis as well as DNA synthesis, leading to scission of the syncytium and budding of nuclei to form mononucleate cells (Endo and Nadal-Ginard, 1989,1998; Iujvidin et al., 1990; Crescenzi et al. 1995; Latella et al., 2000).

The mechanism by which viral oncoproteins induce mitosis in mouse myotubes is not clear. Two important targets of these proteins are pRb and p53 (Ko and Prives, 1996; Crescenzi et al., 1995). Viral oncoproteins bind to and inactivate pRb, but mitosis in *Rb-/-* mouse myotubes, which re-enter S-phase and synthesise DNA, has not been reported (Schneider et al., 1994; Novitch et al; 1996). This suggests that an additional activity of the oncoprotein could be required for traversing the G2 checkpoint. The p53 protein is a key regulator of cellular processes such as differentiation, apoptosis and senescence (reviewed in Ko and Prives, 1996 and Levine, 1997; Morgenbesser et al., 1994; Soddu et al., 1996; Aladjem et al., 1998). One of its better characterised activities is to arrest progression through the cell cycle at several points, including G2, following DNA damage or environmental stress (Kastan et al., 1991; Dulic et al., 1994; Cross et al., 1995; Stewart et al., 1995; Agarwal et al., 1995; Bunz et al., 1998; Taylor et al., 1999). Although there is no absolute requirement for p53 in muscle development (Donehower et al., 1992; Halevy et al., 1995), p53 is transcriptionally activated during *in vitro* myogenesis, and interference with p53 function leads to reduction in myotube size and number (Soddu et al., 1996; Tamir and Bengal, 1998). Therefore, it is possible that p53 function is involved in blocking mitosis in newt myotubes.

I was interested in determining whether overcoming the G2 arrest in newt myotubes would lead to generation of viable mononucleate cells after fragmentation of the syncytium by mitosis and cytokinesis, or possibly lead to cell death. This seemed an important test of the validity of the "M-phase model" for mononucleate cell production. To induce entry to mitosis in the myotubes, I pursued two strategies. The first was treatment with caffeine, which indirectly leads to activation of the M-phase promoting kinase, cyclinB-cdc2, by removal of inhibitory phosphorylations from the kinase subunit (Schlegel and Belinksy, 1990; Yamashita et al., 1990; Steinman et al., 1991; Kumagai et al., 1998). The second was microinjection of the myotubes with plasmids encoding the viral oncogenes SV-40 large T antigen and v-myb. Expression of SV-40 large T antigen in mouse myotubes leads to cell cycle progression and myotube fragmentation (Endo and Nadal-Ginard, 1989,1998), while there is evidence that the myb gene is required in *Drosophila* oocytes for the G2/M transition, and this requirement can be overcome by overexpression of cdc2 (Katzen et al., 1998).

The UV irradiation experiments described in the third part of this chapter were aimed at developing a functional assay for p53 in newt cells. This would allow tools such as mammalian reporter constructs for p53 transcriptional activation or dominant negative proteins to be tested in the newt cells, and subsequently used to study the regulation of p53 in newt myotubes.

Results

4.2 Caffeine treatment of serum stimulated newt myotubes results in the appearance of aberrant nuclei.

Caffeine treatment of newt myotubes 14 days after serum stimulation was expected to induce entry to mitosis. The peak of DNA synthesis in these cells occurred between four and six days, but nuclei were seen to take up BrdU as late as 10 days after addition of serum (Figure 3.2, Chapter 3). The length of S phase in newt myotubes is approximately 48-72 hours (Tanaka et al., 1997), and therefore the nuclei that began S-phase on or before day 11 were expected to complete DNA replication and enter G2 by day 14.

In a preliminary experiment, myotubes were purified and maintained in medium containing 15% FBS for 14 days to maximise the number of nuclei that entered G2. These myotubes were treated with varying concentrations of caffeine and fixed at 8, 16, and 24 hours after the beginning of the treatment (Table 4.1). The myotubes were stained for MHC and Hoechst, and analysed for the presence of mitotic figures, and apoptotic or fragmented nuclei. Several myotubes were observed containing fragmented nuclei as shown in Figure 4.1 A and B, and this has been analysed in more detail; this phenotype was not apparent in other experiments involving long term cultures. The fragmented nuclei observed were reminiscent of the pulverised nuclei observed in mammalian myotubes expressing large T antigen (Endo and Nadal-Ginard, 1998), and in human cells that undergo mitosis prior to completing DNA replication (Heald et al, 1993). Other aberrant nuclear phenotypes were observed infrequently (Figure 4.1 C, D). One MHC-positive cell exiting telophase was observed in the sample treated with 5mM caffeine for 8 hours, but it could also have been two juxtaposed cells. Higher doses of caffeine resulted in cytotoxicity leading to degeneration of myotubes. Myotubes were seen to round up and detach from the culture dish as early as 8 hours after treatment with 25 mM caffeine, and this may account for the smaller number of cells observed after treatment with this dose.

4.3 The appearance of fragmented nuclei depends on traversal of S-phase.

A detailed analysis to record the appearance of aberrant nuclear phenotypes after treatment with caffeine was performed. The percentage of myotubes with fragmented nuclei rose sharply at 4 hours after the addition of 5mM caffeine to the medium, and remained high until 24 hours, returning to control levels at 72 hours (Figure 4.2 A).

Importantly, the appearance of this fragmented phenotype after caffeine treatment was largely dependent on S-phase entry; myotubes that were prevented from entering S-phase, by culture in low serum medium or by contact inhibition in dense cultures, did not show an increase in the percentage of fragmented nuclei relative to untreated, serum-stimulated controls (4.2 A).

Table 4.1. Caffeine treatment results in the appearance of fragmented nuclei in serum stimulated myotubes

	Caffeine dose								
	5mM			10mM			25mM		
Hours of Treatment	number of fragmented nuclei	total nuclei	%	number of fragmented nuclei	total nuclei	%	number of fragmented nuclei	total nuclei	%
8	36	206	17.5	12	109	10.7	8	48	16.8
16	55	252	21.8	22	137	15.9	13	62	21
24	54	263	20.5	18	116	11.3	5	32	15.6

Myotubes were purified and placed in medium containing 15% FBS for 14 days. The medium was refreshed every four days. On day 14 caffeine was added to the cultures and the cells were fixed at the indicated times. Immunostaining was performed for MHC and the nuclei were labelled with Hoechst 33258. The myotubes were analysed for the presence of mitotic figures and other nuclear phenotypes. Note that fragmented nuclei were apparent at all doses of caffeine and that the number of myotubes present in the culture decreased with increasing doses. In other experiments, untreated myotubes did not present fragmented nuclei.

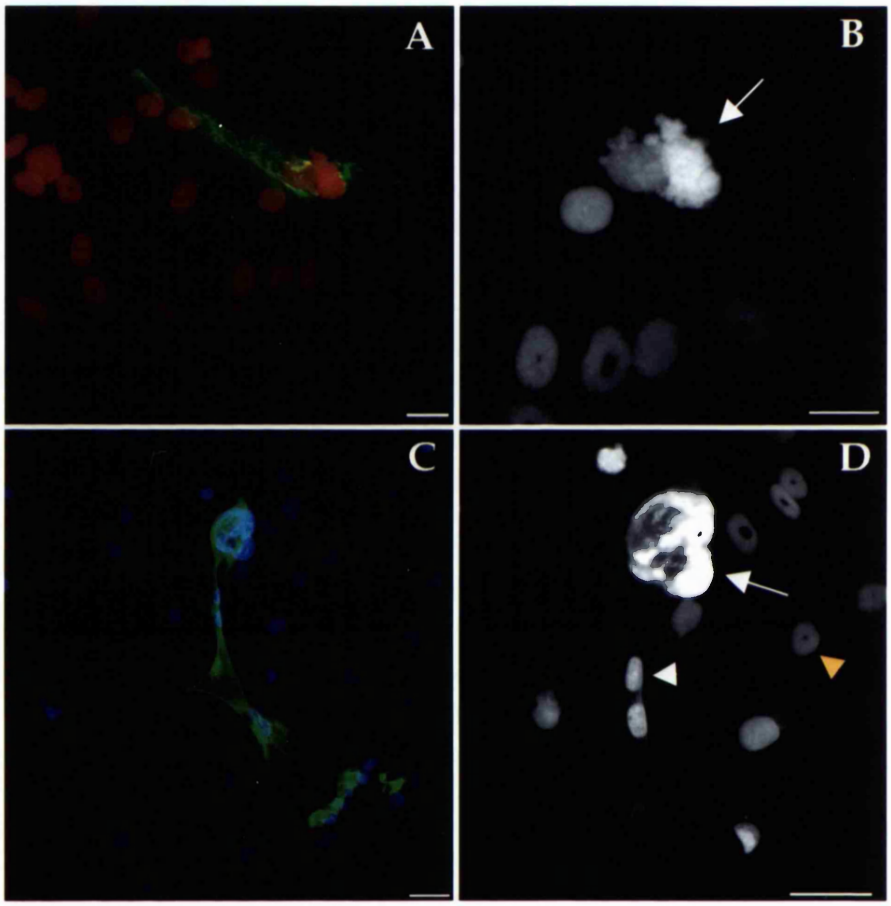


Figure 4.1. Caffeine treatment induces the appearance of aberrant nuclear phenotypes. Cells were prepared as in the legend to Table 1 and treated with 5mM caffeine for 24 hours. (**A, C**) MHC-stained (green) myotubes. (**B, D**) Higher magnification micrographs showing only nuclear staining with Hoechst 33258. (**B**) Fragmented chromatin (arrow) next to a normal nucleus. (**D**) The nuclei in the rounded protrusion of the myotube appear to be coalesced (arrow); the remaining two nuclei appear condensed (white arrowhead) as compared to interphase nuclei in the culture (yellow arrowhead). Scale bars are 50 μm.

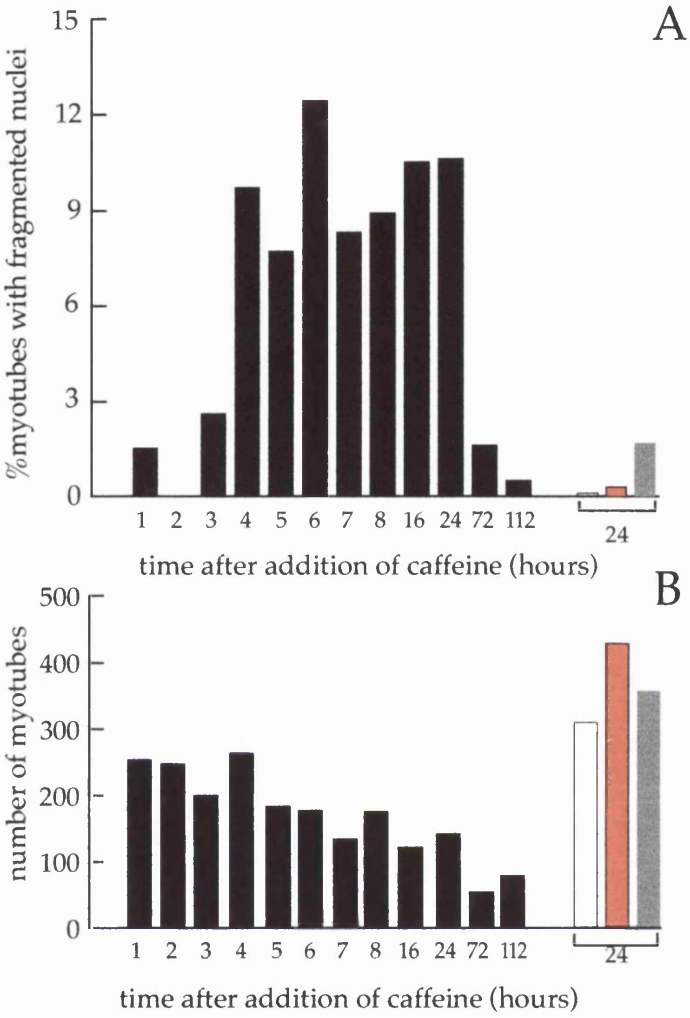


Figure 4.2. Appearance of fragmented nuclei in myotubes after caffeine treatment depends on cell cycle re-entry. Myotubes were purified and maintained in 15% FBS medium for 14 days. On day 14, 5 mM caffeine was added and the cells were fixed at the time points indicated. All the myotubes in the culture were scored. Controls were: serum stimulated, untreated myotubes (white bar); myotubes maintained in medium containing 0.5% FBS and treated with caffeine for 24 hours (grey bar); and myotubes which were contact inhibited by high density culture conditions, serum stimulated, and treated with caffeine for 24 hours (red bar, G1 nuclei). (**A**) Fragmented nuclei appear after 4 hours of treatment with caffeine, but no fragmented nuclei are induced in myotubes with G1 nuclei (red and grey bars). (**B**) The number of myotubes scored in the cultures analysed in A is shown. Note that the number of myotubes present at 72 and 112 hours is less than half the number present in control cultures and in cultures fixed at times points where fragmented nuclei are observed.

This experiment was repeated with a higher dose of caffeine (10mM) with similar results, except that the percentage of fragmented nuclei was lower than that observed with 5mM caffeine. Addition of nocodazole (10 μg/ml) to the medium to prevent progression through mitosis did not block the appearance of fragmented nuclei, but some myotubes with condensed chromatin were observed (Figure 4.3 A and B) as well as one mitotic figure (Figure 4.3 C).

Altogether, 3128 myotubes (MHC-positive, multinucleate cells) were analysed. Only two cells that could be considered reminiscent of mitotic figures were observed; it was not possible to be certain of the number of nuclei in these cells because of the condensed state of the chromatin, but they appeared to be binucleate (Figure 4.3 C).

4.4 v-myb expression induces cell cycle re-entry in A1 mononucleate cells but not in myotubes

Microinjection of a mixture of v-myb and eGFP plasmids into quiescent A1 mononucleate cells induced S-phase entry in 17.5% of eGFP-positive cells after 48 hours; only 2.9% of cells injected with the eGFP plasmid alone were observed to synthesise DNA. The activity of v-myb in respect of S and M-phase entry of myotubes was then analysed. Microinjection of v-myb into myotubes in five separate experiments, entailing an analyses of a total of 411 myotubes, appeared to have no effect on inducing S-phase entry in 1.5% FBS or in dense cultures maintained in 15 % FBS. To test whether v-myb had any effect on G2 events, purified myotubes were microinjected and placed immediately in medium containing 15% serum. The 333 myotubes analysed after 7 days and the 126 myotubes analysed after 5 days did not contain mitotic figures or aberrant nuclear phenotypes. Nocodazole was present in the medium for the 30 hours prior to fixation. In view of the lack of activity of v-myb on myotubes, this line of investigation was not pursued further.

4.5 SV40 large T antigen is active in newt mononucleate cells and myotubes

Expression of wild type T antigen induced DNA synthesis in quiescent mononucleate A1 cells maintained in serum free medium (Figure 4.4). There were

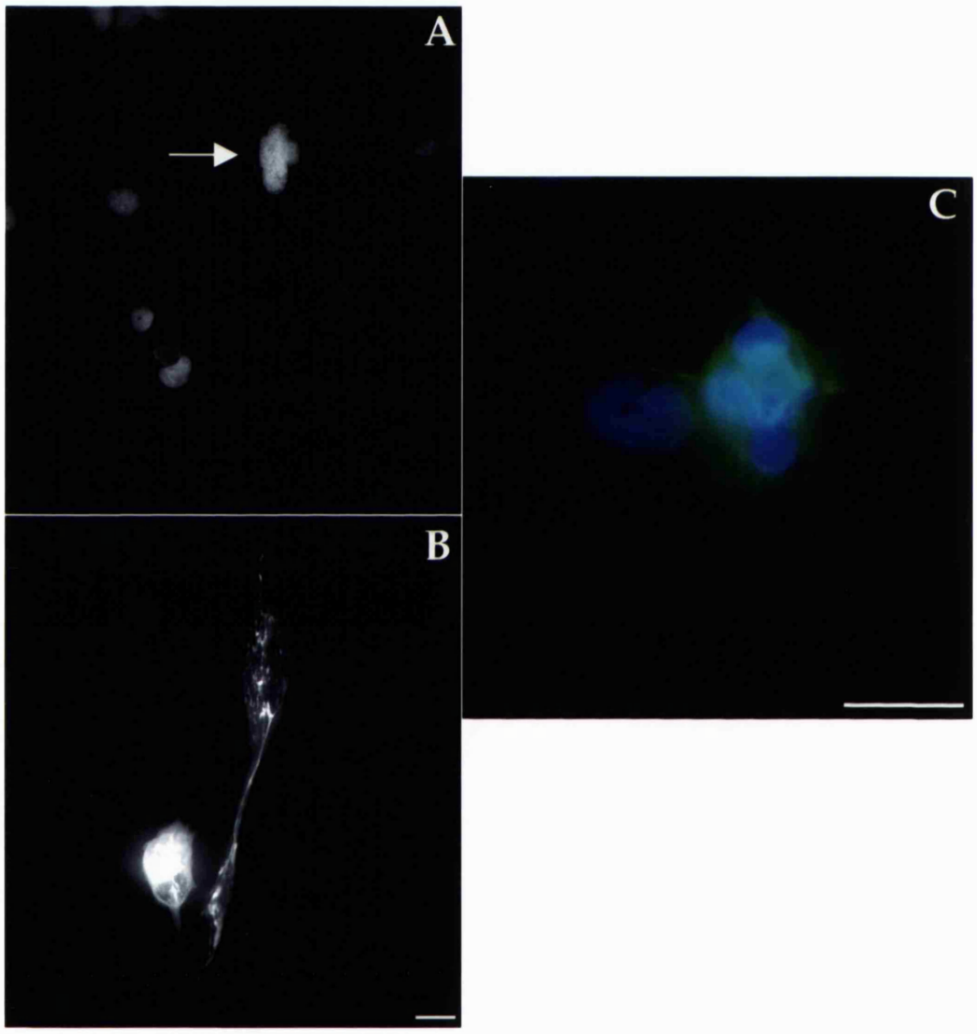


Figure 4.3. Caffeine treatment induces chromosome condensation. Cells were prepared as in the legend to figure 4.2 and treated with 10 mM caffeine in the presence of nocodazole (10 μg/ml) for 24 hours. (**A**) Four nuclei with condensed chromatin (arrow) stained with Hoechst. (**B**) MHC-staining of the myotube shown in A. (**C**) MHC-stained cell (green) undergoing cytokinesis. Note that the chromatin, stained with Hoechst (blue) is condensed. Scale bars are 50 μm.

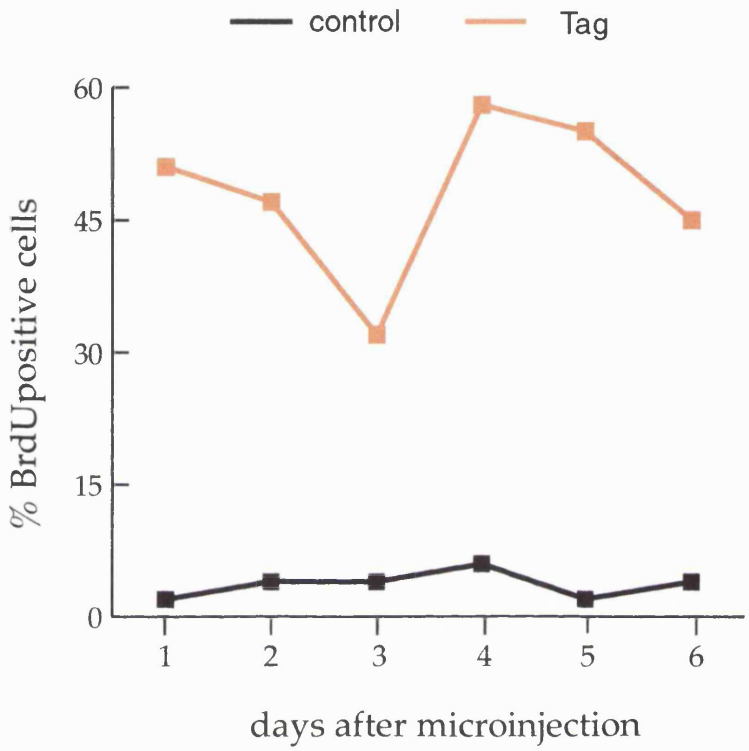


Figure 4.4. T antigen induces cell cycle re-entry in serum starved A1 mononucleate cells. Mononucleate cells were plated at low density in serum free AEMEM for 48 hours, microinjected with the pSE plasmid encoding wild type T antigen, and placed immediately in medium with 1% FBS. BrdU was added for 24 hours prior to fixation at the indicated times. Cells were stained for BrdU and T antigen as described in the Material and methods section. T antigen expressing cells have a labelling index approximately 20 fold greater than that of non-injected cells. Note that there are two peaks in DNA synthesis at day one and day four after microinjection. At least 180 cells were counted for each time point.

two peaks of DNA synthesis observed at 1 day and 4 days after microinjection. This was consistent with a the cycle length of approximately 72 hours for A1 cells. No aberrant nuclear phenotypes were observed in T antigen expressing cells.

Expression of T antigen also resulted in significant BrdU incorporation in contact inhibited myotubes (Figure 4.5), as well as in myotubes maintained in low serum medium (Table 4.2). The stimulation of S-phase entry observed in the latter case was eight fold.

4.6 SV40 large T antigen does not induce the appearance of mitotic figures in myotubes.

Myotubes were purified, microinjected with pSE, placed in high serum medium, and fixed at 3, 4, 5, and 6 days after microinjection. T antigen expressing myotubes began DNA synthesis with a peak response on or before day 3 after microinjection (Figure 4.5). Nocodazole was added to the medium for the 48 hours prior to fixation on days 5 and 6 after serum stimulation. Mitotic figures would have been expected when these myotubes completed S-phase, between four and 6 days after microinjection. No mitotic figures, condensed chromosomes, or aberrant nuclear phenotypes were observed in the 646 myotubes analysed. Between 100 and 150 myotubes were present at each time point.

This result contrasts with those obtained by expression of large T antigen in mammalian myotubes where expression of the oncoprotein leads to DNA synthesis, mitosis, cytokinesis, and cell death (Endo and Nadal-Ginard,1989, 1998). The main methodological difference between these experiments on mammalian cells and those carried out here is that stably transfected mammalian cell lines were used in which T antigen expression was under the control of the metallothinein promoter. Myoblasts were induced to fuse in the absence of T antigen, and its expression was then induced in the differentiated myotubes by addition of $ZnCl_2$ to the medium (Endo and Nadal-Ginard, 1989, 1998).

In order to mimic these conditions more closely, mononucleate A1 cells were transfected with the pMTSVtsA58 plasmid (expressing a temperature sensitive

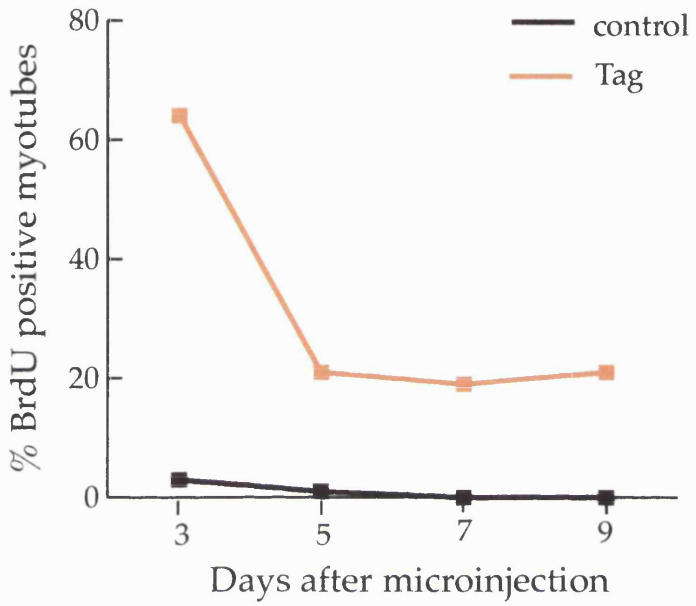


Figure 4.5. T antigen induces DNA synthesis in contact inhibited myotubes. Myotubes were not purified to maintain high density conditions and contact inhibition. Unpurified myotubes were microinjected with pSE and placed in medium containing 15% FBS. BrdU was added to the medium 24 hours prior to fixation at the times indicated. The cells were stained for MHC, BrdU, and T antigen as described in the Material and methods section. T antigen expressing myotubes incorporated BrdU, but non-expressing myotubes did not. Note that BrdU incorporation did not vary significantly at 5, 7, and 9 days after microinjection. At least 90 myotubes were counted for each time point.

Table 4.2. T antigen induces cell cycle re-entry in myotubes in low serum.

T antigen expression	BrdU labelled myotubes	Total myotubes	% BrdU-labelled
+	23	82	55.2
—	3	445	6.7

Myotubes were purified, maintained in medium containing 1.5% serum, and microinjected with pSE (wild type T antigen). BrdU was added four days after microinjection for 24 hours. Cells were fixed and processed for T antigen, BrdU, and MHC immunocytochemistry as described in the Material and methods section. The number of BrdU labelled myotubes in the population was determined. T antigen expression induces an eight fold increase in BrdU incorporation in myotubes maintained in low serum conditions.

mutant of T antigen with wild type activity at temperatures below 37°C) by particle bombardment and induced to fuse 2 days later. After differentiation, purified myotubes were placed in medium containing 15% FBS and 100 mM $ZnCl_2$. Nocodazole was added to the medium for 30 hours prior to fixation on days 4 and 6 after serum stimulation. T antigen could be detected in 286 myotubes (1.2%) by antibody staining, but no mitotic figures or aberrant nuclear phenotypes were observed.

The same experiment was repeated with the pSE plasmid expressing wild type T antigen constitutively. Transfected mononucleate cells fused to form 156 myotubes (2.3%) expressing T antigen (see the discussion), none of which exhibited mitotic figures after 6 days in medium containing 15% FBS. Nocodazole was added to the medium for 30 hours prior to fixation.

To test whether mitosis can be induced in mammalian myotubes under these experimental conditions, pSE was microinjected into C2C12 myotubes. These were placed in medium containing 20% FBS and incubated with BrdU for 24 hours prior to fixation. DNA synthesis was observed in C2C12 myotubes over several days (Figures 4.6 and 4.7), but unexpectedly no mitotic figures or aberrant nuclear phenotypes were observed (see the discussion).

4.7 SV40 large T-antigen induces endoreplication in myotube nuclei.

Although T antigen clearly possessed S-phase re-entry activity in newt myotubes, it did not induce subsequent progression through the G2 and M phases of the cell cycle. Nevertheless, expression of T antigen resulted in a high BrdU labelling index over several days in both contact inhibited A1 myotubes and in C2C12 myotubes (Figures 4.5 and 4.6). This suggested to me that T antigen might be inducing multiple cycles of DNA synthesis in the myotube nuclei.

To test this hypothesis, myotubes were subjected to the double-labelling protocol illustrated in Figure 4.8 A. Three days after plasmid microinjection and serum stimulation, the myotubes were labelled with [^{3}H]-thymidine for 8 hours, washed and left in medium containing 15% FBS. S-phase lasts up to 72 hours in myotubes;

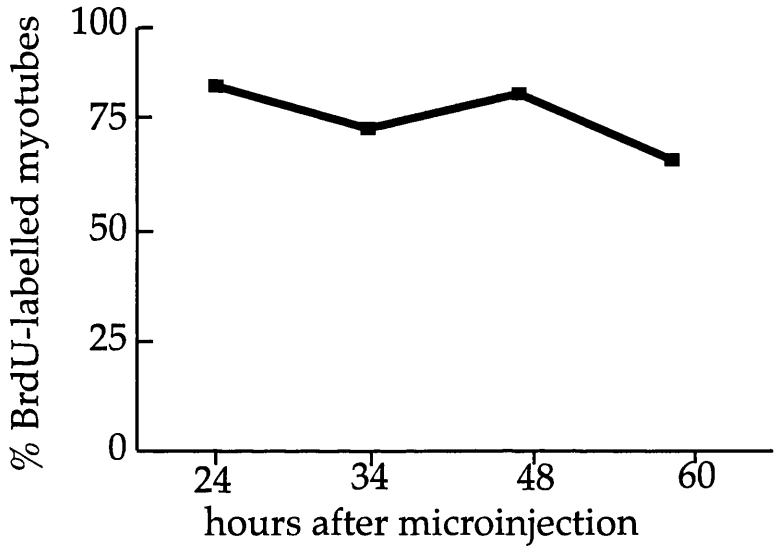


Figure 4.6. T antigen induces DNA-synthesis in C2C12 myotubes. Confluent C2C12 cells were plated on scored dishes and were induced to differentiate by placing in 0.5% FBS DMEM. After 4 days the myotubes were purified by sieving through a 20 μm mesh and microinjected with pSE. BrdU was added to the medium for 24 hours and the cells were fixed at the times indicated. Cells were immunostained for T antigen, BrdU and MHC. At least 70 T antigen expressing and non-expressing myotubes were counted per time point. No BrdU incorporation was observed in myotubes that did not express T antigen.

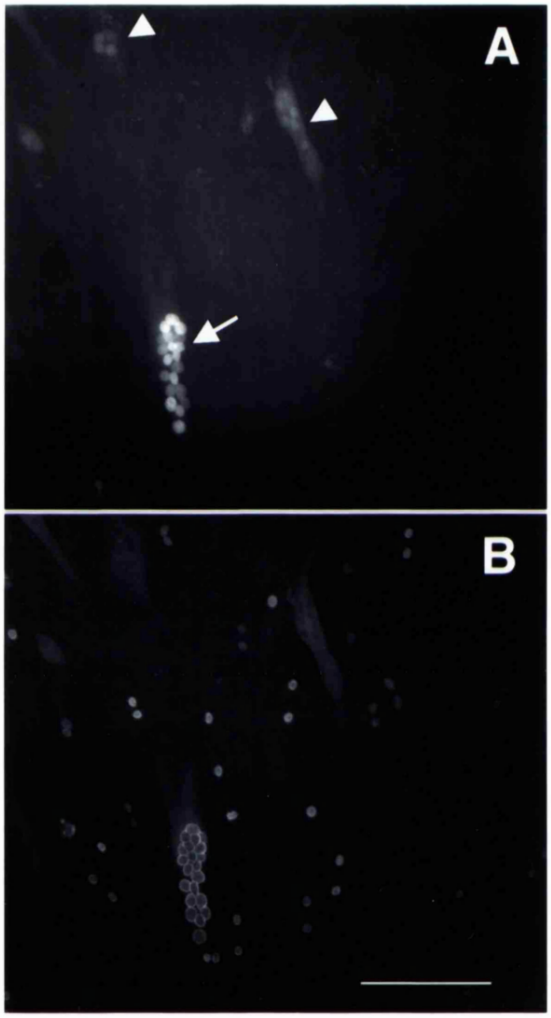


Figure 4.7. T antigen induces DNA synthesis in C2C12 myotubes. C2C12 cells were prepared as in the legend to figure 4.6. (**A**) T antigen staining in myotube nuclei, arrow shows nuclei expressing high levels of T antigen, whereas arrowheads show nuclei expressing lower levels of the protein. (**B**) Same field as A but stained for BrdU. Note many mononucleate cells are positive for BrdU staining, but among the myotubes only the myotube expressing high levels of T antigen, arrowed in A, incorporated BrdU. The scale bar is 50 μm.

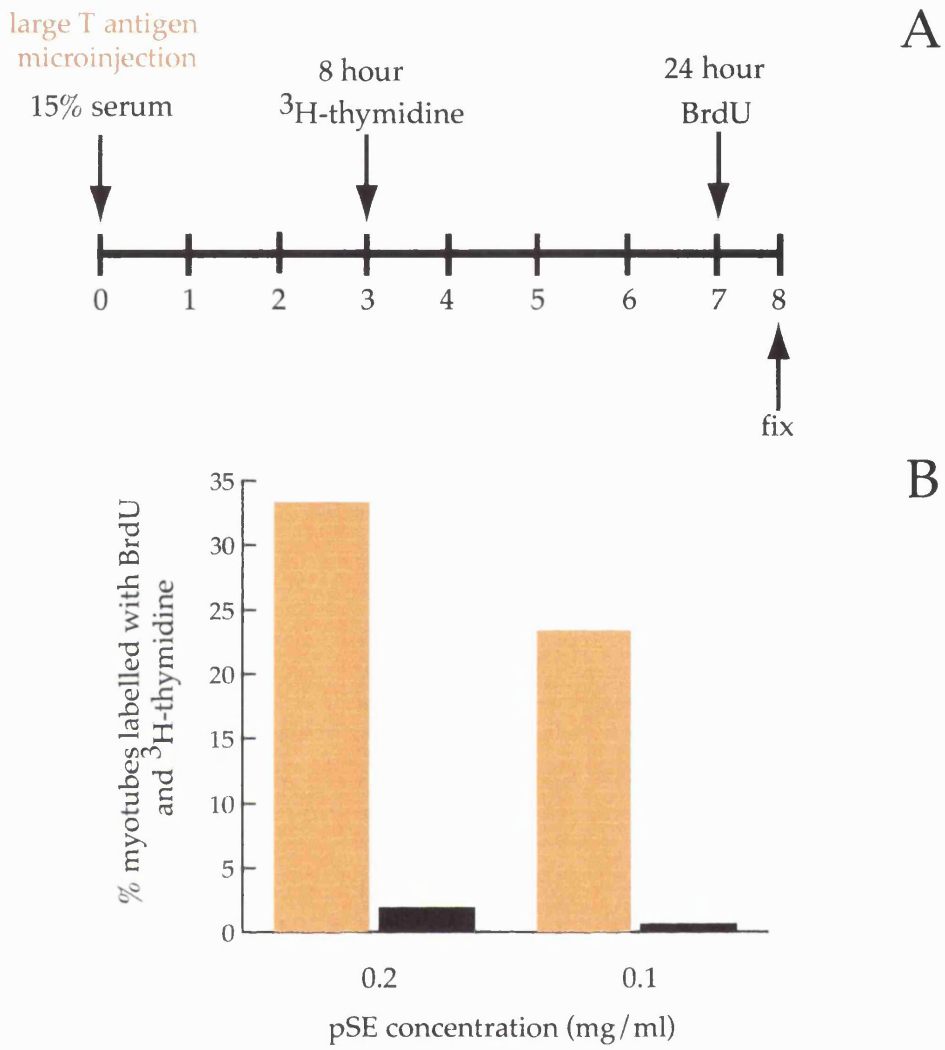


Figure 4.8. T antigen induces endoreplication in A1 myotubes. (**A**) An outline of the experimental design to determine whether myotubes that were in S-phase at 3 days after serum stimulation continue to synthesise DNA at 7 days. Purified myotubes were plated in medium with 15% FCS and were microinjected with the pSE plasmid expressing large T antigen. They were labelled with an 8 hour pulse of [^{3}H]-thymidine after 3 days and rinsed with fresh medium. On day 7, samples were labelled with BrdU for 24 hours and fixed. (**B**) The number of myotubes expressing T antigen and labelled with both BrdU and [^{3}H]-thymidine (orange bars) were compared with the number of double-labelled myotubes not expressing T antigen (black bars). It should be noted that more than 20% of myotubes not expressing T antigen labelled either with [^{3}H]-thymidine or BrdU, but not both. In the absence of T antigen expression, few myotubes synthesised DNA for longer than 96 hours (day 3-7), but T antigen expression resulted in a significant increase in the number of cells synthesising DNA for longer than 96 hours.

cells that had incorporated the thymidine label on the third day after serum stimulation, would have been expected to finish S-phase by the seventh day. One week after serum stimulation myotubes were exposed to a 24 hour BrdU pulse, fixed, stained for MHC and BrdU, and developed for autoradiography. If the nuclei underwent consecutive rounds of DNA synthesis, they would be expected to incorporate both labels. Figure 4.8 B shows the percentage of nuclei that expressed this pattern of labelling as a function of plasmid concentration. At concentrations of 0.2 and 0.1 mg/ml a significant number of T-antigen expressing nuclei incorporated both nucleotide labels as compared to non-expressing myotubes in the same culture. At the lower doses of 0.05 mg/ml and 0.02mg/ml, where T antigen expression was clearly detected in only a minority of nuclei, this response was not observed.

No aberrant nuclear phenotypes were observed in any of the myotubes analysed.

4.8 UV irradiation causes cell cycle arrest and cell death in A1 mononucleate cells and myotubes.

In view of the potential interest of p53 function in newt myotubes, as discussed earlier, I explored the possibility of inducing cell cycle arrest by UV irradiation on both mononucleate cells and A1 myotubes. The p53 protein participates in the response of many cell types to ultraviolet (UV) irradiation.

UV irradiation of A1 mononucleate cells induced cell cycle arrest as ascertained by lack of BrdU incorporation (Figure 4.9). After 24 hours, complete arrest was only observed with the dose of 4500 J/m2. In NIH3T3 fibroblasts, 60 J/m^2 was sufficient to induce complete arrest after 24 hours (data not shown). This dose was effective in arresting A1 mononucleate cells 3 days after irradiation. Cell death was also observed after UV irradiation, as evidenced by a decrease in the number of cells in the culture over time (Figure 4.10).

To determine the response of myotubes to UV irradiation, purified myotubes were exposed to UV at different times before and after serum stimulation. Figure 4.11 shows that the dose of 15 J/m^2 completely inhibited cell cycle re-entry in

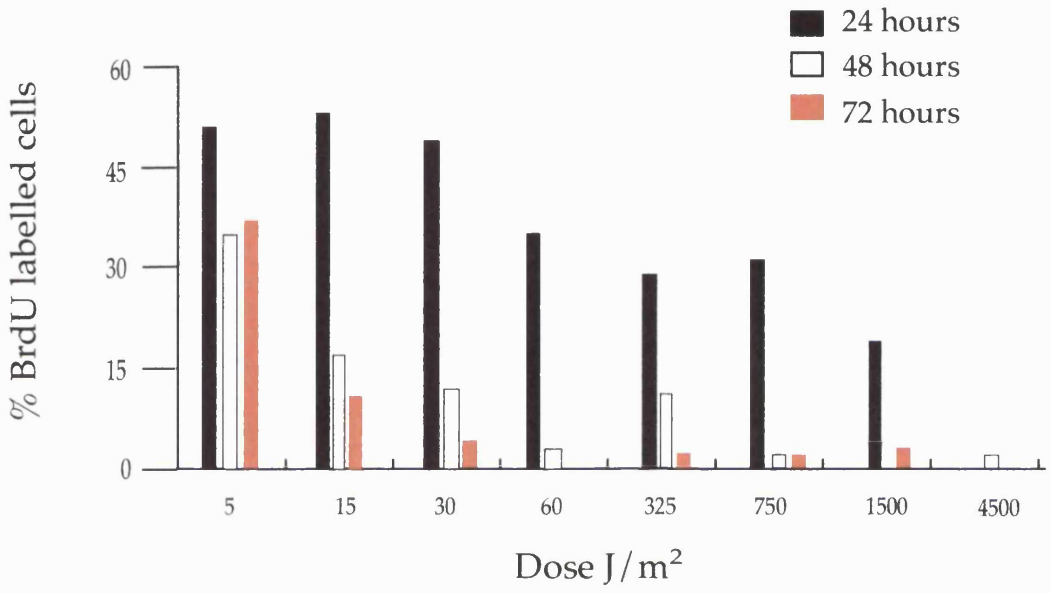


Figure 4.9. Irradiation of mononucleate cells causes cell cycle arrest in a dose and time-dependent manner. Cycling mononucleate cells were UV irradiated with the indicated doses and maintained in medium containing 10% FBS. BrdU was added to the medium for 8 hours prior to fixation. Cells were fixed 24, 48, or 72 hours after irradiation. Note that complete cell cycle arrest at 24 hours was only observed after irradiation with 4500 J/m^2. Inhibition of DNA synthesis was proportional to the dose of irradiation, and irradiation with doses higher than 60 J/m^2 resulted in cell cycle arrest after 3 days.

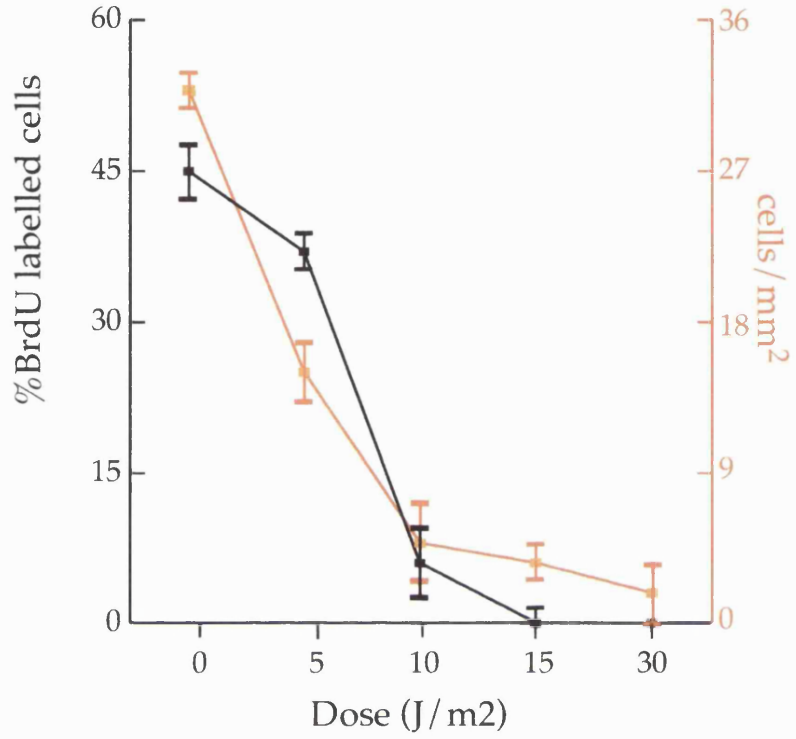


Figure 4.10. UV irradiation of mononucleate cells causes cell cycle arrest and concomitant cell death. Cycling mononucleate cells were irradiated with the indicated doses and maintained in medium containing 15% FBS. BrdU was added to the medium for 16 hours prior to fixation at 96 hours after irradiation. Cells irradiated at 15 J/m^2 arrested after 96 hours. Cell cycle arrest was accompanied by cell death as indicated by the reduction in number of cells on the culture dish relative to non irradiated cells. The experiments were done in triplicate and the points represent the mean ± the standard deviation.

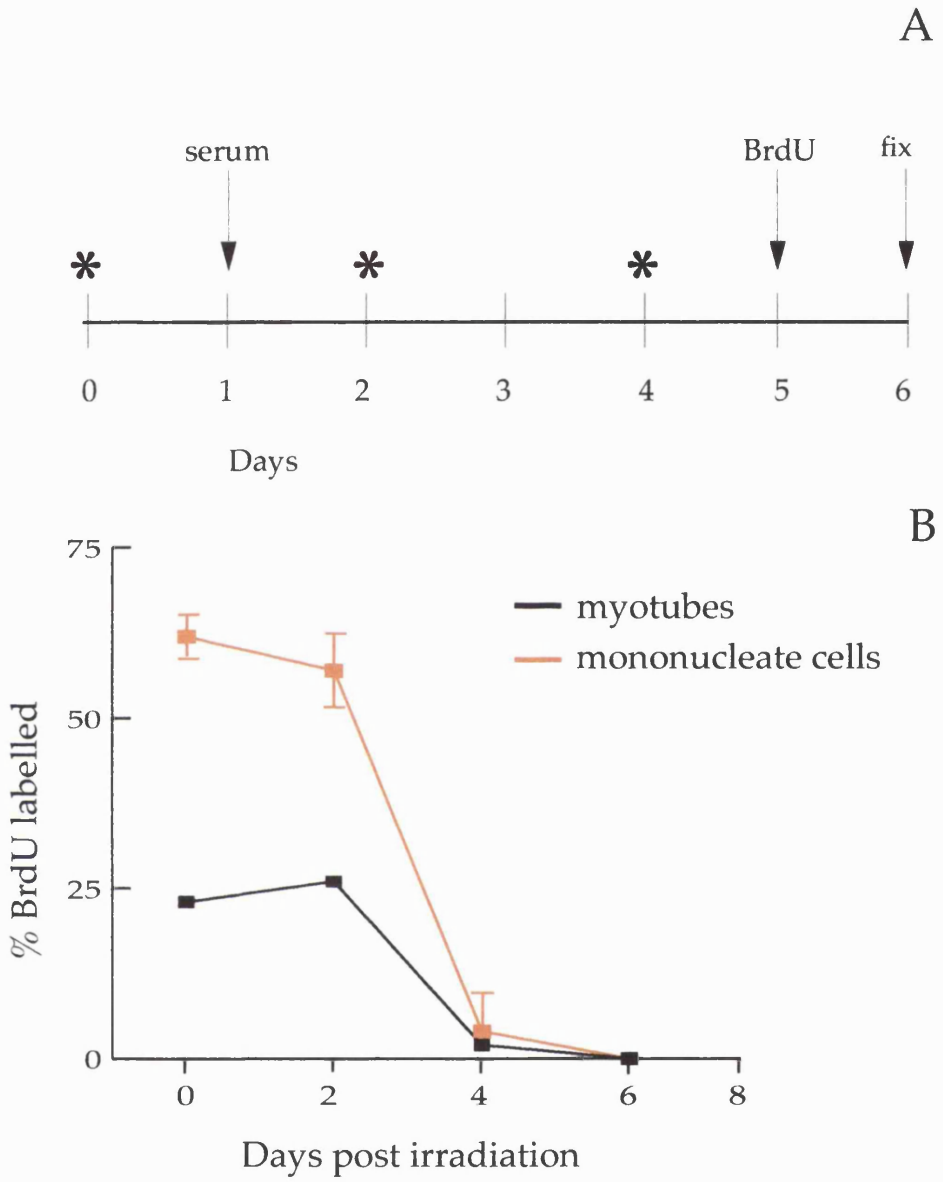


Figure 4.11. UV irradiation inhibits S-phase entry in A1 myotubes. (**A**) An outline of the experimental design to determine whether UV irrradiation inhibits S-phase in newt myotubes. Parallel plates of myotubes were either irradiated (15 J/m^2) and serum stimulated at different time points, as indicated by the asterisks, or only serum stimulated without irradiation. BrdU was added at day 5 and the cells were fixed at day 6 and stained for BrdU and MHC. (**B**) Mononucleate cells and myotubes on the same dish are arrested 4 days after irradiation. The experiments were repeated three times. The error bars on the mononucleate cells denote standard deviation. The black squares give the result of one experiment in which 25% of the control, unirradiated myotubes entered S-phase.

myotubes 4 days after irradiation. A lower dose of 5 J/m^2 had no effect. The myotube response was identical to that of mononucleate A1 cells on the same dish, and the myotubes also began to die 3 to 4 days after irradiation. No myotubes or mononucleate cells were present 10 days after irradiation with 15 J/m^2 of UV, whereas irradiated controls were dense.

The nuclei in the dying mononucleate cells and myotubes that were floating in the medium after irradiation were pycnotic (Figure 4.12), suggesting that cell death occurred by apoptosis, although this possibility was not examined in more detail. I conclude that there is no evidence that myotubes are selectively resistant to the cycle arrest or cell death induced by UV irradiation.

4.9 Discussion

The results presented in this chapter demonstrate that caffeine and SV40 large T antigen do not promote mitosis in newt myotubes. They attest to the stability of the block to M-phase entry in these cells. In addition, differentiation does not confer resistance to UV – induced cell cycle arrest and death.

Caffeine induces aberrant mitoses in newt myotubes

In mammalian cells, caffeine treatment overcomes G2 arrest and induces entry to M-phase by removing inhibitory phosphorylations from the cyclinB-cdc2 kinase (Yamashita et al., 1990; Steinman et al., 1991). Caffeine treatment of serum-stimulated newt myotubes which were arrested in G2 only rarely induced mitotic entry. Instead, myotubes with aberrant and fragmented nuclei were frequently observed (Figures 4.1 and 4.2, Table 4.1). The appearance of fragmented nuclei in serum- stimulated newt myotubes was associated with their distribution in the S and G2 phases of the cell cycle, as reported previously (Tanaka et al., 1997). Myotubes containing G1 nuclei were not sensitive to caffeine treatment, since aberrant nuclear phenotypes were not apparent in contact inhibited myotubes or in myotubes maintained in low serum (Figure 4.2 A). This fragmented phenotype is reminiscent of the pulverised nuclei seen in mammalian cells induced to enter mitosis prematurely by constitutively active cdc2 (Heald et al., 1993).

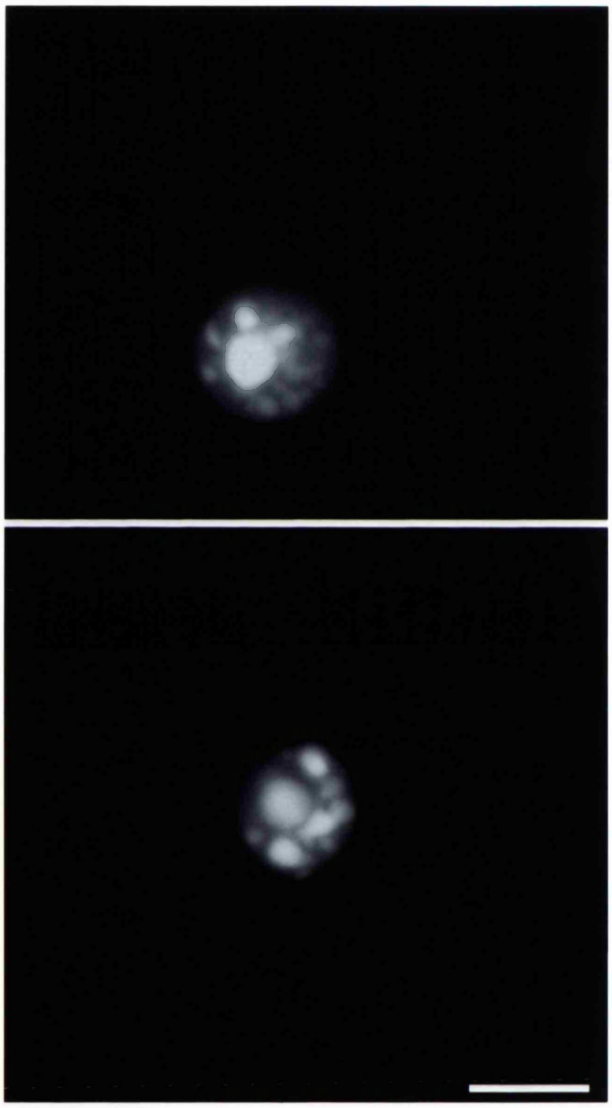


Figure 4.12. Nuclear fragmentation in UV irradiated cells. Cells were treated as in the legend to figure 4.10 and irradiated with a dose of 15 J/m^2. Three days after irradiation, most cells began to die and float in the medium. The Hoechst-stained nuclei in these dying cells are pycnotic and fragmented, and are representative of those observed. Scale bar is 50 μm.

The caffeine sensitivity of the serum-stimulated myotubes, but not of the myotubes with a DNA content of the G1 phase, suggests that inactive newt cyclin B-cdc2 complexes are present in the serum stimulated myotubes. In cycling cells, cdc2 is present throughout the cell cycle, but cyclin B is not present in G1; it is synthesised during S-phase and degraded during the metaphase to anaphase transition (Puri et al., 1999; Pines, 1999). Newly formed cyclin B-cdc2 complexes are kept inactive in late S and in G2 by inhibitory phosphorylations on the kinase subunit (Ohi and Gould, 1999; Pines, 1999; Puri et al., 1999). It is possible that the serum-stimulated newt myotubes synthesise cyclin B when they enter S-phase; in this hypothesis, unstimulated newt myotubes would not contain cyclin B-cdc2 either because they are in G1 or because these proteins are downregulated during myotube differentiation, as occurs in mammalian myogenesis (Okhubo et al., 1994).

Other inferences can be made regarding the regulation of cyclin B and cdc2 in newt myotubes. Myotubes with fragmented nuclei also contain intact nuclei (Figure 4.1 A, C), which suggests that cyclinB-cdc2 activation does not occur in these nuclei. This is consistent with the compartmentalised regulation of cdc2 activity in cycling cells (Heald et al., 1993; Hagting et al., 1998; Jin et al., 1998; Pines, 1999). Active cyclin B-cdc2 complexes may be present in the cytoplasm, but inactivated in some nuclei due to the phosphorylation by the wee1 or chk1 kinases (Heald et al., 1993; Hagting et al., 1998; Kumagai et al., 1998)

Newt myotubes may be susceptible to death resulting from cell cycle progression, as are their mammalian counterparts (Endo and Nadal-Ginard., 1989, 1998; Crescenzi et al., 1995; Latella et al., 2000). The fragmented nuclei observed in serum-stimulated, caffeine treated myotubes may indicate that aberrant mitosis occurred. Myotubes with aberrant nuclei were not observed 72 hours after caffeine treatment, and the number of myotubes present at this time point was reduced relative to the samples treated for shorter times (Figure 4.2 A and B). This indicates that myotubes with fragmented nuclei may degenerate. Caffeine toxicity *per se* is not responsible for reduction in myotube numbers, since samples that were not serum-stimulated and treated with caffeined and samples that were

serum-stimulated but not treated with caffeine contained approximately the same number of myotubes (Figure 4.2 B).

After serum stimulation, not all myotube nuclei progress as far as G2 (Tanaka et al., 1997). The occurrence of fragmented nuclei in caffeine treated, serum stimulated myotubes suggests that nuclei with incompletely replicated DNA entered M-phase. In a multinucleate syncytium, however, normal mitotic spindle formation might not be possible because the coordinate entry to M-phase of closely juxtaposed nuclei would result in pluripolar spindles (Endo and Nadal-Ginard, 1998; Latella et al., 2000); nuclei with a G2 DNA content would undergo fragmentation instead of normal mitosis.

T antigen induces endoreplication in newt myotubes

In mammalian myotubes, SV40 large T antigen induces DNA synthesis and entry to mitosis (Endo and Nadal-Ginard, 1989, 1998). T antigen is also able to overcome G2 arrest in human diploid fibroblasts with incompletely replicated or damaged DNA (Chang et al., 1997). Entry to M-phase, however, is not induced in newt myotubes by T antigen expression. Nevertheless, the pRB binding activity of T antigen is probably intact in newt cells and is sufficient to induce DNA synthesis in the absence of serum stimulation, a context in which pRb is known to be hypophosphorylated and active (Table 4.2; Tanaka et al., 1997).

These observations suggest that functions of large T antigen, other than pRb binding, are required in order to induce mitotic entry, and that the mechanism that operates to induce the G2 arrest in newt myotubes is likely to be pRb independent. In addition to pRb, T antigen also interacts with p107, p130, p300, and p53. In mammalian myotubes, it induces expression of cyclins A and B, cdk2, and cdc2, and inhibits the expression of MyoD family members (Okhubo et al., 1994). If these functions of T antigen are operative in newt myotubes, they are not able to overcome the G2 arrest and induce entry to mitosis.

Other responses of mammalian and newt myotubes to T antigen expression are also different. T antigen expression inhibits fusion of C2C12 myoblasts (Endo and

Nadal-Ginard, 1989), but not of newt A1 cells; T antigen-expressing myotubes form following transfection of mononucleate cells with the pSE plasmid that expresses T antigen constitutively (section 4.5). In the studies carried out on mammalian cells, stably transfected cell lines were used, whereas in the experiments presented here less than 2% of cells expressed T antigen after transfection by particle bombardment. T antigen expression may be required in all the fusion partners for a fusion inhibitory activity to be manifested. In some myotubes that were fixed immediately after purification, T antigen expression was not observed in all of the nuclei (data not shown). Similar results were obtained previously in A1 cells after the expression of the *MSX1* gene which inhibits myogenesis in mammalian myoblasts (Song et al., 1992), but not in newt cells (Crews et al., 1995).

I hypothesised that incomplete differentiation in the stably transfected mammalian cell lines could have occurred in the presence of undetectable amounts of T antigen, due to promoter leakiness, and that this might have affected the results previously obtained (Endo and Nadal Ginard, 1989, 1998) Indeed, microinjection of plasmid DNA into differentiated C2C12 myotubes does not induce the appearance of mitotic figures (Figure 4.7). Neither transfection of myoblasts nor reduction in the concentration of plasmid microinjected into differentiated newt myotubes resulted in induction of mitosis.

T antigen expression does not lead to detectable cell death in newt myotubes. This could be due to a shift to endoreplicative cycles after expression of T antigen as opposed to induction of mitosis; premature entry to mitosis induced by caffeine treatment, on the other hand, seemed to result in cell death.

The evidence supporting a shift to endoreplicative cycles after expression of T antigen is twofold. First and most critically, double labelled nuclei are observed in T antigen expressing myotubes given [^{3}H]-thymidine and BrdU pulses separated by a 96 hour interval. T antigen expressing myotubes therefore continuously synthesise DNA over a period greater than 96 hours (Figure 4.8), but S-phase in newt myotubes normally lasts 48-72 hours (Tanaka et al., 1997). Accordingly,

myotubes that do not express T antigen do not incorporate nucleotide labels for a 96-hour period. Second, after T antigen expression, BrdU incorporation is stable over several days (Figure 4.5). In contrast, a clear peak of DNA synthesis is observed after serum stimulation in myotubes that do not express T antigen (Figure 3.2). The fact that T antigen expression does not alter cell cycle kinetics in mononucleate cells (Figure 4.4) argues against the possibility that T antigen induces a protracted S-phase. It would be necessary to measure the DNA content of T antigen expressing myotubes to establish conclusively that polyploid nuclei are being generated.

In cells which naturally shift from mitotic to endoreplicative cycles, the change is associated with the inability to activate cyclin-B-cdc2 complexes (Datta et al., 1996; MacAuley et al., 1998), as well as with the initiation of waves of cyclin A and E associated activity (Sauer et al., 1995a; MacAuley et al., 1998). If T antigen induces cyclin A and E expression in newt myotubes, as occurs in mammalian myotubes (Okhubo et al., 1994), endoreplication could be induced as a result of the elevated levels of cyclin A/E in a situation where the myotube block to mitosis maintains cyclin B-cdc2 inactive.

It is also possible that T antigen speeds up a natural transition to endoreplicative cycles in the newt myotubes. Indeed there is evidence that nuclei with a greater than 4N DNA content begin accumulating after serum stimulation (Figure 1.5; Tanaka et al., 1997). Evidence for endoreplicative cycles has been reported for *Rb-/-* myocytes (Novitch et al., 1996), myotubes overexpressing E2F transcription factors which induce S-phase entry and cyclin A and E expression (Gill and Hamel, 2000), and myofibres from mice expressing a weak *Rb* allelle in an *Rb* null background (Zacksenhaus et al., 1996). This offers an explanation to why mitotic figures are so rarely observed in myotubes after serum stimulation and caffeine treatment of T antigen expression.

UV irradiation and p53

An involvement of p53 in the block to M-phase entry in newt myotubes is suggested by studies of T antigen expression in senescent fibroblasts. As was

observed in the newt myotubes, in senescent fibroblasts, T antigen induces DNA synthesis without mitosis (Gorman and Cristofalo, 1985; Gire and Wynford-Thomas, 1998). Inactivation of p53 by microinjection of antibodies, on the other hand, leads to mitosis in the fibroblasts. Underlying this difference between T antigen expression and p53 inactivation in fibroblasts may be the regulation of the cdk inhibitor p21. In T antigen expressing fibroblasts p21 levels remain high, but after p53 inactivation, p21 levels are reduced. The p21 gene is a well known transcriptional target of p53 and both p53 and p21 null fibroblasts have impaired G2 arrest responses to DNA damage or inhibition of DNA synthesis (DiLeonardo et al., 1994; Taylor et al., 1999). Although p21 expression is independent of p53 during normal development in mice (Parker et al., 1995; MacLeod et al., 1995), p21 accumulation in most tissues after checkpoint activation, by ionising radiation, depends on p53 (MacLeod et al., 1995; Bunz et al., 1998; Azzam et al., 1997). Additional observations suggest a possible involvement of p21 in G2 arrest. Overexpression of p21 can inhibit entry to mitosis, whereas the absence of p21 results in premature entry to mitosis (Dulic et al., 1998).

The above discussion supports a possible role for p53 in the block to mitotic entry in serum stimulated newt myotubes. This remains to be established, and the UV experiments are a first step in setting up a functional assay for p53 in newt cells. After UV irradiation newt mononucleate cells and myotubes undergo a cell cycle arrest and cell death response that may be p53 dependent. Cell cycle arrest is only observed after 3 days, or one cell cycle, suggesting that the block to DNA synthesis is activated in G1. These responses are observed in cells which undergo arrest and apoptosis in a p53-dependent manner after UV or ionising radiation (Bisonette and Hunting, 1998; Allan and Fried, 1999; Meyer et al., 1999; reviewed in Lakin and Jackson, 1999).

General conclusion

The inability of T antigen to induce mitotic entry and the sensitivity of the serum stimulated myotubes to caffeine treatment suggested to me that cell cycle re-entry activates a strong block to mitosis involving inactivation of cyclin B-cdk1 and possibly p53. In my mind, this undermined the appeal of the model whereby

myotubes give rise to mononucleate cells by entry to M-phase and cytokinesis after implantation into a regenerating limb. It could always be argued that the implanted myotubes are exposed to a blastemal signal that could undermine this block, but my results led me to entertain other possibilities. If an alternative mechanism for generation of mononucleate cells exists, it may not require either cell cycle progression or re-entry. A critical question was therefore to establish whether generation of mononucleate cells following myotube implantation requires cell cycle re-entry. This is addressed in the next chapter.

Chapter 5

Generation of mononucleate cells from cell cycle arrested myotubes

5.1 Introduction

Upon implantation into regenerating limbs, newt myotubes are able to both synthesise DNA and generate mononucleate progeny (Kumar et al., 2000). In culture, myotube nuclei synthesise DNA following serum stimulation, and accumulate with a DNA content corresponding to values for the G2 phase of the cell cycle (Tanaka et al., 1997). Mitotic figures have very rarely been observed in myotubes in culture or *in vivo* (Tanaka et al. 1997; Chapter 4), and attempts to overcome the block to M phase entry in cultured myotubes, as detailed in Chapter 4, suggested that the block is stable. On the other hand, it is possible that implanted myotubes receive signals in the blastema that induce mitosis. Nevertheless, the observation that normal mitosis is not induced in cultured myotubes by caffeine treatment, or by large T antigen expression, led me to question whether cell cycle progression is the mechanism by which mononucleate cells are generated from multinucleate myotubes after implantation. I proposed a model in the Introduction in which mononucleate cells bud from the myotube in the absence of mitosis and cytokinesis. Such a budding mechanism could operate exclusively on nuclei that enter S-phase, or independent of S-phase entry. A critical question is clearly whether cell cycle re-entry is required for generation of mononucleate progeny. The experiments detailed in the following pages address this question by analysis of the fate of cell cycle arrested myotubes in the regenerating limb.

Prior to implantation, cell cycle re-entry was inhibited in the myotubes by two different methods, X-irradiation or expression of the cdk4/6 inhibitor p16. X-irradiation causes cell cycle arrest by activation of DNA damage checkpoints in a variety of cell types (Bae et al., 1995; DiLeonardo et al., 1994; Elledge, 1996; Meyer et al., 1999), whereas p16, a member of the INK4 family, is a specific inhibitor of cdk4/cdk6 and arrests cells in G1 (Sherr and Roberts, 1999). X-irradiated myotubes were labelled by microinjection with a fluorescent lineage tracer, whereas p16 inhibited myotubes were co-injected with a plasmid expressing p16 and one expressing a marker, eGFP. In other experiments myotubes were injected with a bicistronic plasmid expressing both p16 and human placental alkaline

with a bicistronic plasmid expressing both p16 and human placental alkaline phosphatase as a marker. The fluorescent myotubes were implanted into regenerating limbs together with a differentially labelled control population (see Figure 2.4, Chapter 2). The results show that both experimental and control myotubes generate mononucleate progeny upon implantation, thereby excluding a requirement for cell cycle re-entry in the multinucleate to mononucleate transition.

5.2 Results

After myotube implantation into a regenerating limb, mononucleate cells can be observed within 5 days (Kumar et al., 2000), but myotubes are still abundant at this time. After 10 days, few myotubes are present and the mononucleate population predominates. To verify that the generation of mononucleate cells occurred in the absence of cell cycle re-entry, the block to re-entry had to be stable for at least 10 days. As described below, the efficacy of the block to cell cycle progression by X-irradiation and p16 expression was verified by examining cell cycle parameters in culture. Subsequently, the experimental and control myotubes were implanted into limb blastemas and analysed for the presence of mononucleate cells derived from the two implanted populations after 10 days.

5.2 X-irradiation inhibits cell cycle re-entry in mononucleate cells and myotubes for at least 10 days

Mononucleate A1 cells responded to X irradiation by growth arrest in a dose dependent manner (Figure 5.1 A). X-irradiated cells were viable for at least 17 days after irradiation, and DNA synthesis was still inhibited at this time as determined by labelling with BrdU (Figure 5.1 B). X-irradiation was also effective in inhibiting S-phase entry in newt myotubes. Ten days after irradiation with the standard dose of 20 Gray, incorporation of [^{3}H]-thymidine by irradiated myotube nuclei was determined in cells growing in medium containing 15% FBS and was found to be ten fold lower than in unirradiated controls (Table 5.1). This was true

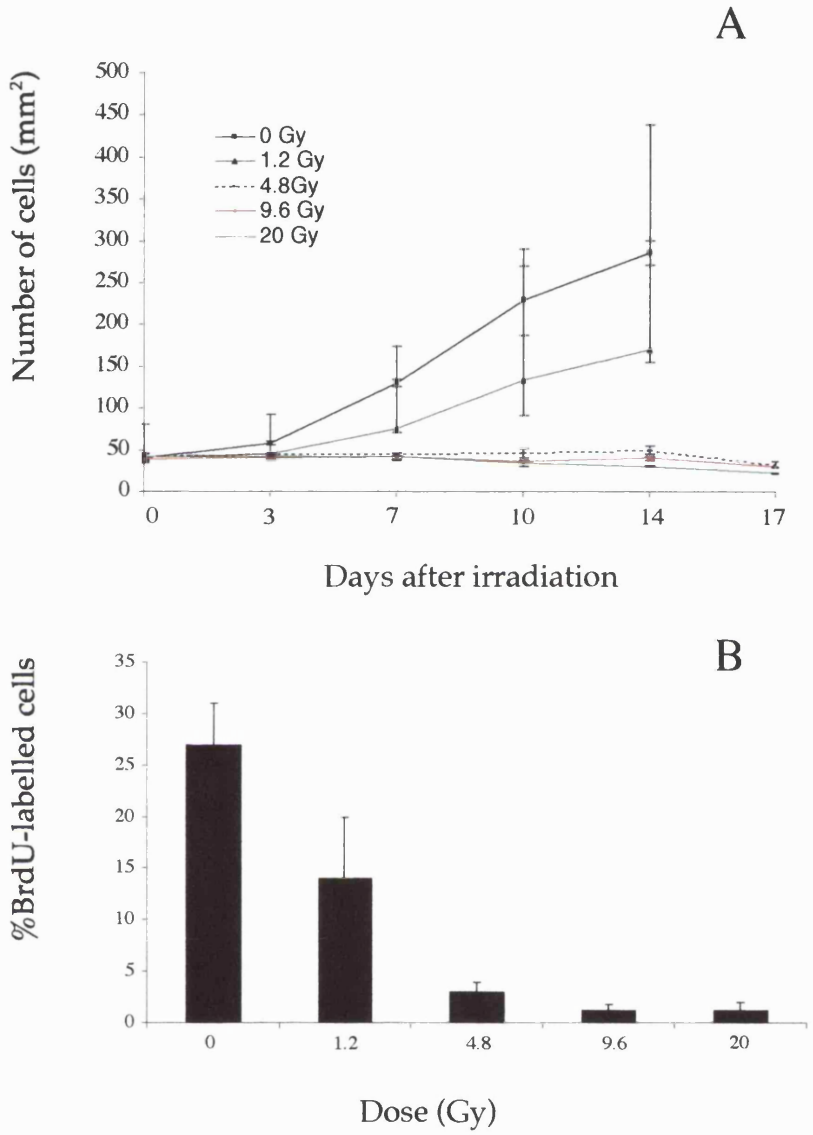


Figure 5.1. A1 mononucleate cells arrest after X-irradiation. Cycling A1 cells were X irradiated at the indicated doses. Adherent cells were counted as detailed in the Material and methods section at the times indicated. Sixteen days after irradiation cells were incubated for 24 hours with BrdU and fixed. (**A**) Population growth curves for various doses of irradiation. Note that after irradiation with 4.8 Gy or greater, cells did not proliferate. (**B**) BrdU incorporation at 17 days after irradiation. The bars represent the standard error of 3 separate experiments.

Table 5.1. X-Irradiation inhibits S phase re-entry by serum stimulated myotubes.

X-ray dose (Gy)	Nuclei in myotubes			Myotubes		
	total	thymidine-labelled	% labelled	total	thymidine-labelled	% labelled
20	532	7	1.3	81	1	1.2
0	506	62	12.2	74	10	13.5

A1 myotubes were purified, irradiated on the following day, and placed in medium containing 15% FBS at 24 hours after irradiation; [^{3}H]thymidine was added after 5 days (see Materials and methods section). The cells were fixed on day 10, and processed for antibody staining to MHC and autoradiography.

The cell cycle arrest was further analysed by measurement of the DNA content of irradiated and control nuclei in mononucleate cells and myotubes. Although this method has an inherent variability because of the Gaussian distribution of DNA content values at different cell cycle phases, the results clearly demonstrated that the irradiated and unirradiated populations were different. FACS analysis could not be used in this case because of the syncytial nature of the myotube. The DNA content was determined by quantitative microscopy after fluorescent labelling of nuclei with Hoechst 33258 (see Materials and methods for detailed description).

In Figure 5.2, the profile of a cycling mononucleate population was compared with that of an irradiated population at ten days after irradiation. It can be seen that the profiles were significantly different; the unirradiated nuclei were distributed in all phases of the cell cycle, whereas most of the irradiated nuclei were in the G1 compartment. In addition, no irradiated cells labelled with the anti phospho-H3 antibody were observed. The phospho-H3 antigen is only present in newt cells from late G2 to late anaphase (see Materials and methods). In Figure 5.3, the DNA content profile of nuclei in irradiated myotubes is compared to that of nuclei in unirradiated myotubes and nuclei in myotubes maintained in low serum. As reported previously, nuclei in serum-stimulated myotubes entered S-phase and accumulated in G2 (Tanaka et al., 1997), but nuclei in irradiated myotubes or in myotubes maintained in low serum did not.

Additional experiments confirmed these observations; the number of irradiated myotube nuclei found in the S+G2 compartments was at least ten-fold less than in unirradiated controls (Figure 5.4). X-irradiation was therefore an effective block to S-phase re-entry and an absolute block to M phase entry.

5.3 X-irradiated myotubes give rise to mononucleate progeny in the blastema

X-irradiated myotubes were microinjected with fluorescein-dextran and implanted into 5 animals, together with a control population microinjected with Texas red-dextran (TR-dextran). In one experiment (2 animals) the labels were reversed with the same results, and the data are therefore presented as the

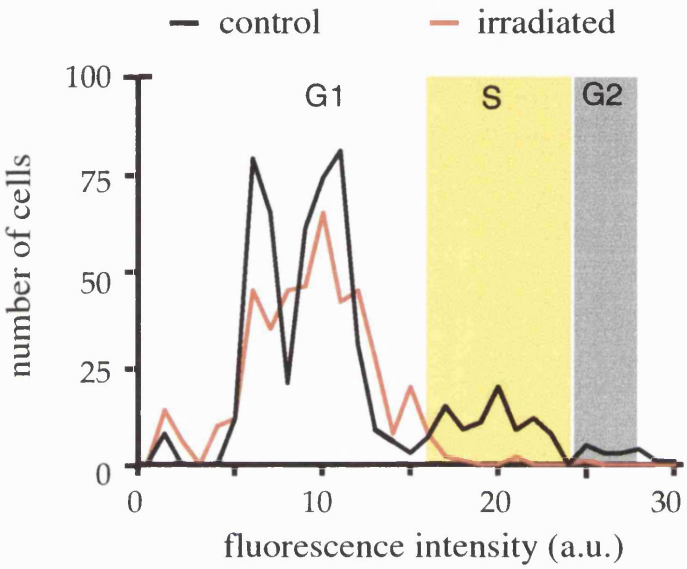


Figure 5.2. X-irradiated, cultured mononucleate A1 cells are blocked in G1. Control A1 mononucleate cells (black), maintained in medium containing 15%FBS, have DNA content distributed between G1, S and G2 values, with 15.7% of cells in the S+G2/M compartment (1.4% in G2/M), compared to only 3% in a parallel irradiated population (red; 0.2% in G2/M). The G2 DNA compartment was determined from the mean value of 10 phospho-H3 positive cells; its distribution is highlighted in grey and labelled G2. The G1 and S phase values were determined as described in the Material and methods section. The area corresponding to cells in S phase is highlighted in yellow. A total of 558 nuclei were analysed in the control population and 434 in the irradiated case.

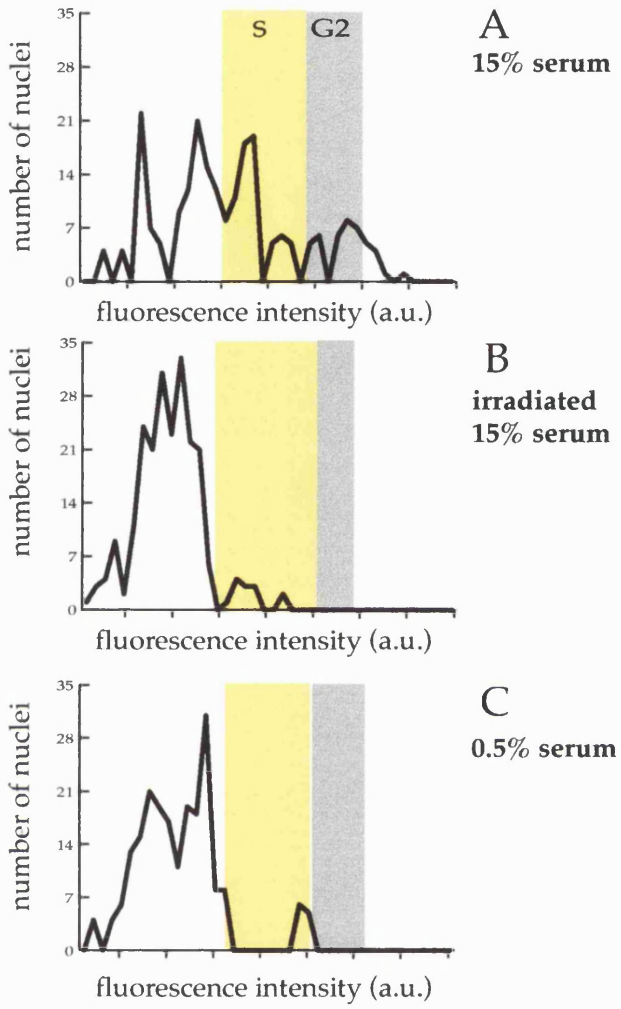


Figure 5.3. X-irradiated A1 myotubes in 15% serum are blocked in G1. Myotubes were prepared as described in the Material and methods section, purified and irradiated with a dose of 20 Gy. After 24 hours, the myotubes were placed in medium containing 15% FBS. (**A**) Serum-stimulated control myotube nuclei have DNA content distributed between G1, S and G2 values, with 42.9% of cells in the S+G2/M compartment. (**B**) Irradiated, serum-stimulated myotube nuclei have DNA content predominantly in the G1 compartment (95.2%). (**C**) Nuclei of myotubes maintained in 0.5% FBS have DNA content predominantly in the G1 compartment (90.8%). The DNA content of 226 nuclei was measured in A, 224 in B, and 205 in C. The G2 values were determined from at least 10 phospho-H3 positive nuclei in a reference cycling population. S and G1 cut off values were determined as indicated in the Material and methods section. S values are highlighted in yellow and G2 values in grey.

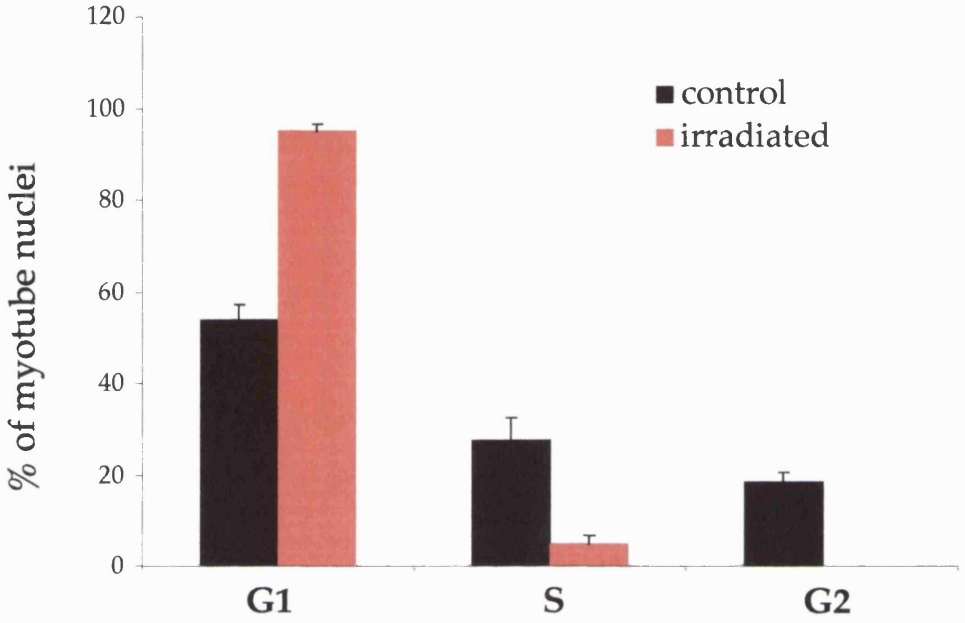


Figure 5.4. Irradiated myotubes in high serum are arrested in G1. Myotubes were prepared as in the legend to Figure 5.3. The G2 values for each experiment were determined from 10 phospho-H3-labelled nuclei in a cycling mononucleate population. S and G1 values were determined as described in the Material and methods section. For control myotubes, the values were determined for 3 separate experiments. For irradiated myotubes values were determined in 4 separate experiments. At least 200 nuclei were measured in each population per experiment. In irradiated myotubes 95 $\pm$ 1.8% of the nuclei were in G1 and none entered the G2 compartment. In control myotubes 54 $\pm$ 3.5 % were in G1, 28 $\pm$ 4.8% were in S, and 18.5 $\pm$ 2.4 % were in G2. The error bars are the standard deviation of the mean.

aggregate of 7 animals. The number of nuclei in each labelled cell was determined before and after implantation and is shown in Figure 5.5. The majority of cells contained more than three nuclei before implantation (Figure 5.5 A) but most of the labelled cells observed after implantation were mononucleate cells, for both irradiated and unirradiated samples (Figure 5.5 B). Approximately equal numbers of nuclei in the two populations were implanted, but the mononucleate progeny of the irradiated myotubes was recovered at 20% of the level of the control myotubes (Figure 5.5 B). Representative sections showing labelled mononucleate cells in the implanted limb blastemas are shown in Figure 5.6.

5.4 Mononucleate cells generated from irradiated myotubes do not proliferate

To test whether the mononucleate cells derived from the irradiated population were themselves arrested, irradiated and unirradiated myotubes were labelled with TR-dextran and implanted separately into regenerating limbs. After 9 days, the animals were injected with BrdU, as described by Kumar et al. (2000), and 24 hours later the limbs were harvested and processed for BrdU staining. The number of nuclei in each labelled cell was determined, before and after implantation, as described above (legend, Figure 5.2). The number of nuclei recovered from the irradiated population after implantation was 25% of control, and was comparable to the double-labelling implantation experiment described in section 5.2. Mononucleate cells derived from irradiated myotubes did not cycle, as evidenced by the low level of BrdU incorporation in these cells compared to unirradiated controls and resident blastemal cells (Figure 5.7, Table 5.2).

5.5 Expression of p16 inhibits cell cycle re-entry in cultured myotubes for at least 10 days

Myotubes were purified and microinjected with a mixture of 0.2 mg/ml pSG5-eGFP and 0.3 mg/ml PTL1-p16 or with the bicistronic plasmid pCAP-p16 0.5 mg/ml (expressing both p16 and alkaline phosphatase). In all plasmids, the expression of the marker and of p16 was under control of the SV40 promoter. The myotubes were placed in medium containing 15% FBS and [^{3}H]-thymidine, 24 hours after microinjection, and labelled continuously for 10 days. Table 5.3 shows

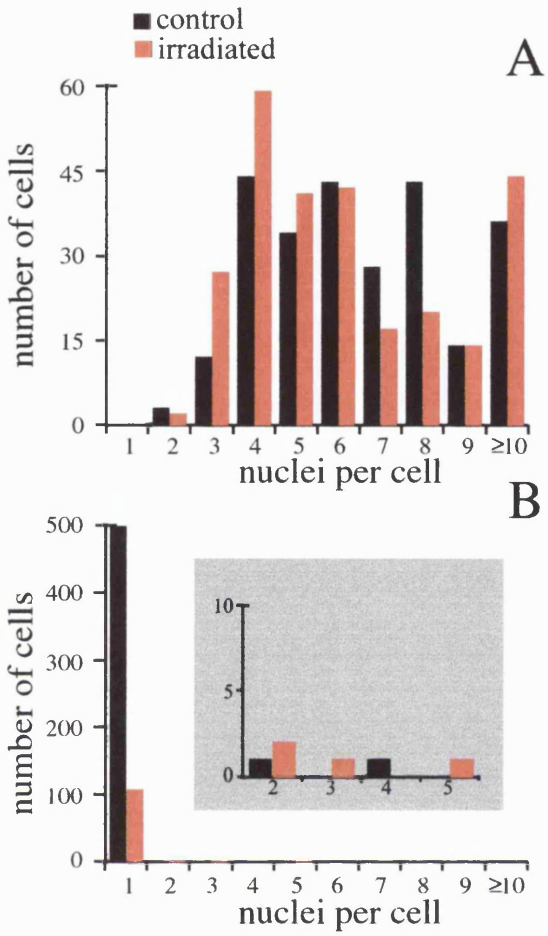


Figure 5.5. Irradiated myotubes give rise to mononucleate cells after implantation into a blastema. A1 myotubes were purified, irradiated and microinjected with fluorescein-conjugated dextran as described in the Materials and methods section (red bars). Unirradiated myotubes were injected in parallel with TR-conjugated dextran (black bars). The distribution of nuclei in the injected populations was determined before implantation and is shown in (**A**). The two populations were mixed and equal numbers of nuclei were implanted into blastemas; a total of 1360 nuclei for control and 1317 for irradiated myotubes were implanted into 7 animals. The limbs were harvested after 10 days and sectioned at 80 µm. Sections were stained with Hoechst, analysed for dextran-labelled cells, and for the number of nuclei in labelled cells. The aggregate data is presented in (**B**); the inset has a contracted scale in order to show residual multinucleate cells. In other experiments, irradiated and control myotubes were injected with TR-conjugated dextran before implantation as separate populations; mononucleate cells were obtained in aggregate from the two populations at a ratio comparable to that shown here (Table 5.2; data not shown).

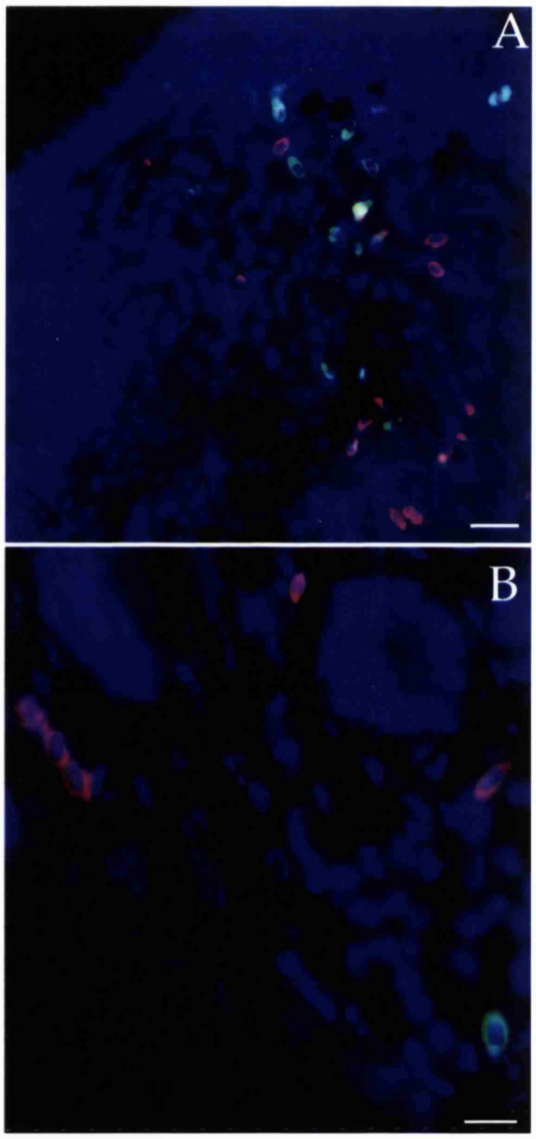


Figure 5.6. Sections of implanted blastemas containing mononucleate cells derived from irradiated and control myotubes. (**A**) Section through a blastema analysed 10 days after implantation of irradiated (green) and control (red) myotubes. The myotubes were labelled by injection of dextrans as described in the legend to Figure 5.5. (**B**) Parallel section to A at higher magnification showing a residual TR-labelled myotube containing 3 nuclei, in addition to mononucleate cells. The scale bars are (A) 100 µm and (B) 50 µm.

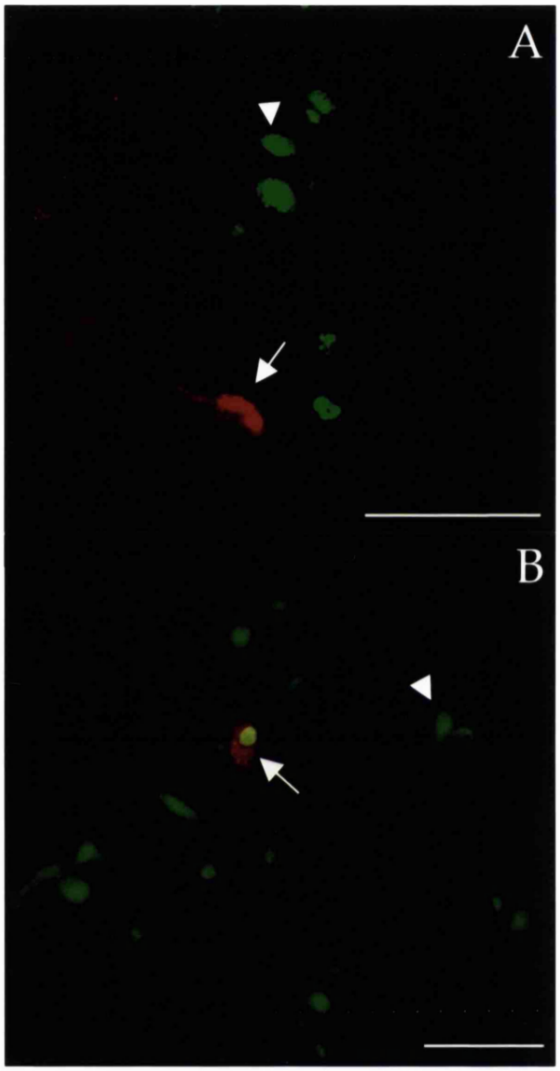


Figure 5.7. Mononucleate cells derived from irradiated myotubes do not incorporate BrdU. Myotube implantation and BrdU labelling are as in the legend to Table 5.2. (**A**) Section through a blastema analysed 10 days after implantation of irradiated myotubes microinjected with TR-dextran. The animal was injected intraperitoneally with BrdU 24 hours prior to harvesting of the blastema. The nucleus in the dextran labelled mononucleate cell did not incorporate BrdU (arrow), but the resident cells in the blastema did (arrowhead). (**B**) Section through a blastema implanted with TR-dextran labelled myotubes which were not irradiated. The nucleus of the dextran-labelled mononucleate cell incorporated BrdU (arrow) as did the resident cells of the blastema (arrowhead). The data is quantitated in Table 5.2. Scale bars are 100 μm.

Table 5.2. Mononucleate cells derived from implantation of irradiated myotubes are arrested from the cell cycle.

X-ray dose (Gy)	Blastemas implanted (N)	mononucleate cells recovered	BrdU-labelled cells	% labelled
20	6	207	2	0.96
0	5	821	120	14.62

A1 myotubes were purified, irradiated and microinjected with TR-conjugated dextran as tracer. The number of nuclei in injected cells was determined, and the cells were implanted into regenerating limbs. After injection with BrdU (see the Materials and methods section) the limbs were harvested and processed for BrdU immunohistochemistry with a fluorescein-conjugated secondary antibody. The labelling of resident nuclei in the blastemas was found to be approximately 15-25%, comparable for implants of irradiated and control cells. A total of 2952 nuclei from labelled irradiated cells and 3140 nuclei from control myotubes were implanted.

Table 5.3. Expression of p16 blocks cell cycle re-entry in serum stimulated A1 myotubes.

Plasmid	Nuclei in myotubes		
	total	thymidine-labelled	% labelled
PTL1-p16	464	0	
uninjected	1572	294	18.7
pCAPp16	467	4	0.8
uninjected	1641	430	26.2
pCAPp16*	1128	10	0.9
untransfected	1470	233	15.8

Myotubes were purified and microinjected with plasmids encoding p16 and eGFP or pCAPp16. *Myotubes were transfected by particle bombardment with pCAPp16 as described in the Material and methods section. After 24 hours the myotubes were stimulated in medium containing 15% FBS with [^{3}H]thymidine for ten days, with change of medium every 3 days. After immunolabelling for MHC, the plates were processed for autoradiography. In control experiments myotubes microinjected with a marker plasmid have the same labelling index after stimulation as parallel uninjected myotubes, as reported previously (Tanaka, 1997).

that nuclei in eGFP-p16 expressing myotubes did not incorporate thymidine, compared with 19% of the nuclei in control myotubes. Incorporation of [^{3}H]-thymidine in the nuclei of myotubes expressing alkaline phosphatase-p16 was also drastically inhibited (Table 5.3). All of the cells expressing markers were differentiated myotubes as assessed by labelling with MHC (not shown).

Co-expression of p16 and eGFP was assayed in myotubes by fluorescent immunocytochemistry, as described in the Materials and methods, and found to be 100% (n=107). Co-expression of alkaline phosphatase and p16 was assayed in mononucleate cells, also by fluorescent immunocytochemistry, and found to be 97% (n=86), in myotubes it was 98.7% (n=33).

The microinjection of the eGFP and p16 plasmids resulted in complete inhibition of S-phase entry and co-expression of both proteins in effectively all of the cells. Most of the experiments with p16 expression were therefore carried out with this system. Nevertheless, the results obtained with the alkaline phosphatase system are also presented, as they corroborate the findings with eGFP.

5.6 Myotubes expressing p16 give rise to mononucleate cells in the blastema

Myotubes were purified and microinjected with a mixture of pSG5-eGFP and PTL1-p16. Myotubes expressing eGFP-p16 were implanted together with a control population injected with TR-dextran, and analysed 10 days after implantation. Figure 5.8A shows the eGFP-p16 myotubes and TR-dextran labelled myotubes before implantation, while mononucleate cells, in sections of the implanted blastemas, expressing the eGFP and dextran labels can be seen in Figure 5.8B. The number of nuclei in eGFP-p16 expressing and dextran-labelled cells was determined before and after implantation and is shown in Figure 5.9. Mononucleate cells were generated from both myotube populations, but cells derived from eGFP-p16 expressing myotubes were recovered at a level of approximately one third of control (Figure 5.9B). The expression of eGFP has no effect on the generation of mononucleate cells by implanted myotubes, as

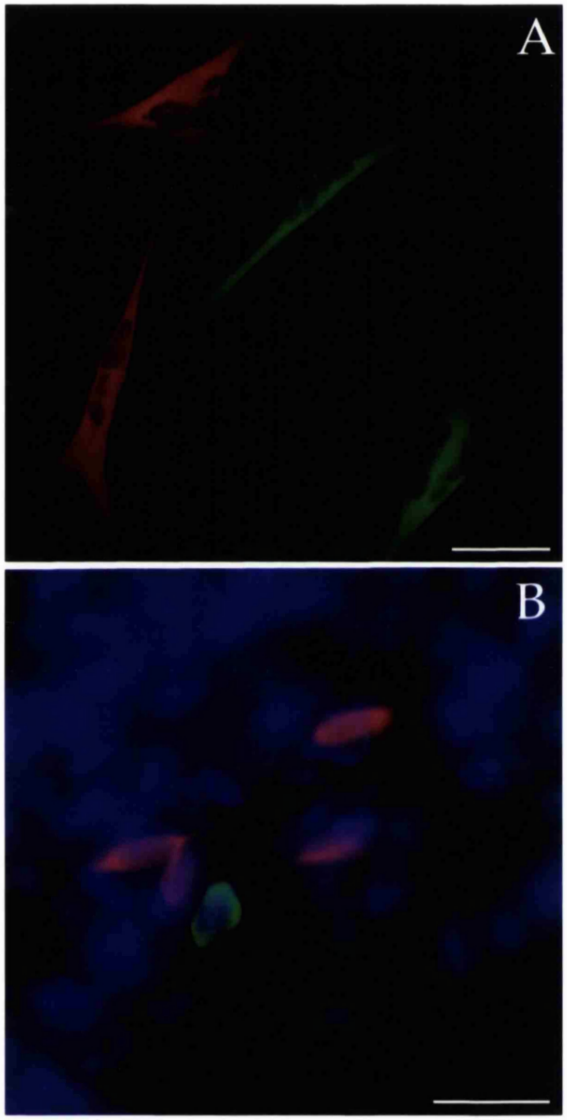


Figure 5.8. Control and p16–expressing myotubes give rise to mononucleate cells after implantation. (**A**) An image of cultured myotubes under fluorescence optics after microinjection of p16 + eGFP plasmids (green), or TR-conjugated dextran (red), at 24 hours after injection. (**B**) Section of implanted blastema showing mononucleate cells either labelled with dextran (red) or expresing eGFP-p16 (green). The scale bars are (A) 100 μm and (B) 50 μm.

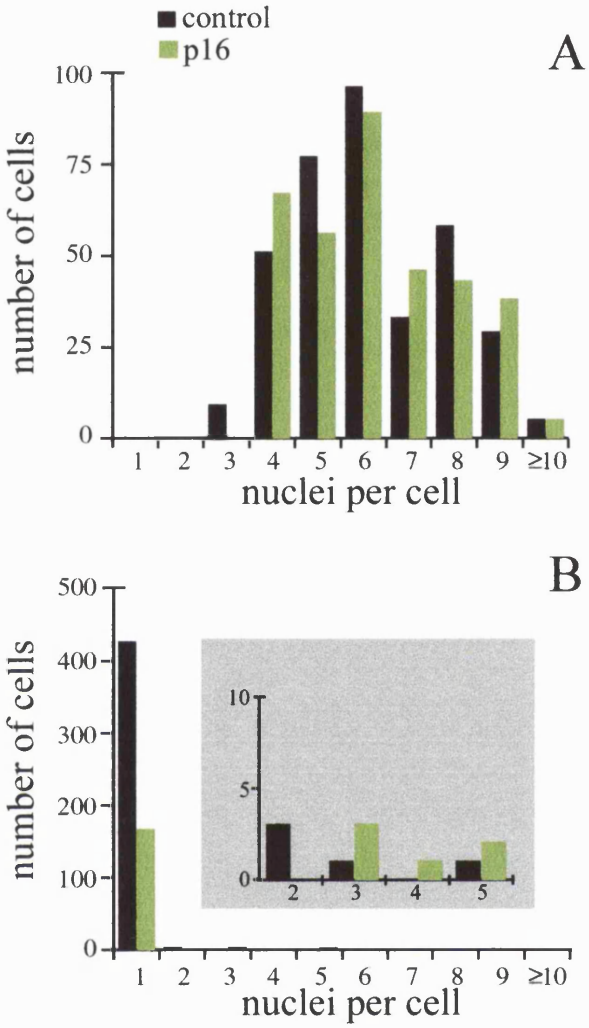


Figure 5.9. Myotubes expressing p16 give rise to mononucleate cells after implantation. Myotubes were purified and microinjected either with TR-conjugated dextran (black bars) or a mixture of plasmids expressing p16 and eGFP (green bars). (**A**) The distribution of nuclei in the injected populations was determined before implantation is shown. Note the absence of cells containing one or two nuclei. (**B**) The aggregate distributions for the two populations post-implantation are shown; the inset has a contracted scale to show residual multinucleate cells. The two populations were mixed and a total of 2296 nuclei for control myotubes, and 2187 for p16-expressing myotubes, were implanted into 11 animals of which two were lost and one was found subsequently not to contain labelled cells. The sections were stained with Hoechst and analysed for dextran-labelled and eGFP-labelled cells, and for the number of nuclei in labelled cells.

assessed in control experiments with microinjection of the eGFP expression plasmid into myotubes followed by implantation (data not shown).

Comparable results were obtained with the use of the alkaline phosphatase marker. Mononucleate progeny were recovered from p-16 expressing myotubes at 37% of the level recovered from control myotubes (Table 5.4). An example of an alkaline phosphatase expressing myotube after microinjection in culture is shown in Figure 5.10 A, and labelled cells in sections of the implanted blastema are shown in Figure 5.10 B.

5.7 Discussion

The results presented in this chapter demonstrate that generation of mononucleate cells from myotubes can occur in the absence of cell cycle re-entry. Three critical aspects of these experiments can be noted. First, the block to cell cycle re-entry was effective and stable, as demonstrated in myotubes expressing eGFP and p16 (Table 5.3). Second, no labelled mononucleate cells were implanted (Figures 5.5A and 5.9A). Third, labelled mononucleate cells were present after the implantation (Figures 5.5B and 5.9B).

X-irradiation completely inhibited entry to mitosis in A1 cells as evidenced by both the lack of staining for phospho-H3 in the irradiated mononucleate cells, and the absence of nuclei in the G2/M compartment for both irradiated mononucleate cells and myotubes (Figure 5.2, 5.3, 5.4 and Table 5.1). After X-irradiation, however, approximately 1% of the myotube nuclei were seen to label with [^{3}H]-thymidine, and it could be suggested that these were the nuclei which gave rise to mononucleate cells after implantation. One result which argues against this interpretation, however, is that 7.5% of the irradiated nuclei were recovered as mononucleate cells (Figure 5.5B), a number too high to be attributed exclusively to nuclei that had entered S-phase. This would also be true if the 1% of nuclei that labelled with [^{3}H]-thymidine had gone through one cell division. Second and critically, the mononucleate cells that were derived from irradiated myotubes were arrested, as discussed below.

Table 5.4. Myotubes expressing alkaline phosphatase and p16 generate mononucleate progeny.

Plasmid	Blastemas analysed	Labelled myotubes implanted	Recovered after implantation	
			myotubes	mononucleates
pCAPp16	2	86	3	54
pCAPp16*	3	111	17	144
pCAP	4	133	11	362
pCAP*	8	404	30	708

Myotubes were purified and microinjected or transfected by particle bombardment (*) with the plasmids pCAP or pCAPp16. For myotubes transfected by particle bombardment, the mononucleate cells were removed from the culture with a micromanipulator prior to implantation as detailed in the Materials and methods section. The total number of myotubes implanted was counted. A sample was fixed and stained for alkaline phosphatase, and the number of transfected myotubes that was implanted was estimated from the transfection efficiency and the total number of myotubes implanted. Blastemas were harvested 10 days after implantation and processed for the hystochemical detection of alkaline phosphatase.

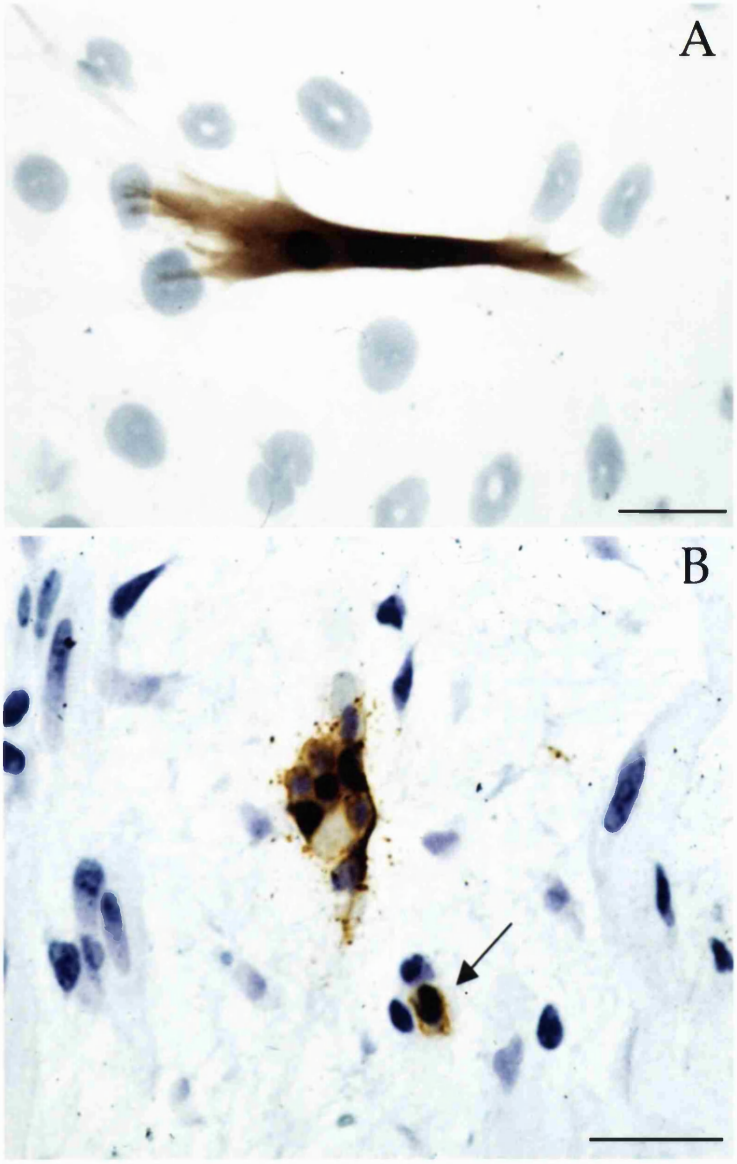


Figure 5.10. Alkaline phosphatase-p16 expressing myotubes give rise to mononucleate cells after implantation. Myotubes were purified, microinjected with pCAPp16, and implanted into regenerating limbs. After 10 days the regenerates were harvested, sectioned, and processed for alkaline phosphatase staining as described in the Material and methods section. (**A**) An image of a cultured myotube in bright field optics stained for alkaline phosphatase, 3 days after injection of the plasmid. (**B**) Section of implanted blastema showing mononucleate cell (arrow) and a multinucleate cell expressing alkaline phosphatase. The scale bars are (A) 50 μm and (B) 100 μm.

The evidence for cell cycle arrest in the mononucleate progeny of irradiated myotubes is twofold. First, they incorporate BrdU at a level comparable to the level of [^{3}H]-thymidine incorporation of the irradiated myotubes in culture. This level is at least ten fold lower than that of controls (Tables 5.1 and 5.2). If all the mononucleate progeny were derived from nuclei that had entered S-phase, they would have been expected to label to the same extent as cells from unirradiated myotubes. Second, three times fewer mononucleate cells were recovered from irradiated than from unirradiated myotubes (Figure 5.5). The cell cycle time of blastemal cells is estimated to be 48-72 hours, as is that of A1 cells and myotubes in culture (Wallace and Maden, 1976; Tanaka et al., 1997). Therefore, in the ten days that elapsed between the implantation and harvest, the cells could have undergone a maximum of three to five population doublings. If the mononucleate progeny of the irradiated myotubes was arrested, the number of cells recovered was expected to be only one third to one fifth of the number of the cycling progeny of unirradiated myotubes. This was indeed the case as shown in Figure 5.5 B.

The results obtained after X-irradiation were supported by the fact that the eGFP-expressing myotubes, which co-expressed p16, also generated mononucleate progeny after implantation. Under the conditions examined here, the block to cell cycle re-entry in eGFP-p16 expressing myotubes was complete (Table 5.3). Furthermore, the eGFP-labelled mononucleate population was roughly one third the number of the TR-dextran-labelled control population implanted at the same time (Figure 5.9B). An extension of the analysis made for the irradiated myotubes above leads to the conclusion that eGFP-positive mononucleates co-expresing p16 were also arrested.

The conclusion that cell cycle re-entry is not required for the generation of mononucleate cells raises the issue of why entry to S-phase is so widespread both in culture, and after implantation (Kumar et al., 2000). Indeed, the observation that DNA synthesis is induced only in differentiated myotubes specifically by a thrombin or plasmin generated activity suggests a role for S-phase entry in

myotube plasticity. In the newt myotubes, S-phase entry is dependent on phosphorylation of pRb (Tanaka et al., 1997). In mammalian myotubes, pRb is required not only for stable cell cycle withdrawal, but also for expression of genes at late stages of muscle differentiation (Novitch et al., 1996, 1999). It is therefore possible that inactivation of pRb and consequent S-phase re-entry is somehow linked to reversal of differentiation.

It is conceivable that the multinucleate to mononucleate transition can be regulated by differential gene expression without a requirement for DNA synthesis. In myotube-fibroblast heterokaryons, muscle gene transcription can be induced in the absence of DNA synthesis (Chiu and Blau, 1984). Moreover, cell cycle withdrawal precedes the upregulation of many genes during myogenesis (Andres and Walsh, 1996). It appears therefore that novel gene and protein expression can occur in stably arrested cells.

The results presented in this chapter, as well as the discussion above, point to a mechanism whereby mononucleate cells are generated by a process of budding independent of DNA synthesis and subsequent mitosis, illustrated schematically in Figure 5.11. Such a mechanism has been observed for cells of the avian osteoclast lineage microinjected with a lineage tracer. In culture, these generate labelled mononucleate cells by budding in the presence of colchicine, a mitotic inhibitor (Solari et al., 1995). Dr. Anoop Kumar has recently observed, in this laboratory, that budding occurs in cultures of primary myofibres from axolotl larvae in the absence of DNA synthesis. I have microinjected these myofibres with the lineage tracer TR-dextran, and we have observed the appearance of labelled mononucleate cells in the culture (unpublished results).

Recent observations in mammalian myotubes also support the budding mechanism. Treatment of C2C12 myotubes with myoseverin, a substituted purine, induces scission of myotubes into viable mononucleate progeny, without concomitant cell cycle re-entry (Rosania et al., 2000). Treatment with myoseverin is also associated with upregulation of a variety of genes required for wound

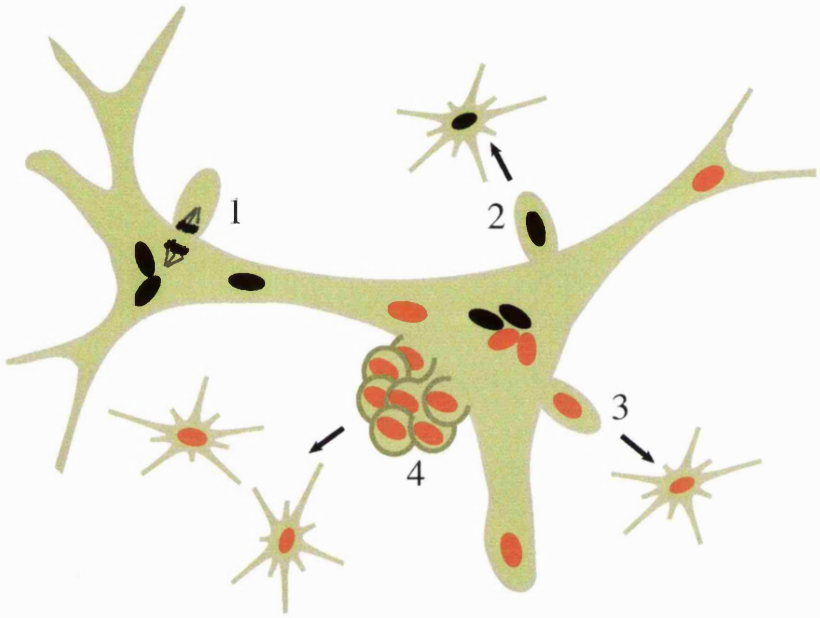


Figure 5.11. Schematic diagram of budding mechanisms that could generate mononucleate progeny from a multinucleate myotube. Nuclei which have re-entered the cell cycle are shown in black, while nuclei in G0 are in red. (**1**) Budding is coupled to mitosis and cytokinesis. The results presented here show that if it operates at all, this cannot be the sole mechanism. (**2**) Budding occurs on nuclei in S phase – this may well take place but the association is not obligatory. (**3**) Budding occurs on nuclei which have not yet entered S phase – the present results establish this possibility. (4) A variant of (3) in which budding occurs on clusters of myotube nuclei. Such "cauliflower" structures were described in the generation of mononucleate cells from osteoclast-like multinucleated giant cells (Solari, 1995), and have been observed in myofibres *in vitro* (Kumar, unpublished).

healing, including matrix metalloproteases, which are upregulated in the regeneration blastema (Miyazaki et al., 1996; Yang and Bryant, 1994), as well as with changes in the tubulin cytoskeleton. Myofibres are compartmentalised cells (Pavlath et al., 1989; Ralston and Hall, 1989) and can respond to local changes in extracellular matrix organisation by localised membrane events (Rossi et al., 2000). For example, receptor declustering in the neuromuscular junction leads to nuclear migration, which involves cytoskeletal changes (Brösamle and Kuffler, 1996). A localised mechanism of budding of nuclei induced by receptor mediated signals in response to the changing extracellular environment is therefore conceivable.

DNA synthesis, although not required for production of mononucleate cells may play a role in other aspects of cell plasticity, such as reversal of differentiation or transdifferentiation. In the jellyfish *Podocoryne*, transdifferentiation of striated muscle into smooth muscle does not require DNA synthesis, but transdifferentiation into neuronal cell types does (Schmid, 1992). In *Xenopus laevis* larvae, the lens regenerates from the outer cornea, the same tissue from which it arises during embryonic development, and there is apparently not a requirement for cell proliferation (Filoni et al., 1995). One could speculate that proliferation is not required when cells transdifferentiate into other cell types of the same or similar developmental origin (muscle to muscle in *Podocoryne*, cornea to lens in *Xenopus*), but may be required for transdifferentiation into cell types that have a different developmental origin.

Further insights into the mechanisms that regulate the generation of mononucleate cells from differentiated myotubes or myofibres in amphibians, as well as identification of similarities and differences in the regulation of the differentiated state in mammalian and newt myotubes, will undoubtedly contribute both to our understanding of the biology of muscle and the puzzle of amphibian limb regeneration and its phylogenetic restriction.

Summary

Urodele amphibians such as the newt are unique among vertebrates in that they are able to regenerate various structures as adults. Underlying the regenerative ability of urodeles may be the plasticity of differentiated tissues at the sites of injury. Why regeneration is absent or curtailed in other vertebrates, including mammals, is not understood. One possibility is that newt and mammalian cells are intrinsically different in their ability to respond to their environment. Alternatively, it is possible that unique signals are generated after injury of newt tissues, which are absent or inhibited in mammalian systems. A context in which to address these issues is the cultured newt myotube. This thesis is concerned with two overarching themes: the relationship between cell cycle re-entry and the plasticity of the differentiated state in newt myotubes, and the comparison of the differentiated state between newt and mouse myotubes.

In culture, newt myotubes are able to respond to serum stimulation and synthesise DNA by virtue of their ability to phosphorylate pRb, and this distinguishes them from mouse and avian myotubes (Gu et al., 1993; Schneider et al., 1994; Novitch et al., 1996; Tanaka et al., 1997). At the time I began my experiments, it was known that the newt myotubes were similar to their mammalian counterparts in one respect: growth factors such as PDGF, which are active on their mononucleate precursors, do not stimulate DNA synthesis in myotubes (Tanaka et al., 1997). The response to serum is activated by an unknown serum component that acts exclusively on the differentiated newt myotube. Further characterisation of this response could provide a cellular correlate for the difference in regenerative ability between newts and mammals. An important question in this regard was whether the same intracellular mechanism that is operative in newt myotubes is able to induce cell cycle re-entry in differentiated, post-mitotic mammalian myotubes.

In the first year of my PhD, I attempted to answer this question by investigating the serum response of nuclei in heterokaryon myotubes formed by fusion of mouse C2C12 and newt A1 cells, as described in Chapter 3. Although the response of the A1 nuclei in heterokaryons and in homokaryon myotubes

under the culture conditions used was low, DNA synthesis was observed in the C2C12 nuclei in the heterokaryon myotubes. This was an important result. It showed that the newt pathway can act across species, presumably on molecules that are conserved between the two, to elicit a response in mouse nuclei. In light of the results obtained by Tanaka et al. (1999), this observation can now be extended to establish conclusively that DNA synthesis cannot be induced in heterokaryon myotubes by purified growth factors, and that the stimulus for cell cycle re-entry is the thrombin-activated component of serum that is active on newt A1 myotubes. Furthermore, the results support the effort to establish an S-phase entry assay for thrombin-induced activity in C2C12 myotubes by expression of newt cDNAs in these cells to clone genes involved in cell cycle re-entry. This approach is now being used in this laboratory and has the advantage that it circumvents the problems associated with culturing newt cells at high temperature and inappropriate osmolarity.

When I concluded the heterokaryon experiments, the working hypothesis in this laboratory was that cell cycle re-entry and morphological plasticity of myotubes were directly linked. Thus S-phase entry and subsequent cell cycle progression, leading to mitosis and cytokinesis, was thought to be the mechanism by which mononucleate cells are generated from multinucleate myotubes after implantation into regenerating limbs. Cultured myotubes arrest in G2 after serum stimulation. I therefore attempted to overcome the G2 arrest of serum-stimulated A1 myotubes in culture and induce mitosis. My attempts, as described in Chapter 4, led me to conclude that the G2 block was stable and to question the validity of this 'M-phase entry model'. My doubts were reinforced by the response of mammalian myotubes to viral oncogene expression: progression through mitosis and cytokinesis followed by cleavage and cell death (Endo and Nadal-Ginard, 1989, 1998; Iujvidin et al., 1990; Crescenzi et al., 1995; Latella et al., 2000). The possibility remained, however, that signals that are present and might induce mitosis in the blastema cannot be reproduced in cultured newt myotubes by expression of SV-40 large T antigen. An attempt to mimic the blastemal environment more closely could have been made by culturing the myotubes on

slices of blastemal or stump tissues, but I thought that a different point needed to be established.

A critical question was whether cell cycle re-entry and generation of mononucleate cells after implantation were interdependent phenomena. This question was addressed by the experiments described in Chapter 5. Cell cycle re-entry was inhibited in myotubes that were labelled with markers and implanted into regenerating limbs. Dr. Elly Tanaka had established that S-phase entry could be inhibited in newt myotubes by expression of the cdk inhibitor $p16^{INK4}$ (Tanaka et al., 1997). I had observed that X-irradiation caused cell cycle arrest without affecting cell viability. This was an extension of my interest in p53 regulation in newt myotubes, discussed in Chapter 4, and subsequent efforts to characterise the response of newt A1 mononucleate cells and myotubes to DNA damaging agents. The generation of mononucleate cells following implantation of X-irradiated or p16-expressing myotubes established that cell cycle re-entry is not required for the morphological plasticity of newt myotubes. The 'M-phase entry' model was excluded and we now favour a mechanism whereby nuclei 'bud' from the syncytial myotube after implantation. It is possible that nuclei that enter S-phase can participate in this budding mechanism, but S-phase entry is not required.

An important question in terms of blastema formation is the plasticity of resident limb myofibres. As discussed in the introduction, the contribution of muscle tissue to the blastema has not been observed directly. In an attempt to establish primary cultures of myofibres for labelling and implantation, Dr. Anoop Kumar, in this laboratory, observed that myofibres from axolotl (*Ambystoma mexicanum*) and salamander (*Ambystoma maculatum*) larvae undergo a budding process in culture to generate mononucleate cells. Very few attached mononucleate cells are observed on the myofibre surfaces. I have microinjected these myofibres with lineage tracer TR-dextran and we have observed labelled mononucleate cells in the culture. Furthermore, Dr. Kumar has incubated these budding myofibres with [^{3}H]-thymidine and has observed that their nuclei do not incorporate the label, but that mononucleate cells in the culture do. These current

observations support the hypothesis that implanted myotubes generate mononucleate cells after implantation by a process of budding that occurs in the absence of DNA synthesis.

What is the significance of cell cycle re-entry in newt myotubes? Cell cycle re-entry is not required for the generation of mononucleate cells. Nevertheless, cell cycle re-entry and progression could be relevant to other phenomena such as dedifferentiation and transdifferentiation which occur in and are important for amphibian regeneration. It is possible that the mononucleate cells that are generated from cell cycle arrested myotubes are not dedifferentiated cells. Biochemical differentiation of muscle can occur in the absence of morphological differentiation, i.e. fusion into myotubes. Mononucleate myocytes are stably arrested from the cell cycle, and genes characteristic of late stages of myogenesis, such as myosin heavy chain and muscle creatine kinase are expressed (Novitch et al., 1996; Russo et al., 1998). Cell cycle re-entry involves phosphorylation and functional inactivation of the Rb protein. The function of Rb in the G1 checkpoint and inhibiting DNA synthesis through binding of E2F transcription factors is well established. More recently, a role for Rb in transcriptional regulation of late markers of muscle differentiation has become evident (Novitch et al., 1996, 1999). Dr. Yutaka Imokawa, in this laboratory, has observed that the myogenic regulatory factor Myf 5 is not expressed in mononucleate A1 cells, but is upregulated in A1 myotubes. Following serum stimulation Myf 5 is downregulated exclusively in myotubes in which the nuclei incorporate [^{3}H]-thymidine. It would be important to determine the phenotype and fate of mononucleate cells derived from cell cycle arrested myotubes after implantation.

During limb regeneration, cell cycle re-entry and dedifferentiation of tissues occur concomitantly. My results suggest that they are not interdependent, at least in the muscle system, although both are required for regeneration. Other studies had already indicated that this might be the case. Cells with a mesenchymal phenotype accumulate in the amputated limb in situations where blastema formation does not occur due to lack of cell proliferation, i.e. following

X-irradiation, denervation, or inhibition of wound epithelium formation (Wallace, 1981; Stocum, 1995). Extensive analysis of gene expression in the 'dedifferentiated' cells has not been undertaken in these cases, but observations with the 22/18 antigen in are informative. The 22/18 antigen is expressed for up to two months following denervation or replacement of the wound epithelium with a skin flap (Gordon and Brockes, 1988). The appearance of 22/18 reactivity after limb amputation is due to a rearrangement of the network of intermediate filaments, rather than changes in transcription or translation of the antigenic protein (Ferretti and Brockes, 1990; 1991). It is possible that cytoskeletal rearrangements induced by the changing extracellular matrix composition after amputation results in budding of nuclei from myofibres. This could occur in the absence of cell cycle re-entry, new protein synthesis or novel gene transcription, and this could be tested in the cultured myofibres described above. Nevertheless, the time course of mononucleate cell generation as well as cell cycle re-entry in myotubes after implantation (5-10 days, Lo et al., 1993; Kumar et al., 2000) suggest that novel gene transcription and protein synthesis are likely to be occurring.

Are the stimuli for cell cycle re-entry in myotubes and mononucleate cell generation the same? There seems to be an agreement that amputation is the stimulus that initiates blastema formation by triggering both cell cycle re-entry and dedifferentiation (meaning the appearance of mesenchymal progenitor cells), but how is this stimulus transmitted in molecular terms? One possible answer may be provided in terms of thrombin activity in the blastema. A membrane overlay assay has shown that thrombin activity is present in the blastema at a time when histological dedifferentiation is observed (8 days after amputation), but not in later blastemas (25 days after amputation) when dedifferentiation is thought to have ceased and differentiation to have begun (Tanaka et al., 1999 and unpublished). The acute events of clotting occur immediately after amputation, and the continued presence of thrombin activity after 8 days may be suggestive of a continued requirement for it at this later time. In preliminary experiments, Dr. Yutaka Imokawa has identified thrombin activity in the dorsal iris, the origin of progenitor cells for regeneration of the lens, after lentectomy. No thrombin

activity was observed in the ventral iris. Lentectomy does not generally lead to extensive bleeding and clotting as occurs in the limb after amputation, and it may be correct to interpret the lens and limb data as indicating that thrombin activity in these regenerating structures is not due exclusively to clotting cascade events. It would be interesting to determine whether myotubes can generate mononucleate cells after implantation into late limb blastemas, but there are technical problems associated with this manipulations. Alternatively, it would be interesting to determine whether thrombin activity is present in denervated limbs, a context in which dedifferentiation but not cell proliferation occurs, and whether myotubes can generate mononucleate cells after implantation into these limbs. If thrombin is the signal that triggers both cell cycle re-entry and dedifferentiation, the localised activation and inactivation of thrombin is important for restricting these processes to the site of injury. It is possible that subsequent signals (emanating from the wound epithelium or the nerve supply) are required for continued proliferation and further biochemical dedifferentiation, but not for morphological dedifferentiation.

It is also possible, however, that thrombin is not the signal that induces generation of mononucleate cells; Dr. Kumar has observed that budding of nuclei from axolotl myofibres occurs in serum free medium. Nevertheless, like thrombin, the signal that triggers generation of mononucleate cells must be localised to the site of amputation and may arise as a result of extracellular matrix remodelling. The signal may be a soluble factor, since myofibre dedifferentiation can be observed in culture, but the culture system also supports the theory that inhibitory signals or stabilising influences are removed when tissue structure is disrupted.

What is the relevance of newt myotube plasticity from a 'mammalian' point of view? The mononucleate cells generated from multinucleate myofibres after implantation can proliferate and contribute to tissues in the regenerate (Lo et al., 1993). Multinucleate C2C12 myotubes can give rise to viable, proliferating mononucleate cells in culture by treatment with a compound called myoseverin, a

substituted purine that causes microtubule depolymerisation (Rosania et al., 2000). Scission of myotubes occurs in the absence of DNA synthesis. The parallels to the implantated newt myotube are striking. It seems that dissecting the signalling pathways that operate to induce cell cycle re-entry and generation of mononucleate cells in the newt myotube may have more parallels than expected with mammalian systems. The example of myoseverin suggests that there might be numerous applications for therapeutic interventions based on activating differentiated cells. The mononucleate cells derived from the myotubes treated with myoseverin can fuse into myotubes in culture (Rosania, personal communication). It is important to determine, both for basic research and for therapeutic applications, whether the mononucleate cells can also fuse into myofibres after implantation into an animal. Moreover, it is unclear if myoseverin would elicit a proliferative response or a regenerative response after injection into muscle. The urodele strategy of local dedifferentiation may have the advantage of allowing blastemal cells to derive important cues from their differentiated precursors, for example on the proximodistal axis, and this might be applicable in future in the mammalian context.

Other lessons can be learned from the newt and these might have potential applications in the repair and regeneration of tissues in mammals. In the regenerating newt limb, where tissue plasticity is elevated and where connective tissue fibroblasts are a major source of cells (Muneoka et al., 1986), no scar formation occurs. Do the signals that trigger plasticity and/or cell cycle re-entry of differentiated cells inhibit scarring? Are the fibroblasts inhibited from synthesising collagen and induced to synthesise fibronectin? Is differentiation into myofibroblasts inhibited? In the newt heart, where cardiomyocytes re-enter the cell cycle but no blastema formation is observed, scars do form (Oberpriller et al., 1995). Scar formation may be one obstacle to regeneration in higher vertebrates and it is a source of functional impairment in many lesioned tissues.

It seems clear that additional insights can be gained from the study of the newt. Although differentiated tissues do participate in blastema formation, a stem

cell contribution cannot be discounted, and processes important in blastema formation may be relevant to the activation of stem cell compartments in general. Investigation of the similarities and differences between limb development and regeneration may help us to understand how embryonic programmes controlling growth and morphogenesis can be reactivated in an adult. The newt is thus a valuable model system. The comparison of regenerative phenomena in newts and other model organisms more amenable to genetic manipulations, such as the zebrafish, as well as the cell biological approaches described above, will hopefully lead to an understanding of how regeneration occurs and why it is restricted among tetrapod vertebrates to the urodele amphibians.

References

Abmayr, S. M., and Keller, C. A. (1998). Drosophila myogenesis and insights into the role of nautilus. *Curr Top Dev Biol*, 38: 35-80.

Abrieu, A., Brassac, T., Galas, S., Fisher, D., Labbe, J. C., and Doree, M. (1998). The Polo-like kinase Plx1 is a component of the MPF amplification loop at the G2/M-phase transition of the cell cycle in Xenopus eggs. *J Cell Sci*, 111: 1751-1757.

Agarwal, M. L., Agarwal, A., Taylor, W. R., and Stark, G. R. (1995). p53 controls both the G2/M and the G1 cell cycle checkpoints and mediates reversible growth arrest in human fibroblasts. *Proc Natl Acad Sci U S A*, 92: 8493-8497.

Agata, K., Kobayashi, H., Itoh, Y., Mochii, M., Sawada, K., and Eguchi, G. (1993). Genetic characterization of the multipotent dedifferentiated state of pigmented epithelial cells in vitro. *Development*, 118: 1025-1030.

Aladjem, M. I., Spike, B. T., Rodewald, L. W., Hope, T. J., Klemm, M., Jaenisch, R., and Wahl, G. M. (1998). ES cells do not activate p53-dependent stress responses and undergo p53- independent apoptosis in response to DNA damage. *Curr Biol*, 8: 145-155.

Alberts, B. B., D.; Lewis, J.; Raff, J.; Roberts, K.; Watson, J. D. (1994). "Molecular biology of the cell." Garland Publishing Inc, New Yrok.

Allan, L. A., and Fried, M. (1999). p53-dependent apoptosis or growth arrest induced by different forms of radiation in U2OS cells: p21WAF1/CIP1 repression in UV induced apoptosis. *Oncogene*, 18: 5403-5412.

Allen, R. E., and Rankin, L. L. (1990). Regulation of satellite cells during skeletal muscle growth and development. *Proc Soc Exp Biol Med*, 194: 81-86.

Alt, F. W., Blackwell, T. K., and Yancopoulos, G. D. (1987). Development of the primary antibody repertoire. *Science*, 238: 1079-1087.

Anand, P., McGregor, G. P., Gibson, S. J., Maden, M., Polak, J. M., and Bloom, S. R. (1987). Increase of substance P-like immunoreactivity in the peripheral nerve of the axolotl after injury. *Neurosci Lett*, 82: 241-245.

Andres, V., and Walsh, K. (1996). Myogenin expression, cell cycle withdrawal, and phenotypic differentiation are temporally separable events that precede cell fusion upon myogenesis. *J Cell Biol*, 132: 657-666.

Arnold, H. H., and Braun, T. (1996). Targeted inactivation of myogenic factor genes reveals their role during mouse myogenesis: a review. *Int J Dev Biol*, 40: 345-353.

Arnold, H. H., and Braun, T. (2000). Genetics of muscle determination and development. *Curr Top Dev Biol*, 48: 129-164.

Aurade, F., Pinset, C., Chafey, P., Gros, F., and Montarras, D. (1994). Myf5, MyoD, myogenin and MRF4 myogenic derivatives of the embryonic mesenchymal cell line C3H10T1/2 exhibit the same adult muscle phenotype. *Differentiation*, 55: 185-192.

Azuma-Hara, M., Taniura, H., Uetsuki, T., Niinobe, M., and Yoshikawa, K. (1999). Regulation and deregulation of E2F1 in postmitotic neurons differentiated from embryonal carcinoma P19 cells. *Exp Cell Res*, 251: 442-451.

Azzam, E. I., de Toledo, S. M., Pykett, M. J., Nagasawa, H., and Little, J. B. (1997). CDC2 is down-regulated by ionizing radiation in a p53-dependent manner. *Cell Growth Differ*, 8: 1161-1169.

Bae, I., Smith, M. L., and Fornace, A. J., Jr. (1995). Induction of p53-, MDM2-, and WAF1/CIP1-like molecules in insect cells by DNA-damaging agents. *Exp Cell Res*, 217: 541-545.

Baroffio, A., Bochaton-Piallat, M. L., Gabbiani, G., and Bader, C. R. (1995). Heterogeneity in the progeny of single human muscle satellite cells. *Differentiation*, 59: 259-268.

Baroffio, A., Hamann, M., Bernheim, L., Bochaton-Piallat, M. L., Gabbiani, G., and Bader, C. R. (1996). Identification of self-renewing myoblasts in the progeny of single human muscle satellite cells. *Differentiation*, 60: 47-57.

Baserga, R. (1999). Introduction to the cell cycle. *In* "The molecular basis of cell cycle control" (G. Stein, R. Baserga, A. Giordano, and D. T. Denhardt, Eds.). Wiley Liss, New York.

Bischoff, R., and Heintz, C. (1994). Enhancement of skeletal muscle regeneration. *Dev Dyn*, 201: 41-54.

Bissonnette, N., and Hunting, D. J. (1998). p21-induced cycle arrest in G1 protects cells from apoptosis induced by UV-irradiation or RNA polymerase II blockage. *Oncogene*, 16: 3461-3469.

Black, B. L., and Olson, E. N. (1998). Transcriptional control of muscle development by myocyte enhancer factor-2 (MEF2) proteins. *Annu Rev Cell Dev Biol*, 14: 167-196.

Bladt, F., Riethmacher, D., Isenmann, S., Aguzzi, A., and Birchmeier, C. (1995). Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud [see comments]. *Nature*, 376: 768-771.

Blasina, A., de Weyer, I. V., Laus, M. C., Luyten, W. H., Parker, A. E., and McGowan, C. H. (1999). A human homologue of the checkpoint kinase Cds1 directly inhibits Cdc25 phosphatase. *Curr Biol*, 9: 1-10.

Blau, H. M., Chiu, C. P., and Webster, C. (1983). Cytoplasmic activation of human nuclear genes in stable heterocaryons. *Cell*, 32: 1171-1180.

Blau, H. M., Pavlath, G. K., Hardeman, E. C., Chiu, C. P., Silberstein, L., Webster, S. G., Miller, S. C., and Webster, C. (1985). Plasticity of the differentiated state. *Science*, 230: 758-766.

Blaveri, K., Heslop, L., Yu, D. S., Rosenblatt, J. D., Gross, J. G., Partridge, T. A., and Morgan, J. E. (1999). Patterns of repair of dystrophic mouse muscle: studies on isolated fibers. *Dev Dyn*, 216: 244-256.

Block, N. E., and Miller, J. B. (1992). Expression of MRF4, a myogenic helix-loop-helix protein, produces multiple changes in the myogenic program of BC3H-1 cells. *Mol Cell Biol*, 12: 2484-2492.

Bober, E., Franz, T., Arnold, H. H., Gruss, P., and Tremblay, P. (1994). Pax-3 is required for the development of limb muscles: a possible role for the migration of dermomyotomal muscle progenitor cells. *Development*, 120: 603-612.

Bober, E., Lyons, G. E., Braun, T., Cossu, G., Buckingham, M., and Arnold, H. H. (1991). The muscle regulatory gene, Myf-6, has a biphasic pattern of expression during early mouse development. *J Cell Biol*, 113: 1255-1265.

Boilly, B., and Albert, P. (1988). Control of blastema cell proliferation during axolotl limb regeneration: in vitro cell culture study. *Monogr Dev Biol*, 21: 1-8.

Boilly, B., Cavanaugh, K. P., Thomas, D., Hondermarck, H., Bryant, S. V., and Bradshaw, R. A. (1991). Acidic fibroblast growth factor is present in regenerating limb blastemas of axolotls and binds specifically to blastema tissues. *Dev Biol*, 145: 302-310.

Boilly, B., Oudkhir, M., and Lassalle, B. (1985). Control of the blastemal cell cycle by the peripheral nervous system during newt limb regeneration; continuous labeling analysis. *Biol Cell*, 55: 107-111.

Booher, R. N., Holman, P. S., and Fattaey, A. (1997). Human Myt1 is a cell cycle-regulated kinase that inhibits Cdc2 but not Cdk2 activity. *J Biol Chem*, 272: 22300-22306.

Brand-Saberi, B., and Christ, B. (1999). Genetic and epigenetic control of muscle development in vertebrates. *Cell Tissue Res*, 296: 199-212.

Braun, T., and Arnold, H. H. (1995). Inactivation of Myf-6 and Myf-5 genes in mice leads to alterations in skeletal muscle development. *Embo J*, 14: 1176-1186.

Braun, T., and Arnold, H. H. (1996). Myf-5 and myoD genes are activated in distinct mesenchymal stem cells and determine different skeletal muscle cell lineages. *Embo J*, 15: 310-318.

Braun, T., Buschhausen-Denker, G., Bober, E., Tannich, E., and Arnold, H. H. (1989). A novel human muscle factor related to but distinct from MyoD1 induces myogenic conversion in 10T1/2 fibroblasts. *Embo J*, 8: 701-709.

Braun, T., Rudnicki, M. A., Arnold, H. H., and Jaenisch, R. (1992). Targeted inactivation of the muscle regulatory gene Myf-5 results in abnormal rib development and perinatal death. *Cell*, 71: 369-382.

Brehm, A., Miska, E. A., McCance, D. J., Reid, J. L., Bannister, A. J., and Kouzarides, T. (1998). Retinoblastoma protein recruits histone deacetylase to repress transcription [see comments]. *Nature*, 391: 597-601.

Brennan, T. J., Chakraborty, T., and Olson, E. N. (1991). Mutagenesis of the myogenin basic region identifies an ancient protein motif critical for activation of myogenesis. *Proc Natl Acad Sci USA*, 88: 5675-5679.

Briggs, R., and King, T. J. (1952). Transplantation of living nuclei from blastula cells into enucleated frog's eggs. *Prc Nat Acad Sci USA*, 38: 455-463.

Briggs, R., and King, T. J. (1960). Nuclear transplantation studies on the early gastrula (*Rana pipiens*). *Dev Biol*, 2: 252-270.

Brockes, J. (1994). Cell differentiation. Muscle escapes from a jelly mould. *Curr Biol*, 4: 1030-1032.

Brockes, J. P. (1997). Amphibian limb regeneration: rebuilding a complex structure. *Science*, 276: 81-87.

Brockes, J. P. (1998). Regeneration and cancer. *Biochim Biophys Acta*, 1377: M1-11.

Brockes, J. P., and Kintner, C. R. (1986). Glial growth factor and nerve-dependent proliferation in the regeneration blastema of Urodele amphibians. *Cell*, 45: 301-306.

Brodsky, W. Y., Arefyeva, A. M., and Uryvaeva, I. V. (1980). Mitotic polyploidization of mouse heart myocytes during the first postnatal week. *Cell Tissue Res*, 210: 133-144.

Brosamle, C., and Kuffler, D. P. (1996). Rapid dispersal of clustered postsynaptic nuclei following dissociation of skeletal muscle fibers. *J Exp Biol*, 199: 2359-2367.

Brunet, A., Roux, D., Lenormand, P., Dowd, S., Keyse, S., and Pouyssegur, J. (1999). Nuclear translocation of p42/p44 mitogen-activated protein kinase is required for growth factor-induced gene expression and cell cycle entry. *Embo J*, 18: 664-674.

Bryant, S. V., and Gardiner, D. M. (1992). Retinoic acid, local cell-cell interactions, and pattern formation in vertebrate limbs. *Dev Biol*, 152: 1-25.

Buchberger, A., Ragge, K., and Arnold, H. H. (1994). The myogenin gene is activated during myocyte differentiation by pre- existing, not newly synthesized transcription factor MEF-2. *J Biol Chem*, 269: 17289-17296.

Buckingham, M. (1992). Making muscle in mammals. *Trends Genet*, 8: 144-148.

Bunz, F., Dutriaux, A., Lengauer, C., Waldman, T., Zhou, S., Brown, J. P., Sedivy, J. M., Kinzler, K. W., and Vogelstein, B. (1998). Requirement for p53 and p21 to sustain G2 arrest after DNA damage. *Science*, 282: 1497-1501.

Buskin, J. N., and Hauschka, S. D. (1989). Identification of a myocyte nuclear factor that binds to the muscle-specific enhancer of the mouse muscle creatine kinase gene. *Mol Cell Biol*, 9: 2627-2640.

Butler, E. G. (1935). Studies on limb regeneration in X-rayed *Amblystoma* larvae. *Anat Rec*, 62: 295-307.

Butler, E. G. (1955). Regeneration of the urodele forelimb after reversal of its proximo-distal axis. *J Morphol*, 96: 265-281.

Butler, E. G., and O'Brien, J. P. (1942). Effects of localized X-radiation on regeneration of the urodele limb. *Anat Rec*, 84: 407-413.

Cameron, J. A., Hilgers, A. R., and Hinterberger, T. J. (1986). Evidence that reserve cells are a source of regenerated sdult newt muscle in vitro. *Nature*, 321: 607-610.

Carlone, R. L., and Foret, J. E. (1979). Stimulation of mitosis in cultured limb blastemata of the newt, *Notophthalmus viridescens*. *J Exp Zool*, 210: 245-252.

Carlson, M. R., Bryant, S. V., and Gardiner, D. M. (1998). Expression of Msx-2 during development, regeneration, and wound healing in axolotl limbs. *J Exp Zool*, 282: 715-723.

Casimir, C. M., Gates, P. B., Patient, R. K., and Brockes, J. P. (1988). Evidence for dedifferentiation and metaplasia in amphibian limb regeneration from inheritance of DNA methylation. *Development*, 104: 657-668.

Cathieni, M., and Tabin, C. H. (1992). Cyclic nucleotide fluctuations during newt limb regeneration depend on injury and nerve action. *Monongr Dev Biol*, 23: 85-92.

Chalkley, D. T. (1954). A quantitative histological analysis of forlimb regeneration in *Triturus visidescens*. *J Morphol*, 94: 21-70.

Chang, T. H., Ray, F. A., Thompson, D. A., and Schlegel, R. (1997). Disregulation of mitotic checkpoints and regulatory proteins following acute expression of SV40 large T antigen in diploid human cells. *Oncogene*, 14: 2383-2393.

Chapron, C. (1974). [Evidence for the role of an apical cap glycoprotein in amphibian regeneration: cytochemical and autoradiographic electron-microscopic studies (author's transl)]. *J Embryol Exp Morphol*, 32: 133-145.

Chellappan, S. P., Hiebert, S., Mudryj, M., Horowitz, J. M., and Nevins, J. R. (1991). The E2F transcription factor is a cellular target for the RB protein. *Cell*, 65: 1053-1061.

Chen, H. C., Appeddu, P. A., Isoda, H., and Guan, J. L. (1996a). Phosphorylation of tyrosine 397 in focal adhesion kinase is required for binding phosphatidylinositol 3-kinase. *J Biol Chem*, 271: 26329-26334.

Chen, P. L., Riley, D. J., Chen, Y., and Lee, W. H. (1996b). Retinoblastoma protein positively regulates terminal adipocyte differentiation through direct interaction with C/EBPs. *Genes Dev*, 10: 2794-2804.

Cheng, T. C., Wallace, M. C., Merlie, J. P., and Olson, E. N. (1993). Separable regulatory elements governing myogenin transcription in mouse embryogenesis. *Science*, 261: 215-218.

Chiu, C. P., and Blau, H. M. (1984). Reprogramming cell differentiation in the absence of DNA synthesis. *Cell*, 37: 879-887.

Choi, J., Costa, M. L., Mermelstein, C. S., Chagas, C., Holtzer, S., and Holtzer, H. (1990). MyoD converts primary dermal fibroblasts, chondroblasts, smooth muscle, and retinal pigmented epithelial cells into striated mononucleated myoblasts and multinucleated myotubes. *Proc Natl Acad Sci U S A*, 87: 7988-7992.

Christ, B., and Ordahl, C. P. (1995). Early stages of chick somite development. *Anat Embryol (Berl)*, 191: 381-396.

Clarke, A. R., Maandag, E. R., van Roon, M., van der Lugt, N. M., van der Valk, M., Hooper, M. L., Berns, A., and te Riele, H. (1992). Requirement for a functional Rb-1 gene in murine development [see comments]. *Nature*, 359: 328-330.

Clarke, D. J., and Gimenez-Abian, J. F. (2000). Checkpoints controlling mitosis. *Bioessays*, 22: 351-363.

Clegg, C. H., and Hauschka, S. D. (1987). Heterokaryon analysis of muscle differentiation: regulation of the postmitotic state. *J Cell Biol*, 105: 937-947.

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

Clute, P., and Pines, J. (1999). Temporal and spatial control of cyclin B1 destruction in metaphase. *Nat Cell Biol*, 1: 82-87.

Coleman, T. R., and Dunphy, W. G. (1994). Cdc2 regulatory factors. *Curr Opin Cell Biol*, 6: 877-882.

Cornelison, D. D., and Wold, B. J. (1997). Single-cell analysis of regulatory gene expression in quiescent and activated mouse skeletal muscle satellite cells. *Dev Biol*, 191: 270-283.

Couly, G. F., Coltey, P. M., and Le Douarin, N. M. (1993). The triple origin of skull in higher vertebrates: a study in quail- chick chimeras. *Development*, 117: 409-429.

Crescenzi, M., Crouch, D. H., and Tato, F. (1994). Transformation by myc prevents fusion but not biochemical differentiation of C2C12 myoblasts: mechanisms of phenotypic correction in mixed culture with normal cells. *J Cell Biol*, 125: 1137-1145.

Crescenzi, M., Soddu, S., and Tato, F. (1995). Mitotic cycle reactivation in terminally differentiated cells by adenovirus infection. *J Cell Physiol*, 162: 26-35.

Crews, L., Gates, P. B., Brown, R., Joliot, A., Foley, C., Brockes, J. P., and Gann, A. A. (1995). Expression and activity of the newt Msx-1 gene in relation to limb regeneration. *Proc R Soc Lond B Biol Sci*, 259: 161-171.

Cross, S. M., Sanchez, C. A., Morgan, C. A., Schimke, M. K., Ramel, S., Idzerda, R. L., Raskind, W. H., and Reid, B. J. (1995). A p53-dependent mouse spindle checkpoint. *Science*, 267: 1353-1356.

Cserjesi, P., Lilly, B., Hinkley, C., Perry, M., and Olson, E. N. (1994). Homeodomain protein MHox and MADS protein myocyte enhancer-binding factor-2 converge on a common element in the muscle creatine kinase enhancer. *J Biol Chem*, 269: 16740-16745.

Cserjesi, P., and Olson, E. N. (1991). Myogenin induces the myocyte-specific enhancer binding factor MEF-2 independently of other muscle-specific gene products. *Mol Cell Biol*, 11: 4854-4862.

Cusella De Angelis, M. G., Molinari, S., Le Donne, A., Coletta, M., Vivarelli, E., Bouche, M., Molinaro, M., Ferrari, S., and Cossu, G. (1994). Differential response of embryonic and fetal myoblasts to TGF beta: a possible regulatory mechanism of skeletal muscle histogenesis. *Development*, 925-933.

Dang, V. D., Benedik, M. J., Ekwall, K., Choi, J., Allshire, R. C., and Levin, H. L. (1999). A new member of the Sin3 family of corepressors is essential for cell viability and required for retroelement propagation in fission yeast. *Mol Cell Biol*, 19: 2351-2365.

Daston, G., Lamar, E., Olivier, M., and Goulding, M. (1996). Pax-3 is necessary for migration but not differentiation of limb muscle precursors in the mouse. *Development*, 122: 1017-1027.

Datta, N. S., Williams, J. L., Caldwell, J., Curry, A. M., Ashcraft, E. K., and Long, M. W. (1996). Novel alterations in CDK1/cyclin B1 kinase complex formation occur during the acquisition of a polyploid DNA content. *Mol Biol Cell*, 7: 209-223.

Davidson, D. R., and Hill, R. E. (1991). Msh-like genes: a family of homeobox genes with wide-ranging expression during vertebrate development. *Semin Dev Biol*, 2: 405-412.

Davidson, E. H. (1986). "Gene activity in early development." Academic Press, Orlando.

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

Denetclaw, W. F., Jr., Bi, G., Pham, D. V., and Steinhardt, R. A. (1993). Heterokaryon myotubes with normal mouse and Duchenne nuclei exhibit sarcolemmal dystrophin staining and efficient intracellular free calcium control. *Mol Biol Cell*, 4: 963-972.

Denetclaw, W. F., Jr., Christ, B., and Ordahl, C. P. (1997). Location and growth of epaxial myotome precursor cells. *Development*, 124: 1601-1610.

Deng, C., Zhang, P., Harper, J. W., Elledge, S. J., and Leder, P. (1995). Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. *Cell*, 82: 675-684.

Depoortere, F., Pirson, I., Bartek, J., Dumont, J. E., and Roger, P. P. (2000). Transforming growth factor beta(1) selectively inhibits the cyclic AMP- dependent proliferation of primary thyroid epithelial cells by preventing the association of cyclin D3-cdk4 with nuclear p27(kip1). *Mol Biol Cell*, 11: 1061-1076.

Desai, D., Gu, Y., and Morgan, D. O. (1992). Activation of human cyclin-dependent kinases in vitro. *Mol Biol Cell*, 3: 571-582.

Devault, A., Martinez, A. M., Fesquet, D., Labbe, J. C., Morin, N., Tassan, J. P., Nigg, E. A., Cavadore, J. C., and Doree, M. (1995). MAT1 ('menage a trois') a new RING finger protein subunit stabilizing cyclin H-cdk7 complexes in starfish and Xenopus CAK. *Embo J*, 14: 5027-5036.

Di Leonardo, A., Linke, S. P., Clarkin, K., and Wahl, G. M. (1994). DNA damage triggers a prolonged p53-dependent G1 arrest and long-term induction of Cip1 in normal human fibroblasts. *Genes Dev*, 8: 2540-2551.

Diehl, J. A., Cheng, M., Roussel, M. F., and Sherr, C. J. (1998). Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. *Genes Dev*, 12: 3499-3511.

Diehl, J. A., and Sherr, C. J. (1997). A dominant-negative cyclin D1 mutant prevents nuclear import of cyclin- dependent kinase 4 (CDK4) and its phosphorylation by CDK-activating kinase. *Mol Cell Biol*, 17: 7362-7374.

Diehl, J. A., Zindy, F., and Sherr, C. J. (1997). Inhibition of cyclin D1 phosphorylation on threonine-286 prevents its rapid degradation via the ubiquitin-proteasome pathway. *Genes Dev*, 11: 957-972.

DiMario, J. X., Fernyak, S. E., and Stockdale, F. E. (1993). Myoblasts transferred to the limbs of embryos are committed to specific fibre fates. *Nature*, 362: 165-167.

Donehower, L. A., Harvey, M., Slagle, B. L., McArthur, M. J., Montgomery, C. A., Jr., Butel, J. S., and Bradley, A. (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature*, 356: 215-221.

Dou, Q. P., Markell, P. J., and Pardee, A. B. (1992). Thymidine kinase transcription is regulated at G1/S phase by a complex that contains retinoblastoma-like protein and a cdc2 kinase. *Proc Natl Acad Sci U S A*, 89: 3256-3260.

Dresden, M. H. (1969). Denervation effects on newt limb regeneration: DNA, RNA, and protein synthesis. *Dev Biol*, 19: 311-320.

Dulic, V., Kaufmann, W. K., Wilson, S. J., Tlsty, T. D., Lees, E., Harper, J. W., Elledge, S. J., and Reed, S. I. (1994). p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G1 arrest. *Cell*, 76: 1013-1023.

Dunaief, J. L., Strober, B. E., Guha, S., Khavari, P. A., Alin, K., Luban, J., Begemann, M., Crabtree, G. R., and Goff, S. P. (1994). The retinoblastoma protein and BRG1 form a complex and cooperate to induce cell cycle arrest. *Cell*, 79: 119-130.

Dunis, D. A., and Namenwirth, M. (1977). The role of grafted skin in the regeneration of x-irradiated axolotl limbs. *Dev Biol*, 56: 97-109.

Dusterhoft, S., Yablonka-Reuveni, Z., and Pette, D. (1990). Characterization of myosin isoforms in satellite cell cultures from adult rat diaphragm, soleus and tibialis anterior muscles. *Differentiation*, 45: 185-191.

Dyson, N. (1998). The regulation of E2F by pRB-family proteins. *Genes Dev*, 12: 2245-2262.

Dyson, N., Buchkovich, K., Whyte, P., and Harlow, E. (1989). The cellular 107K protein that binds to adenovirus E1A also associates with the large T antigens of SV40 and JC virus. *Cell*, 58: 249-255.

Edmondson, D. G., and Olson, E. N. (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 [published erratum appears in Genes Dev 1990 Aug;4(8):1450]. *Genes Dev*, 3: 628-640.

Egar, M. W. (1988). Accessory limb production by nerve-induced cell proliferation. *Anat Rec*, 221: 550-564.

Eguchi, G. (1988). Cellular and molecular background of wolffian lens regeneration. *Cell Differ Dev*, 25 Suppl: 147-158.

Eguchi, G., Abe, S. I., and Watanabe, K. (1974). Differentiation of lens-like structures from newt iris epithelial cells in vitro. *Proc Natl Acad Sci U S A*, 71: 5052-5056.

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

El-Deiry, W. S. (1998). p21/p53, cellular growth control and genomic integrity. *Curr Top Microbiol Immunol*, 227: 121-137.

El-Deiry, W. S., Harper, J. W., O'Connor, P. M., Velculescu, V. E., Canman, C. E., Jackman, J., Pietenpol, J. A., Burrell, M., Hill, D. E., Wang, Y., and et al. (1994). WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. *Cancer Res*, 54: 1169-1174.

Elledge, S. J. (1996). Cell cycle checkpoints: preventing an identity crisis. *Science*, 274: 1664-1672.

Endo, T., and Nadal-Ginard, B. (1989). SV40 large T antigen induces reentry of terminally differentiated myotubes into the cell cycle. *In* "Cellular and molecular biology of muscle development" (F. Stockdale and L. Kedes, Eds.). Alan R Liss Iinc, New York.

Endo, T., and Nadal-Ginard, B. (1998). Reversal of myogenic terminal differentiation by SV40 large T antigen results in mitosis and apoptosis. *J Cell Sci*, 111: 1081-1093.

Fekete, D. M., and Brockes, J. P. (1987). A monoclonal antibody detects a difference in the cellular composition of developing and regenerating limbs of newts. *Development*, 99: 589-602.

Fekete, D. M., and Brockes, J. P. (1988). Evidence that the nerve controls molecular identity of progenitor cells for limb regeneration. *Development*, 103: 567-573.

Feldman, J. L., and Stockdale, F. E. (1991). Skeletal muscle satellite cell diversity: satellite cells form fibers of different types in cell culture. *Dev Biol*, 143: 320-334.

Ferrari, G., Cusella-De Angelis, G., Coletta, M., Paolucci, E., Stornaiuolo, A., Cossu, G., and Mavilio, F. (1998). Muscle regeneration by bone marrow-derived myogenic progenitors [see comments] [published erratum appears in Science 1998 Aug 14;281(5379):923]. *Science*, 279: 1528-1530.

Ferretti, P., and Brockes, J. P. (1988). Culture of newt cells from different tissues and their expression of a regeneration-associated antigen. *J Exp Zool*, 247: 77-91.

Ferretti, P., and Brockes, J. P. (1990). The monoclonal antibody 22/18 recognizes a conformational change in an intermediate filament of the newt, Notophthalmus viridescens, during limb regeneration. *Cell Tissue Res*, 259: 483-493.

Ferretti, P., and Brockes, J. P. (1991). Cell origin and identity in limb regeneration and development. *Glia*, 4: 214-224.

Fields, A. P., and Thompson, L. J. (1995). The regulation of mitotic nuclear envelope breakdown: a role for multiple lamin kinases. *Prog Cell Cycle Res*, 1: 271-286.

Filoni, S., Cannata, S. M., Bernardini, S., and La Mesa, G. (1995). The inhibition of cell proliferation by mitomycin C does not prevent transdifferentiation of outer cornea into lens in larval Xenopus laevis. *Differentiation*, 58: 195-203.

Fischman, D. A. (1967). An electron microscope study of myofibril formation in embryonic chick skeletal muscle. *J Cell Biol*, 32: 557-575.

Fisher, R. P., and Morgan, D. O. (1994). A novel cyclin associates with MO15/CDK7 to form the CDK-activating kinase. *Cell*, 78: 713-724.

Florini, J. R., Ewton, D. Z., and Magri, K. A. (1991). Hormones, growth factors, and myogenic differentiation. *Annu Rev Physiol*, 53: 201-216.

Flucher, B. E. (1992). Structural analysis of muscle development: transverse tubules, sarcoplasmic reticulum, and the triad. *Dev Biol*, 154: 245-260.

Franklin, D. S., and Xiong, Y. (1996). Induction of p18INK4c and its predominant association with CDK4 and CDK6 during myogenic differentiation. *Mol Biol Cell*, 7: 1587-1599.

Fuchtbauer, E. M., and Westphal, H. (1992). MyoD and myogenin are coexpressed in regenerating skeletal muscle of the mouse. *Dev Dyn*, 193: 34-39.

Fukuda, M., Gotoh, I., Adachi, M., Gotoh, Y., and Nishida, E. (1997). A novel regulatory mechanism in the mitogen-activated protein (MAP) kinase cascade. Role of nuclear export signal of MAP kinase kinase. *J Biol Chem*, 272: 32642-32648.

Furnari, B., Blasina, A., Boddy, M. N., McGowan, C. H., and Russell, P. (1999). Cdc25 inhibited in vivo and in vitro by checkpoint kinases Cds1 and Chk1. *Mol Biol Cell*, 10: 833-845.

Furst, D. O., Osborn, M., and Weber, K. (1989). Myogenesis in the mouse embryo: differential onset of expression of myogenic proteins and the involvement of titin in myofibril assembly. *J Cell Biol*, 109: 517-527.

Furuno, N., den Elzen, N., and Pines, J. (1999). Human cyclin A is required for mitosis until mid prophase. *J Cell Biol*, 147: 295-306.

Galaktionov, K., and Beach, D. (1991). Specific activation of cdc25 tyrosine phosphatases by B-type cyclins: evidence for multiple roles of mitotic cyclins. *Cell*, 67: 1181-1194.

Galaktionov, K., Lee, A. K., Eckstein, J., Draetta, G., Meckler, J., Loda, M., and Beach, D. (1995). CDC25 phosphatases as potential human oncogenes. *Science*, 269: 1575-1577.

Gardiner, D. M., Carlson, M. R., and Roy, S. (1999). Towards a functional analysis of limb regeneration. *Semin Cell Dev Biol*, 10: 385-393.

Gardiner, D. M., Muneoka, K., and Bryant, S. V. (1986). The migration of dermal cells during blastema formation in axolotls. *Dev Biol*, 118: 488-493.

Geng, Y., Eaton, E. N., Picon, M., Roberts, J. M., Lundberg, A. S., Gifford, A., Sardet, C., and Weinberg, R. A. (1996). Regulation of cyclin E transcription by E2Fs and retinoblastoma protein. *Oncogene*, 12: 1173-1180.

George-Weinstein, M., Gerhart, J., Reed, R., Flynn, J., Callihan, B., Mattiacci, M., Miehle, C., Foti, G., Lash, J. W., and Weintraub, H. (1996). Skeletal myogenesis: the preferred pathway of chick embryo epiblast cells in vitro. *Dev Biol*, 173: 279-291.

Geraudie, J., and Singer, M. (1978). Nerve dependent macromolecular synthesis in the epidermis and blastema of the adult newt regenerate. *J Exp Zool*, 203: 455-460.

Ghosh, S., Thorogood, P., and Ferretti, P. (1994). Regenerative ability of upper and lower jaws in the newt. *Int. J. Dev. Biol.*, 38: 479-490.

Gill, R. M., and Hamel, P. A. (2000). Subcellular compartmentalization of E2F family members is required for maintenance of the postmitotic state in terminally differentiated muscle. *J Cell Biol*, 148: 1187-1201.

Girard, F., Strausfeld, U., Fernandez, A., and Lamb, N. J. (1991). Cyclin A is required for the onset of DNA replication in mammalian fibroblasts. *Cell*, 67: 1169-1179.

Gire, V., and Wynford-Thomas, D. (1998). Reinitiation of DNA synthesis and cell division in senescent human fibroblasts by microinjection of anti-p53 antibodies. *Mol Cell Biol*, 18: 1611-1621.

Globus, M., and Vethamany-Globus, S. (1977). Transfilter mitogenic effect of dorsal root ganglia on cultured regeneration blastemata, in the newt, Notophthalmus viridescens. *Dev Biol*, 56: 316-328.

Globus, M., Vethamany-Globus, S., and Lee, Y. C. (1980). Effect of apical epidermal cap on mitotic cycle and cartilage differentiation in regeneration blastemata in the newt, Notophthalmus viridescens. *Dev Biol*, 75: 358-372.

Goichberg, P., and Geiger, B. (1998). Direct involvement of N-cadherin-mediated signaling in muscle differentiation. *Mol Biol Cell*, 9: 3119-3131.

Goldhamer, D. J., Tomlinson, B. L., and Tassava, R. A. (1989). A developmentally regulated wound epithelial antigen of the newt limb regenerate is also present in a variety of secretory/transport cell types. *Dev Biol*, 135: 392-404.

Gordon, H., and Brockes, J. P. (1988). Appearance and regulation of an antigen associated with limb regeneration in Notophthalmus viridescens. *J Exp Zool*, 247: 232-243.

Gorman, S. D., and Cristofalo, V. J. (1985). Reinitiation of cellular DNA synthesis in BrdU-selected nondividing senescent WI-38 cells by simian virus 40 infection. *J Cell Physiol*, 125: 122-126.

Goss, R. J. (1969). "Principles of regeneration." Academic Press, New York.

Gossett, L. A., Kelvin, D. J., Sternberg, E. A., and Olson, E. N. (1989). A new myocyte-specific enhancer-binding factor that recognizes a conserved element associated with multiple muscle-specific genes. *Mol Cell Biol*, 9: 5022-5033.

Gould, K. L., and Nurse, P. (1989). Tyrosine phosphorylation of the fission yeast cdc2+ protein kinase regulates entry into mitosis. *Nature*, 342: 39-45.

Gregorio, C. C., Granzier, H., Sorimachi, H., and Labeit, S. (1999). Muscle assembly: a titanic achievement? *Curr Opin Cell Biol*, 11: 18-25.

Griffin, K. J., Fekete, D. M., and Carlson, B. M. (1987). A monoclonal antibody stains myogenic cells in regenerating newt muscle. *Development*, 101: 267-277.

Grounds, M. D. (1998). Age-associated changes in the response of skeletal muscle cells to exercise and regeneration. *Ann N Y Acad Sci*, 854: 78-91.

Gu, W., Schneider, J. W., Condorelli, G., Kaushal, S., Mahdavi, V., and Nadal-Ginard, B. (1993). Interaction of myogenic factors and the retinoblastoma protein mediates muscle cell commitment and differentiation. *Cell*, 72: 309-324.

Guan, K. L., Jenkins, C. W., Li, Y., Nichols, M. A., Wu, X., O'Keefe, C. L., Matera, A. G., and Xiong, Y. (1994). Growth suppression by p18, a p16INK4/MTS1- and p14INK4B/MTS2-related CDK6 inhibitor, correlates with wild-type pRb function. *Genes Dev*, 8: 2939.

Gulati, A. K., Zalewski, A. A., and Reddi, A. H. (1983). An immunofluorescent study of the distribution of fibronectin and laminin during limb regeneration in the adult newt. *Dev Biol*, 96: 355-365.

Guo, K., and Walsh, K. (1997). Inhibition of myogenesis by multiple cyclin-Cdk complexes. Coordinate regulation of myogenesis and cell cycle activity at the level of E2F. *J Biol Chem*, 272: 791-797.

Guo, K., Wang, J., Andres, V., Smith, R. C., and Walsh, K. (1995). MyoD-induced expression of p21 inhibits cyclin-dependent kinase activity upon myocyte terminal differentiation. *Mol Cell Biol*, 15: 3823-3829.

Gurdon, J. B. (1962a). Adult frogs derived from the nuclei of single somatic cells. *Dev Biol*, 4: 256-273.

Gurdon, J. B. (1962b). The developmental capacity of nuclei taken from intestinal epithelium cells of feeding tadpoles. *J Embryol Exp Morphol*, 10: 622-640.

Gurdon, J. B., Brennan, S., Fairman, S., and Mohun, T. J. (1984). Transcription of muscle-specific actin genes in early Xenopus development: nuclear transplantation and cell dissociation. *Cell*, 38: 691-700.

Gurdon, J. B., and Uehlinger, V. (1966). "Fertile" intestine nuclei. *Nature*, 210: 1240-1241.

Gussoni, E., Soneoka, Y., Strickland, C. D., Buzney, E. A., Khan, M. K., Flint, A. F., Kunkel, L. M., and Mulligan, R. C. (1999). Dystrophin expression in the mdx mouse restored by stem cell transplantation. *Nature*, 401: 390-394.

Hache, R. J., Tse, R., Reich, T., Savory, J. G., and Lefebvre, Y. A. (1999). Nucleocytoplasmic trafficking of steroid-free glucocorticoid receptor. *J Biol Chem*, 274: 1432-1439.

Hagting, A., Karlsson, C., Clute, P., Jackman, M., and Pines, J. (1998). MPF localization is controlled by nuclear export. *Embo J*, 17: 4127-4138.

Halevy, O., Novitch, B. G., Spicer, D. B., Skapek, S. X., Rhee, J., Hannon, G. J., Beach, D., and Lassar, A. B. (1995). Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD [see comments]. *Science*, 267: 1018-1021.

Hannon, G. J., and Beach, D. (1994). p15INK4B is a potential effector of TGF-beta-induced cell cycle arrest [see comments]. *Nature*, 371: 257-261.

Harbour, J. W., and Dean, D. C. (2000). Rb function in cell-cycle regulation and apoptosis. *Nat Cell Biol*, 2: E65-67.

Harbour, J. W., Luo, R. X., Dei Santi, A., Postigo, A. A., and Dean, D. C. (1999). Cdk phosphorylation triggers sequential intramolecular interactions that progressively block Rb functions as cells move through G1. *Cell*, 98: 859-869.

Harper, J. W., and Elledge, S. J. (1996). Cdk inhibitors in development and cancer. *Curr Opin Genet Dev*, 6: 56-64.

Hartley, R. S., Bandman, E., and Yablonka-Reuveni, Z. (1991). Myoblasts from fetal and adult skeletal muscle regulate myosin expression differently. *Dev Biol*, 148: 249-260.

Hartwell, L. H., and Kastan, M. B. (1994). Cell cycle control and cancer. *Science*, 266: 1821-1828.

Hasty, P., Bradley, A., Morris, J. H., Edmondson, D. G., Venuti, J. M., Olson, E. N., and Klein, W. H. (1993). Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene [see comments]. *Nature*, 364: 501-506.

Hatakeyama, M., and Weinberg, R. A. (1995). The role of RB in cell cycle control. *Prog Cell Cycle Res*, 1: 9-19.

Hauschka, S. D. (1974). Clonal analysis of vertebrate myogenesis. 3. Developmental changes in the muscle-colony-forming cells of the human fetal limb. *Dev Biol*, 37: 345-368.

Haushcka, S. D., Linkhart, T. A., Clegg, C., and Merrill, G. (1979). Clonal studies of human and mouse muscle. *In* "Muscle regeneration" (A. Mauro, Ed.). Raven Press, New York.

Hay, E. D. (1959). Electron microscopic observations of muscle dedifferentiation in regenerating *Amblystoma* limbs. *Dev Biol*, 1: 555-585.

Hay, E. D. (1962). Cytological studies of dedifferentiation and differentiation in regenerating amphibian limbs. *In* "Regeneration", pp. 177-210. Ronald Press, New York.

Hay, E. D. (1979). Reversibility of muscle differentiation in newt limb regeneration. *In* "Muscle regeneration" (A. Mauro, Ed.). Raven Press, New York.

Hay, E. D., and Fischman, D. A. (1961). Origin of the blastema in regenerating limbs of the newt *Triturus viridescens*. *Dev Biol*, 3: 26-59.

Heald, R., McLoughlin, M., and McKeon, F. (1993). Human wee1 maintains mitotic timing by protecting the nucleus from cytoplasmically activated Cdc2 kinase. *Cell*, 74: 463-474.

Hendzel, M. J., Wei, Y., Mancini, M. A., Van Hooser, A., Ranalli, T., Brinkley, B. R., Bazett-Jones, D. P., and Allis, C. D. (1997). Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. *Chromosoma*, 106: 348-360.

Hill, R. E., Jones, P. F., Rees, A. R., Sime, C. M., Justice, M. J., Copeland, N. G., Jenkins, N. A., Graham, E., and Davidson, D. R. (1989). A new family of homeobox-containing genes: molecular structure, chromosomal location , and developmental expression of Hox-7. *Genes Dev*, 3: 26-37.

Hinchcliffe, E. H., Cassels, G. O., Rieder, C. L., and Sluder, G. (1998). The coordination of centrosome reproduction with nuclear events of the cell cycle in the sea urchin zygote. *J Cell Biol*, 140: 1417-1426.

Hinterberger, T. J., Sassoon, D. A., Rhodes, S. J., and Konieczny, S. F. (1991). Expression of the muscle regulatory factor MRF4 during somite and skeletal myofiber development. *Dev Biol*, 147: 144-156.

Hondermarck, H., and Boilly, B. (1992). Characterization of fibroblast growth factor binding in regenerating limb blastemas of axolotls. *Monogr Dev Biol*, 23: 110-115.

Howard, A., and Pelc, S. R. (1951). Nuclear incorporation of P^{32} as demonstrated by autoradiographs. *Exp Cell Res*, 2: 178-187.

Howe, A., Aplin, A. E., Alahari, S. K., and Juliano, R. L. (1998). Integrin signaling and cell growth control. *Curr Opin Cell Biol*, 10: 220-231.

Hua, X. H., and Newport, J. (1998). Identification of a preinitiation step in DNA replication that is independent of origin recognition complex and cdc6, but dependent on cdk2. *J Cell Biol*, 140: 271-281.

Huang, R., Zhi, Q., Neubuser, A., Muller, T. S., Brand-Saberi, B., Christ, B., and Wilting, J. (1996). Function of somite and somitocoele cells in the formation of the vertebral motion segment in avian embryos. *Acta Anat*, 155: 231-241.

Hughes, S. M., and Salinas, P. C. (1999). Control of muscle fibre and motoneuron diversification. *Curr Opin Neurobiol*, 9: 54-64.

Imokawa, Y., and Yoshizato, K. (1997). Expression of Sonic hedgehog gene in regenerating newt limb blastemas recapitulates that in developing limb buds. *Proc Natl Acad Sci U S A*, 94: 9159-9164.

Ingham, P. W., and Fietz, M. J. (1995). Quantitative effects of hedgehog and decapentaplegic activity on the patterning of the Drosophila wing. *Curr Biol*, 5: 432-440.

Iten, L. E., and Bryant, S. V. (1973). Forelimb regeneration from different levels of amputation in the newt, *Notophthalmus viridescens*: length, rate and stages. *Roux' Archiv für Entwicklungsmechanik der Organismen*, 173: 263-282.

Itoh, M., Takeda, S., Yamamoto, H., Izumi, S., Tomino, S., and Eguchi, M. (1991). Cloning and sequence analysis of membrane-bound alkaline phosphatase cDNA of the silkworm, Bombyx mori. *Biochim Biophys Acta*, 1129: 135-138.

Itoh, Y., and Eguchi, G. (1986). In vitro analysis of cellular metaplasia from pigmented epithelial cells to lens phenotypes: a unique model system for studying cellular and molecular mechanisms of "transdifferentiation". *Dev Biol*, 115: 353-362.

Ivjvidin, S., Fuchs, O., Nudel, U., and Yaffe, D. (1990). SV40 immortalizes myogenic cells: DNA synthesis and mitosis in differentiating myotubes. *Differentiation*, 43: 192-203.

Jacks, T., Fazeli, A., Schmitt, E. M., Bronson, R. T., Goodell, M. A., and Weinberg, R. A. (1992). Effects of an Rb mutation in the mouse [see comments]. *Nature*, 359: 295-300.

Jaffrey, P. D., Russo, A. A., and Polyak, K. (1995). Mechanism of cdk activation revealed byuu the structure of a cyclin A-cdk2 complex. *Nature*, 376: 313-320.

Jahn, L., Sadoshima, J., and Izumo, S. (1994). Cyclins and cyclin-dependent kinases are differentially regulated during terminal differentiation of C2C12 muscle cells. *Exp Cell Res*, 212: 297-307.

Jallepalli, P. V., Brown, G. W., Muzi-Falconi, M., Tien, D., and Kelly, T. J. (1997). Regulation of the replication initiator protein p65cdc18 by CDK phosphorylation. *Genes Dev*, 11: 2767-2779.

Jin, P., Gu, Y., and Morgan, D. O. (1996). Role of inhibitory CDC2 phosphorylation in radiation-induced G2 arrest in human cells. *J Cell Biol*, 134: 963-970.

Jin, P., Hardy, S., and Morgan, D. O. (1998). Nuclear localization of cyclin B1 controls mitotic entry after DNA damage. *J Cell Biol*, 141: 875-885.

Johnson, D. G., Schwarz, J. K., Cress, W. D., and Nevins, J. R. (1993). Expression of transcription factor E2F1 induces quiescent cells to enter S phase. *Nature*, 365: 349-352.

Johnson, R. T., and Rao, P. N. (1970). Mammalian cell fusion: induction of premature chromosome condensation in interphase nuclei. *Nature*, 226: 717-722.

Kablar, B., Krastel, K., Ying, C., Asakura, A., Tapscott, S. J., and Rudnicki, M. A. (1997). MyoD and Myf-5 differentially regulate the development of limb versus trunk skeletal muscle. *Development*, 124: 4729-4738.

Kaelin, W. G. J. (1999). Functions of the retinoblastoma protein. *Bioessays*, 21: 950-958.

Kamrin, A. A., and Singer, M. (1959). The growth influence of spinal ganglia implanted into the denervated forelimb regenerate of the newt, *Triturus*. *J Morphol*, 104: 415-439.

Karaiskou, A., Jessus, C., Brassac, T., and Ozon, R. (1999). Phosphatase 2A and polo kinase, two antagonistic regulators of cdc25 activation and MPF auto-amplification. *J Cell Sci*, 112: 3747-3756.

Karlsson, C., Katich, S., Hagting, A., Hoffmann, I., and Pines, J. (1999). Cdc25B and Cdc25C differ markedly in their properties as initiators of mitosis. *J Cell Biol*, 146: 573-584.

Kastan, M. B., Onyekwere, O., Sidransky, D., Vogelstein, B., and Craig, R. W. (1991). Participation of p53 protein in the cellular response to DNA damage. *Cancer Res*, 51: 6304-6311.

Kato, H., Taniguchi, Y., Kurooka, H., Minoguchi, S., Sakai, T., Nomura-Okazaki, S., Tamura, K., and Honjo, T. (1997). Involvement of RBP-J in biological functions of mouse Notch1 and its derivatives. *Development*, 124: 4133-4141.

Katzen, A. L., Jackson, J., Harmon, B. P., Fung, S. M., Ramsay, G., and Bishop, J. M. (1998). Drosophila myb is required for the G2/M transition and maintenance of diploidy. *Genes Dev*, 12: 831-843.

Kaufman, S. J., George-Weinstein, M., and Foster, R. F. (1991). In vitro development of precursor cells in the myogenic lineage. *Dev Biol*, 146: 228-238.

Kaushal, S., Schneider, J. W., Nadal-Ginard, B., and Mahdavi, V. (1994). Activation of the myogenic lineage by MEF2A, a factor that induces and cooperates with MyoD. *Science*, 266: 1236-1240.

Keller, C. A., Grill, M. A., and Abmayr, S. M. (1998). A role for nautilus in the differentiation of muscle precursors. *Dev Biol*, 202: 157-171.

Kelly, D. J., and Tassava, R. A. (1973). Cell division and ribonucleic acid synthesis during the initiation of limb regeneration in larval axolotls (Ambystoma mexicanum). *J Exp Zool*, 185: 45-54.

King, R. W., Peters, J. M., Tugendreich, S., Rolfe, M., Hieter, P., and Kirschner, M. W. (1995). A 20S complex containing CDC27 and CDC16 catalyzes the mitosis-specific conjugation of ubiquitin to cyclin B. *Cell*, 81: 279-288.

Kintner, C. R., and Brockes, J. P. (1984). Monoclonal antibodies identify blastemal cells derived from dedifferentiating limb regeneration. *Nature*, 308: 67-69.

Kintner, C. R., and Brockes, J. P. (1985). Monoclonal antibodies to the cells of a regenerating limb. *J Embryol Exp Morphol*, 89: 37-55.

Kirshenbaum, L. A., Abdellatif, M., Chakraborty, S., and Schneider, M. D. (1996). Human E2F-1 reactivates cell cycle progression in ventricular myocytes and represses cardiac gene transcription. *Dev Biol*, 179: 402-411.

Kitzmann, M., Carnac, G., Vandromme, M., Primig, M., Lamb, N. J., and Fernandez, A. (1998). The muscle regulatory factors MyoD and myf-5 undergo distinct cell cycle-specific expression in muscle cells. *J Cell Biol*, 142: 1447-1459.

Klatt, K. P., Yang, E. V., and Tassava, R. A. (1992). Monoclonal antibody MT2 identifies an extracellular matrix glycoprotein that is co-localized with tenascin during adult newt limb regeneration. *Differentiation*, 50: 133-140.

Knudsen, E. S., and Wang, J. Y. (1996). Differential regulation of retinoblastoma protein function by specific Cdk phosphorylation sites. *J Biol Chem*, 271: 8313-8320.

Ko, L. J., and Prives, C. (1996). p53: puzzle and paradigm. *Genes Dev*, 10: 1054-1072.

Koishi, K., Zhang, M., McLennan, I. S., and Harris, A. J. (1995). MyoD protein accumulates in satellite cells and is neurally regulated in regenerating myotubes and skeletal muscle fibers. *Dev Dyn*, 202: 244-254.

Komarova, E. A., Zelnick, C. R., Chin, D., Zeremski, M., Gleiberman, A. S., Bacus, S. S., and Gudkov, A. V. (1997). Intracellular localization of p53 tumor suppressor protein in gamma- irradiated cells is cell cycle regulated and determined by the nucleus. *Cancer Res*, 57: 5217-5220.

Koshiba, K., Kuroiwa, A., Yamamoto, H., Tamura, K., and Ide, H. (1998). Expression of Msx genes in regenerating and developing limbs of axolotl. *J Exp Zool*, 282: 703-714.

Krek, W., Ewen, M. E., Shirodkar, S., Arany, Z., Kaelin, W. G., Jr., and Livingston, D. M. (1994). Negative regulation of the growth-promoting transcription factor E2F-1 by a stably bound cyclin A-dependent protein kinase. *Cell*, 78: 161-172.

Krude, T., Jackman, M., Pines, J., and Laskey, R. A. (1997). Cyclin/Cdk-dependent initiation of DNA replication in a human cell-free system. *Cell*, 88: 109-119.

Kujawa, M. J., Pechak, D. G., Fiszman, M. Y., and Caplan, A. I. (1986). Hyaluronic acid bonded to cell culture surfaces inhibits the program of myogenesis. *Dev Biol*, 113: 10-16.

Kujawa, M. J., and Tepperman, K. (1983). Culturing chick muscle cells on glycosaminoglycan substrates: attachment and differentiation. *Dev Biol*, 99: 277-286.

Kumagai, A., and Dunphy, W. G. (1996). Purification and molecular cloning of Plx1, a Cdc25-regulatory kinase from Xenopus egg extracts. *Science*, 273: 1377-1380.

Kumagai, A., and Dunphy, W. G. (1997). Regulation of Xenopus Cdc25 protein. *Methods Enzymol*, 283: 564-571.

Kumagai, A., Guo, Z., Emami, K. H., Wang, S. X., and Dunphy, W. G. (1998). The Xenopus Chk1 protein kinase mediates a caffeine-sensitive pathway of checkpoint control in cell-free extracts. *J Cell Biol*, 142: 1559-1569.

Kumar, A., Velloso, C. P., Imokawa, Y., and Brockes, J. P. (2000). Plasticity of retrovirus-labelled myotubes in the newt limb regeneration blastema. *Dev Biol*, 218: 125-136.

La Rocca, S. A., Crouch, D. H., and Gillespie, D. A. (1994). c-Myc inhibits myogenic differentiation and myoD expression by a mechanism which can be dissociated from cell transformation. *Oncogene*, 9: 3499-3508.

La Thangue, N. B. (1994). DP and E2F proteins: components of a heterodimeric transcription factor implicated in cell cycle control. *Curr Opin Cell Biol*, 6: 443-450.

LaBaer, J., Garrett, M. D., Stevenson, L. F., Slingerland, J. M., Sandhu, C., Chou, H. S., Fattaey, A., and Harlow, E. (1997). New functional activities for the p21 family of CDK inhibitors. *Genes Dev*, 11: 847-862.

Lakin, N. D., and Jackson, S. P. (1999). Regulation of p53 in response to DNA damage. *Oncogene*, 18: 7644-7655.

Lassar, A., and Munsterberg, A. (1994). Wiring diagrams: regulatory circuits and the control of skeletal myogenesis. *Curr Opin Cell Biol*, 6: 432-442.

Lassar, A. B., Buskin, J. N., Lockshon, D., Davis, R. L., Apone, S., Hauschka, S. D., and Weintraub, H. (1989). MyoD is a sequence-specific DNA binding protein requiring a region of myc homology to bind to the muscle creatine kinase enhancer. *Cell*, 58: 823-831.

Lassar, A. B., Davis, R. L., Wright, W. E., Kadesch, T., Murre, C., Voronova, A., Baltimore, D., and Weintraub, H. (1991). Functional activity of myogenic HLH proteins requires hetero- oligomerization with E12/E47-like proteins in vivo. *Cell*, 66: 305-315.

Lassar, A. B., Skapek, S. X., and Novitch, B. (1994). Regulatory mechanisms that coordinate skeletal muscle differentiation and cell cycle withdrawal. *Curr Opin Cell Biol*, 6: 788-794.

Latella, L., Sacchi, A., and Crescenzi, M. (2000). Long-term fate of terminally differentiated skeletal muscle cells following E1A-initiated cell cycle reactivation. *Cell Death Differ*, 7: 145-154.

Lavoie, J. N., L'Allemain, G., Brunet, A., Muller, R., and Pouyssegur, J. (1996). Cyclin D1 expression is regulated positively by the p42/p44MAPK and negatively by the p38/HOGMAPK pathway. *J Biol Chem*, 271: 20608-20616.

Leatherwood, J. (1998). Emerging mechanisms of eukaryotic DNA replication initiation. *Curr Opin Cell Biol*, 10: 742-748.

Lee, E. Y., Chang, C. Y., Hu, N., Wang, Y. C., Lai, C. C., Herrup, K., Lee, W. H., and Bradley, A. (1992). Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis [see comments]. *Nature*, 359: 288-294.

Lee, M. H., Reynisdottir, I., and Massague, J. (1995). Cloning of p57KIP2, a cyclin-dependent kinase inhibitor with unique domain structure and tissue distribution. *Genes Dev*, 9: 639-649.

Lehner, C. F., and O'Farrell, P. H. (1989). Expression and function of Drosophila cyclin A during embryonic cell cycle progression. *Cell*, 56: 957-968.

Leone, G., DeGregori, J., Yan, Z., Jakoi, L., Ishida, S., Williams, R. S., and Nevins, J. R. (1998). E2F3 activity is regulated during the cell cycle and is required for the induction of S phase. *Genes Dev*, 12: 2120-2130.

Levine, A. J. (1997). p53, the cellular gatekeeper for growth and division. *Cell*, 88: 323-331.

Lew, D. J., and Kornbluth, S. (1996). Regulatory roles of cyclin dependent kinase phosphorylation in cell cycle control. *Curr Opin Cell Biol*, 8: 795-804.

Lheureux, E., and Carey, F. (1988). The irradiated epidermis inhibits newt limb regeneration by preventing blastema growth. A histological study. *Biol Struct Morphog*, 1: 49-57.

Li, J., Meyer, A. N., and Donoghue, D. J. (1997). Nuclear localization of cyclin B1 mediates its biological activity and is regulated by phosphorylation. *Proc Natl Acad Sci U S A*, 94: 502-507.

Lilly, B., Zhao, B., Ranganayakulu, G., Paterson, B. M., Schulz, R. A., and Olson, E. N. (1995). Requirement of MADS domain transcription factor D-MEF2 for muscle formation in Drosophila. *Science*, 267: 688-693.

Lin, M. H., Bour, B. A., Abmayr, S. M., and Storti, R. V. (1997). Ectopic expression of MEF2 in the epidermis induces epidermal expression of muscle genes and abnormal muscle development in Drosophila. *Dev Biol*, 182: 240-255.

Lo, D. C., Allen, F., and Brockes, J. P. (1993). Reversal of muscle differentiation during urodele limb regeneration. *Proc Natl Acad Sci U S A*, 90: 7230-7234.

Lopez-Girona, A., Furnari, B., Mondesert, O., and Russell, P. (1999). Nuclear localization of Cdc25 is regulated by DNA damage and a 14-3-3 protein [see comments]. *Nature*, 397: 172-175.

Lopez-Girona, A., Mondesert, O., Leatherwood, J., and Russell, P. (1998). Negative regulation of Cdc18 DNA replication protein by Cdc2. *Mol Biol Cell*, 9: 63-73.

Ludlow, J. W., DeCaprio, J. A., Huang, C. M., Lee, W. H., Paucha, E., and Livingston, D. M. (1989). SV40 large T antigen binds preferentially to an underphosphorylated member of the retinoblastoma susceptibility gene product family. *Cell*, 57-65.

Lukas, J., Parry, D., Aagaard, L., Mann, D. J., Bartkova, J., Strauss, M., Peters, G., and Bartek, J. (1995). Retinoblastoma-protein-dependent cell-cycle inhibition by the tumour suppressor p16. *Nature*, 375: 503-506.

Lukas, J., Petersen, B. O., Holm, K., Bartek, J., and Helin, K. (1996). Deregulated expression of E2F family members induces S-phase entry and overcomes p16INK4A-mediated growth suppression. *Mol Cell Biol*, 16: 1047-1057.

Lundberg, A. S., and Weinberg, R. A. (1998). Functional inactivation of the retinoblastoma protein requires sequential modification by at least two distinct cyclin-cdk complexes. *Mol Cell Biol*, 18: 753-761.

MacAuley, A., Cross, J. C., and Werb, Z. (1998). Reprogramming the cell cycle for endoreduplication in rodent trophoblast cells. *Mol Biol Cell*, 9: 795-807.

MacLeod, K. F., Sherry, N., Hannon, G., Beach, D., Tokino, T., Kinzler, K., Vogelstein, B., and Jacks, T. (1995). p53-dependent and independent expression of p21 during cell growth, differentiation, and DNA damage. *Genes Dev*, 9: 935-944.

Maden, M. (1979). Neurotrophic and x-ray blocks in the blastemal cell cycle. *J Embryol Exp Morphol*, 50: 169-173.

Magnaghi-Jaulin, L., Groisman, R., Naguibneva, I., Robin, P., Lorain, S., Le Villain, J. P., Troalen, F., Trouche, D., and Harel-Bellan, A. (1998). Retinoblastoma protein represses transcription by recruiting a histone deacetylase [see comments]. *Nature*, 391: 601-605.

Mailman, M. L., and Dresden, M. H. (1976). Collagen metabolism in the regenerating forelimb of Notophthalmus viridescens: synthesis, accumulation, and maturation. *Dev Biol*, 50: 378-394.

Mal, A., Chattopadhyay, D., Ghosh, M. K., Poon, R. Y., Hunter, T., and Harter, M. L. (2000). p21 and retinoblastoma protein control the absence of DNA replication in terminally differentiated muscle cells. *J Cell Biol*, 149: 281-292.

Mankoo, B. S., Collins, N. S., Ashby, P., Grigorieva, E., Pevny, L. H., Candia, A., Wright, C. V., Rigby, P. W., and Pachnis, V. (1999). Mox2 is a component of the genetic hierarchy controlling limb muscle development. *Nature*, 400: 69-73.

Maroto, M., Reshef, R., Munsterberg, A. E., Koester, S., Goulding, M., and Lassar, A. B. (1997). Ectopic Pax-3 activates MyoD and Myf-5 expression in embryonic mesoderm and neural tissue. *Cell*, 89: 139-148.

Matsuoka, S., Edwards, M. C., Bai, C., Parker, S., Zhang, P., Baldini, A., Harper, J. W., and Elledge, S. J. (1995). p57KIP2, a structurally distinct member of the p21CIP1 Cdk inhibitor family, is a candidate tumor suppressor gene. *Genes Dev*, 9: 650-662.

Mauro, A. (1961). Satellite cells of skeletal muscle fibers. *J Biophys Biochem Cytol*, 9: 493-495.

McLellan, W. R., and Schneider, M. D. (1999). The cardiac cell cycle. *In* "Heart Development" (R. P. Harvey and N. Rosenthal, Eds.). Academic Press, New York.

Medema, R. H., Herrera, R. E., Lam, F., and Weinberg, R. A. (1995). Growth suppression by p16ink4 requires functional retinoblastoma protein. *Proc Natl Acad Sci U S A*, 92: 6289-6293.

Megeney, L. A., Kablar, B., Garrett, K., Anderson, J. E., and Rudnicki, M. A. (1996). MyoD is required for myogenic stem cell function in adult skeletal muscle. *Genes Dev*, 10: 1173-1183.

Mescher, A. L., and Cox, C. A. (1988). Hyaluronate accumulation and nerve-dependent growth during regeneration of larval Ambystoma limbs. *Differentiation*, 38: 161-168.

Mescher, A. L., and Tassava, R. A. (1975). Denervation effects on DNA replication and mitosis during the initiation of limb regeneration in adult newts. *Dev Biol*, 44: 187-197.

Meyer, K. M., Hess, S. M., Tlsty, T. D., and Leadon, S. A. (1999). Human mammary epithelial cells exhibit a differential p53-mediated response following exposure to ionizing radiation or UV light. *Oncogene*, 18: 5795-5805.

Michael, W. M., and Newport, J. (1998). Coupling of mitosis to the completion of S phase through Cdc34-mediated degradation of Wee1 [published errata appear in Science 1999 Jan 1;283(5398):35 and 1999 Mar 26;283(5410):2102]. *Science*, 282: 1886-1889.

Miller, J. B., Crow, M. T., and Stockdale, F. E. (1985). Slow and fast myosin heavy chain content defines three types of myotubes in early muscle cell cultures. *J Cell Biol*, 101: 1643-1650.

Miller, J. B., Schaefer, L., and Dominov, J. A. (1999). Seeking muscle stem cells. *Curr Top Dev Biol*, 43: 191-219.

Miner, J. H., and Wold, B. (1990). Herculin, a fourth member of the MyoD family of myogenic regulatory genes. *Proc Natl Acad Sci U S A*, 87: 1089-1093.

Minshull, J., Pines, J., Golsteyn, R., Standart, N., Mackie, S., Colman, A., Blow, J., Ruderman, J. V., Wu, M., and Hunt, T. (1989). The role of cyclin synthesis, modification and destruction in the control of cell division. *J Cell Sci Suppl*, 12: 77-97.

Miyazaki, K., Uchiyama, K., Imokawa, Y., and Yoshizato, K. (1996). Cloning and characterization of cDNAs for matrix metalloproteinases of regenerating newt limbs. *Proc Natl Acad Sci U S A*, 93: 6819-6824.

Molkentin, J. D., Black, B. L., Martin, J. F., and Olson, E. N. (1995). Cooperative activation of muscle gene expression by MEF2 and myogenic bHLH proteins. *Cell*, 83: 1125-1136.

Montarras, D., Chelly, J., Bober, E., Arnold, H., Ott, M. O., Gros, F., and Pinset, C. (1991). Developmental patterns in the expression of Myf5, MyoD, myogenin, and MRF4 during myogenesis. *New Biol*, 3: 592-600.

Mook-Jung, I., and Gordon, H. (1996). Acetylcholine receptor clustering associates with proteoglycan biosynthesis in C2 variant and heterkaryon muscle cells. *J Neurobiol*, 31: 210-218.

Moore, J. D., Yang, J., Truant, R., and Kornbluth, S. (1999). Nuclear import of Cdk/cyclin complexes: identification of distinct mechanisms for import of Cdk2/cyclin E and Cdc2/cyclin B1. *J Cell Biol*, 144: 213-224.

Morgan, D. O. (1995). Principles of CDK regulation. *Nature*, 374: 131-134.

Morgan, D. O. (1997). Cyclin-dependent kinases: engines, clocks, and microprocessors. *Annu Rev Cell Dev Biol*, 13: 261-291.

Morgenbesser, S. D., Williams, B. O., Jacks, T., and DePinho, R. A. (1994). p53-dependent apoptosis produced by Rb-deficiency in the developing mouse lens [see comments]. *Nature*, 371: 72-74.

Moss, F. P., and Leblond, C. P. (1971). Satellite cells as the source of nuclei in muscles of growing rats. *Anat Rec*, 170: 421-435.

Mueller, P. R., Coleman, T. R., Kumagai, A., and Dunphy, W. G. (1995). Myt1: a membrane-associated inhibitory kinase that phosphorylates Cdc2 on both threonine-14 and tyrosine-15. *Science*, 270: 86-90.

Mullen, L. M., Bryant, S. V., Torok, M. A., Blumberg, B., and Gardiner, D. M. (1996). Nerve dependency of regeneration: the role of Distal-less and FGF signaling in amphibian limb regeneration. *Development*, 122: 3487-3497.

Mulligan, G., and Jacks, T. (1998). The retinoblastoma gene family: cousins with overlapping interests. *Trends Genet*, 14: 223-229.

Munaim, S. I., and Mescher, A. L. (1986). Transferrin and the trophic effect of neural tissue on amphibian limb regeneration blastemas. *Dev Biol*, 116: 138-142.

Muneoka, K., and Bryant, S. V. (1984). Cellular contribution to supernumerary limbs in the axolotl, Ambystoma mexicanum. *Dev Biol*, 105: 166-178.

Muneoka, K., Fox, W. F., and Bryant, S. V. (1986). Cellular contribution from dermis and cartilage to the regenerating limb blastema in axolotls. *Dev Biol*, 116: 256-260.

Muneoka, K., and Sassoon, D. (1992). Molecular aspects of regeneration in developing vertebrate limbs. *Dev Biol*, 152: 37-49.

Nabeshima, Y., Hanaoka, K., Hayasaka, M., Esumi, E., Li, S., and Nonaka, I. (1993). Myogenin gene disruption results in perinatal lethality because of severe muscle defect [see comments]. *Nature*, 364: 532-535.

Nadal-Ginard, B. (1978). Commitment, fusion and biochemical differentiation of a myogenic cell line in the absence of DNA synthesis. *Cell*, 15: 855-864.

Naidu, P. S., Ludolph, D. C., To, R. Q., Hinterberger, T. J., and Konieczny, S. F. (1995). Myogenin and MEF2 function synergistically to activate the MRF4 promoter during myogenesis. *Mol Cell Biol*, 15: 2707-2718.

Namenwirth, M. (1974). The inheritance of cell differentiation during limb regeneration in the axolotl. *Dev Biol*, 41: 42-56.

Nasmyth, K. (1996). Viewpoint: putting the cell cycle in order. *Science*, 274: 1643-1645.

Nasmyth, K. (1999). Separating sister chromatids. *Trends Biochem Sci*, 24: 98-104.

Nead, M. A., Baglia, L. A., Antinore, M. J., Ludlow, J. W., and McCance, D. J. (1998). Rb binds c-Jun and activates transcription. *Embo J*, 17: 2342-2352.

Neufeld, D. A., and Aulthouse, A. L. (1986). Association of mesenchyme with attenuated basement membranes during morphogenetic stages of newt limb regeneration. *Am J Anat*, 176: 411-421.

Niswander, L., Tickle, C., Vogel, A., and Martin, G. (1994). Function of FGF-4 in limb development. *Mol Reprod Dev*, 39: 83-88; discussion 88-89.

Novitch, B. G., Mulligan, G. J., Jacks, T., and Lassar, A. B. (1996). Skeletal muscle cells lacking the retinoblastoma protein display defects in muscle gene expression and accumulate in S and G2 phases of the cell cycle. *J Cell Biol*, 135: 441-456.

Novitch, B. G., Spicer, D. B., Kim, P. S., Cheung, W. L., and Lassar, A. B. (1999). pRb is required for MEF2-dependent gene expression as well as cell- cycle arrest during skeletal muscle differentiation. *Curr Biol*, 9: 449-459.

Nurse, P. (1997). Checkpoint pathways come of age. *Cell*, 91: 865-867.

Oberpriller, J. O., and Oberpriller, J. C. (1974). Response of the adult newt ventricle to injury. *J Exp Zool*, 187: 249-253.

Oberpriller, J. O., Oberpriller, J. C., Matz, D. G., and Soonpaa, M. H. (1995). Stimulation of proliferative events in the adult amphibian cardiac myocyte. *Ann N Y Acad Sci*, 752: 30-46.

Ogris, E., Rotheneder, H., Mudrak, I., Pichler, A., and Wintersberger, E. (1993). A binding site for transcription factor E2F is a target for trans activation of murine thymidine kinase by polyomavirus large T antigen and plays an important role in growth regulation of the gene. *J Virol*, 67: 1765-1771.

Ohi, R., and Gould, K. L. (1999). Regulating the onset of mitosis. *Curr Opin Cell Biol*, 11: 167-173.

Ohkubo, Y., Kishimoto, T., Nakata, T., Yasuda, H., and Endo, T. (1994). SV40 large T antigen reinduces the cell cycle in terminally differentiated myotubes through inducing Cdk2, Cdc2, and their partner cyclins. *Exp Cell Res*, 214: 270-278.

Ohtsubo, M., and Roberts, J. M. (1993). Cyclin-dependent regulation of G1 in mammalian fibroblasts. *Science*, 259: 1908-1912.

Ohtsubo, M., Theodoras, A. M., Schumacher, J., Roberts, J. M., and Pagano, M. (1995). Human cyclin E, a nuclear protein essential for the G1-to-S phase transition. *Mol Cell Biol*, 15: 2612-2624.

Okada, T. S. (1991). "Transdifferentiation." Clarendon Press, Oxford.

Okada, T. S., Yasuda, K., Araki, M., and Eguchi, G. (1979). Possible demonstration of multipotential nature of embryonic neural retina by clonal cell culture. *Dev Biol*, 68: 600-617.

Okazaki, K., and Holtzer, H. (1966). Myogenesis: fusion, myosin synthesis, and the mitotic cycle. *Proc Natl Acad Sci U S A*, 56: 1484-1490.

Olsen, C. L., and Tassava, R. A. (1984). Cell cycle and histological effects of reinnervation in denervated forelimb stumps of larval Ambystoma. *J Exp Zool*, 229: 247-258.

Olson, E. N. (1992). Interplay between proliferation and differentiation within the myogenic lineage. *Dev Biol*, 154: 261-272.

Olson, E. N., Arnold, H. H., Rigby, P. W., and Wold, B. J. (1996). Know your neighbors: three phenotypes in null mutants of the myogenic bHLH gene MRF4. *Cell*, 85: 1-4.

Olson, E. N., and Klein, W. H. (1994). bHLH factors in muscle development: dead lines and commitments, what to leave in and what to leave out. *Genes Dev*, 8: 1-8.

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

Olson, E. N., and Williams, R. S. (2000). Remodeling muscles with calcineurin [In Process Citation]. *Bioessays*, 22: 510-519.

Onda, H., Goldhamer, D. J., and Tassava, R. A. (1990). An extracellular matrix molecule of newt and axolotl regenerating limb blastemas and embryonic limb buds: immunological relationship of MT1 antigen with tenascin. *Development*, 108: 657-668.

Onda, H., Poulin, M. L., Tassava, R. A., and Chiu, I. M. (1991). Characterization of a newt tenascin cDNA and localization of tenascin mRNA during newt limb regeneration by in situ hybridization. *Dev Biol*, 148: 219-232.

Onda, H., and Tassava, R. A. (1991). Expression of the 9G1 antigen in the apical cap of axolotl regenerates requires nerves and mesenchyme. *J Exp Zool*, 257: 336-349.

Opas, M., and Dziak, E. (1998). Direct transdifferentiation in the vertebrate retina. *Int J Dev Biol*, 42: 199-206.

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

Ordahl, C. P., Williams, B. A., and Denetclaw, W. (2000). Determination and morphogenesis in myogenic progenitor cells: an experimental embryological approach. *Curr Top Dev Biol*, 48: 319-367.

Ornatsky, O. I., Andreucci, J. J., and McDermott, J. C. (1997). A dominant-negative form of transcription factor MEF2 inhibits myogenesis. *J Biol Chem*, 272: 33271-33278.

Ornatsky, O. I., and McDermott, J. C. (1996). MEF2 protein expression, DNA binding specificity and complex composition, and transcriptional activity in muscle and non-muscle cells. *J Biol Chem*, 271: 24927-24933.

Ott, M. O., Bober, E., Lyons, G., Arnold, H., and Buckingham, M. (1991). Early expression of the myogenic regulatory gene, myf-5, in precursor cells of skeletal muscle in the mouse embryo. *Development*, 111: 1097-1107.

Pajalunga, D., Tognozzi, D., Tiainen, M., D'Angelo, M., Ferrantelli, F., Helin, K., Sacchi, A., and Crescenzi, M. (1999). E2F activates late-G1 events but cannot replace E1A in inducing S phase in terminally differentiated skeletal muscle cells. *Oncogene*, 18: 5054-5062.

Panganiban, G., Irvine, S. M., Lowe, C., Roehl, H., Corley, L. S., Sherbon, B., Grenier, J. K., Fallon, J. F., Kimble, J., Walker, M., Wray, G. A., Swalla, B. J., Martindale, M. Q., and Carroll, S. B. (1997). The origin and evolution of animal appendages. *Proc Natl Acad Sci U S A*, 94: 5162-5166.

Pardee, A. B. (1989). G1 events and regulation of cell proliferation. *Science*, 246: 603-608.

Parker, S. B., Eichele, G., Zhang, P., Rawls, A., Sands, A. T., Bradley, A., Olson, E. N., Harper, J. W., and Elledge, S. J. (1995). p53-independent expression of p21Cip1 in muscle and other terminally differentiating cells [see comments]. *Science*, 267: 1024-1027.

Pavlath, G. K., Rich, K., Webster, S. G., and Blau, H. M. (1989). Localization of muscle gene products in nuclear domains. *Nature*, 337: 570-573.

Peng, C. Y., Graves, P. R., Thoma, R. S., Wu, Z., Shaw, A. S., and Piwnica-Worms, H. (1997). Mitotic and G2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216 [see comments]. *Science*, 277: 1501-1505.

Peng, H. B., Wolosewick, J. J., and Cheng, P. C. (1981). The development of myofibrils in cultured muscle cells: a whole-mount and thin-section electron microscopic study. *Dev Biol*, 88: 121-136.

Pescitelli, M. J., Jr., and Stocum, D. L. (1980). The origin of skeletal structures during intercalary regeneration of larval Ambystoma limbs. *Dev Biol*, 79: 255-275.

Petersen, B. O., Lukas, J., Sorensen, C. S., Bartek, J., and Helin, K. (1999). Phosphorylation of mammalian CDC6 by cyclin A/CDK2 regulates its subcellular localization. *Embo J*, 18: 396-410.

Phelps, D. E., Hsiao, K. M., Li, Y., Hu, N., Franklin, D. S., Westphal, E., Lee, E. Y., and Xiong, Y. (1998). Coupled transcriptional and translational control of cyclin-dependent kinase inhibitor p18INK4c expression during myogenesis. *Mol Cell Biol*, 18: 2334-2343.

Pines, J. (1996). Cyclin from sea urchins to HeLas: making the human cell cycle. *Biochem Soc Trans*, 24: 15-33.

Pines, J. (1999a). Cell cycle. Checkpoint on the nuclear frontier [news; comment]. *Nature*, 397: 104-105.

Pines, J. (1999b). Four-dimensional control of the cell cycle. *Nat Cell Biol*, 1: E73-79.

Pines, J., and Hunter, T. (1991). Human cyclins A and B1 are differentially located in the cell and undergo cell cycle-dependent nuclear transport. *J Cell Biol*, 115: 1-17.

Polyak, K., Lee, M. H., Erdjument-Bromage, H., Koff, A., Roberts, J. M., Tempst, P., and Massague, J. (1994). Cloning of p27Kip1, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. *Cell*, 78: 59-66.

Popiela, H. (1976). Muscle satellite cells in urodele amphibians: faciliatated identification of satellite cells using ruthenium red staining. *J Exp Zool*, 198: 57-64.

Poulin, M. L., and Chiu, I. M. (1995). Re-programming of expression of the KGFR and bek variants of fibroblast growth factor receptor 2 during limb regeneration in newts (Notophthalmus viridescens). *Dev Dyn*, 202: 378-387.

Poulin, M. L., Patrie, K. M., Botelho, M. J., Tassava, R. A., and Chiu, I. M. (1993). Heterogeneity in the expression of fibroblast growth factor receptors during limb regeneration in newts (Notophthalmus viridescens). *Development*, 119: 353-361.

Powell, J. A. (1969). Analysis of histogenesis and regenerative ability of denervated forelimb regenerates of Triturus viridescens. *J Exp Zool*, 170: 125-147.

Puri, P. L., Balsano, C., Burgio, V. L., Chirillo, P., Natoli, G., Ricci, L., Mattei, E., Graessmann, A., and Levrero, M. (1997). MyoD prevents cyclinA/cdk2 containing E2F complexes formation in terminally differentiated myocytes. *Oncogene*, 14: 1171-1184.

Puri, P. L., Cimino, L., Fulco, M., Zimmerman, C., La Thangue, N. B., Giordano, A., Graessmann, A., and Levrero, M. (1998). Regulation of E2F4 mitogenic activity during terminal differentiation by its heterodimerization partners for nuclear translocation. *Cancer Res*, 58: 1325-1331.

Puri, P. L., MacLachlan, M., Levrero., M., and Giordano, A. (1999). The intrinsic cell cycle: from yeast to mammals. *In* "The molecular basis of cell cycle control" (G. Stein, R. Baserga, A. Giordano, and D. T. Denhardt, Eds.). Wiley Liss, New York.

Qian, Y. W., Erikson, E., and Maller, J. L. (1998). Purification and cloning of a protein kinase that phosphorylates and activates the polo-like kinase Plx1. *Science*, 282: 1701-1704.

Qin, X. Q., Livingston, D. M., Ewen, M., Sellers, W. R., Arany, Z., and Kaelin, W. G., Jr. (1995). The transcription factor E2F-1 is a downstream target of RB action. *Mol Cell Biol*, 15: 742-755.

Qin, X. Q., Livingston, D. M., Kaelin, W. G., Jr., and Adams, P. D. (1994). Deregulated transcription factor E2F-1 expression leads to S-phase entry and p53-mediated apoptosis. *Proc Natl Acad Sci U S A*, 91: 10918-10922.

Ragsdale, C. W., Jr., Petkovich, M., Gates, P. B., Chambon, P., and Brockes, J. P. (1989). Identification of a novel retinoic acid receptor in regenerative tissues of the newt. *Nature*, 341: 654-657.

Ralston, E., and Hall, Z. W. (1989). Intracellular and surface distribution of a membrane protein (CD8) derived from a single nucleus in multinucleated myotubes. *J Cell Biol*, 109: 2345-2352.

Rantanen, J., Hurme, T., Lukka, R., Heino, J., and Kalimo, H. (1995). Satellite cell proliferation and the expression of myogenin and desmin in regenerating skeletal muscle: evidence for two different populations of satellite cells. *Lab Invest*, 72: 341-347.

Rao, P. N., and Johnson, R. T. (1970). Mammalian cell fusion: studies on the regulation of DNA synthesis and mitosis. *Nature*, 225: 159-164.

Rao, S. S., Chu, C., and Kohtz, D. S. (1994). Ectopic expression of cyclin D1 prevents activation of gene transcription by myogenic basic helix-loop-helix regulators. *Mol Cell Biol*, 14: 5259-5267.

Rawls, A., Morris, J. H., Rudnicki, M., Braun, T., Arnold, H. H., Klein, W. H., and Olson, E. N. (1995). Myogenin's functions do not overlap with those of MyoD or Myf-5 during mouse embryogenesis [published erratum appears in Dev Biol 1996 Mar 15;174(2):453]. *Dev Biol*, 172: 37-50.

Rawls, A., Valdez, M. R., Zhang, W., Richardson, J., Klein, W. H., and Olson, E. N. (1998). Overlapping functions of the myogenic bHLH genes MRF4 and MyoD revealed in double mutant mice. *Development*, 125: 2349-2358.

Repesh, L. A., Fitzgerald, T. J., and Furcht, L. T. (1982). Changes in the distribution of fibronectin during limb regeneration in newts using immunocytochemistry. *Differentiation*, 22: 125-131.

Repesh, L. A., and Oberpriller, J. C. (1978). Scanning electron microscopy of epidermal cell migration in wound healing during limb regeneration in the adult newt, Notophthalmus viridescens. *Am J Anat*, 151: 539-555.

Repesh, L. A., and Oberpriller, J. C. (1980). Ultrastructural studies on migrating epidermal cells during the wound healing stage of regeneration in the adult newt, Notophthalmus viridescens. *Am J Anat*, 159: 187-208.

Reshef, R., Maroto, M., and Lassar, A. B. (1998). Regulation of dorsal somitic cell fates: BMPs and Noggin control the timing and pattern of myogenic regulator expression. *Genes Dev*, 12: 290-303.

Resnitzky, D., Gossen, M., Bujard, H., Reed, S. I., and CITATION), S. B. (1994). Acceleration of the G1/S phase transition by expression of cyclins D1 and E with an inducible system. *Mol Cell Biol*, 14: 1669-1679.

Reyer, R. W. (1954). Regeneration of the lens in the amphibian eye. *Quart. Rev. Biol.*, 29: 1-46.

Reynisdottir, I., and Massague, J. (1997). The subcellular locations of p15(Ink4b) and p27(Kip1) coordinate their inhibitory interactions with cdk4 and cdk2. *Genes Dev*, 11: 492-503.

Rhodes, S. J., and Konieczny, S. F. (1989). Identification of MRF4: a new member of the muscle regulatory factor gene family. *Genes Dev*, 3: 2050-2061.

Rieder, C. L., Schultz, A., Cole, R., and Sluder, G. (1994). Anaphase onset in vertebrate somatic cells is controlled by a checkpoint that monitors sister kinetochore attachment to the spindle. *J Cell Biol*, 127: 1301-1310.

Ringertz, N. R., Nyman, U., and Bergman, M. (1985). DNA replication and H5 histone exchange during reactivation of chick erythrocyte nuclei in heterokaryons. *Chromosoma*, 91: 391-396.

Rosania, G. R., Chang, Y. T., Perez, O., Sutherlin, D., Dong, H., Lockhart, D. J., and Schultz, P. G. (2000). Myoseverin, a microtubule-binding molecule with novel cellular effects. *Nat Biotechnol*, 18: 304-308.

Rosenblatt, J. D., Parry, D. J., and Partridge, T. A. (1996). Phenotype of adult mouse muscle myoblasts reflects their fiber type of origin. *Differentiation*, 60: 39-45.

Rossi, S. G., Vazquez, A. E., and Rotundo, R. L. (2000). Local control of acetylcholinesterase gene expression in multinucleated skeletal muscle fibers: individual nuclei respond to signals from the overlying plasma membrane. *J Neurosci*, 20: 919-928.

Rudnicki, M. A., Braun, T., Hinuma, S., and Jaenisch, R. (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.

Rudnicki, M. A., Schnegelsberg, P. N., Stead, R. H., Braun, T., Arnold, H. H., and Jaenisch, R. (1993). MyoD or Myf-5 is required for the formation of skeletal muscle. *Cell*, 75: 1351-1359.

Russo, S., Tomatis, D., Collo, G., Tarone, G., and Tato, F. (1998). Myogenic conversion of NIH3T3 cells by exogenous MyoD family members: dissociation of terminal differentiation from myotube formation. *J Cell Sci*, 111: 691-700.

Sabourin, L. A., Girgis-Gabardo, A., Seale, P., Asakura, A., and Rudnicki, M. A. (1999). Reduced differentiation potential of primary MyoD-/- myogenic cells derived from adult skeletal muscle. *J Cell Biol*, 144: 631-643.

Sanchez, Y., Wong, C., Thoma, R. S., Richman, R., Wu, Z., Piwnica-Worms, H., and Elledge, S. J. (1997). Conservation of the Chk1 checkpoint pathway in mammals: linkage of DNA damage to Cdk regulation through Cdc25 [see comments]. *Science*, 277: 1497-1501.

Sassoon, D., Lyons, G., Wright, W. E., Lin, V., Lassar, A., Weintraub, H., and Buckingham, M. (1989). Expression of two myogenic regulatory factors myogenin and MyoD1 during mouse embryogenesis. *Nature*, 341: 303-307.

Sastry, S. K., Lakonishok, M., Thomas, D. A., Muschler, J., and Horwitz, A. F. (1996). Integrin alpha subunit ratios, cytoplasmic domains, and growth factor synergy regulate muscle proliferation and differentiation. *J Cell Biol*, 133: 169-184.

Sastry, S. K., Lakonishok, M., Wu, S., Truong, T. Q., Huttenlocher, A., Turner, C. E., and Horwitz, A. F. (1999). Quantitative changes in integrin and focal adhesion signaling regulate myoblast cell cycle withdrawal. *J Cell Biol*, 144: 1295-1309.

Schafer, B. W., Blakely, B. T., Darlington, G. J., and Blau, H. M. (1990). Effect of cell history on response to helix-loop-helix family of myogenic regulators. *Nature*, 344: 454-458.

Scherer, S. S., Kamholz, J., and Jakowlew, S. B. (1993). Axons modulate the expression of transforming growth factor-betas in Schwann cells. *Glia*, 8: 265-276.

Schilthuis, J. G., Gann, A. A., and Brockes, J. P. (1993). Chimeric retinoic acid/thyroid hormone receptors implicate RAR-alpha 1 as mediating growth inhibition by retinoic acid. *Embo J*, 12: 3459-3466.

Schlegel, R., Belinsky, G. S., and Harris, M. O. (1990). Premature mitosis induced in mammalian cells by the protein kinase inhibitors 2-aminopurine and 6-dimethylaminopurine. *Cell Growth Differ*, 1: 171-178.

Schmid, V. (1992). Transdifferentiation in medusae. *Int Rev Cytol*, 142: 213-261.

Schmidt, A. J. (1968). "Cellular biology of vertebrate regeneration and repair." University of Chicago Press, Chicago.

Schneider, J. W., Gu, W., Zhu, L., Mahdavi, V., and Nadal-Ginard, B. (1994). Reversal of terminal differentiation mediated by p107 in Rb-/- muscle cells. *Science*, 264: 1467-1471.

Schultz, E. (1996). Satellite cell proliferative compartments in growing skeletal muscles. *Dev Biol*, 175: 84-94.

Schwab, I. A., and Luger, O. (1980). Reinitiation of DNA synthesis in postmitotic nuclei of myotubes by virus-mediated fusion with embryonic fibroblasts. *Differentiation*, 16: 93-99.

Schwartz, M. A., and Baron, V. (1999). Interactions between mitogenic stimuli, or, a thousand and one connections. *Curr Opin Cell Biol*, 11: 197-202.

Seale, P., and Rudnicki, M. A. (2000). A new look at the origin, function, and "stem-cell" status of muscle satellite cells. *Dev Biol*, 218: 115-124.

Serizawa, H., Makela, T. P., Conaway, J. W., Conaway, R. C., Weinberg, R. A., and Young, R. A. (1995). Association of Cdk-activating kinase subunits with transcription factor TFIIH. *Nature*, 374: 280-282.

Shawber, C., Nofziger, D., Hsieh, J. J., Lindsell, C., Bogler, O., Hayward, D., and Weinmaster, G. (1996). Notch signaling inhibits muscle cell differentiation through a CBF1- independent pathway. *Development*, 122: 3765-3773.

Sherr, C. J. (1996). Cancer cell cycles. *Science*, 274: 1672-1677.

Sherr, C. J., and Roberts, J. M. (1999). CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev*, 13: 1501-1512.

Shimizu-Nishikawa, K., Tazawa, I., Uchiyama, K., and Yoshizato, K. (1999). Expression of helix-loop-helix type negative regulators of differentiation during limb regeneration in urodeles and anurans. *Dev Growth Differ*, 41: 731-743.

Shiyanov, P., Bagchi, S., Adami, G., Kokontis, J., Hay, N., Arroyo, M., Morozov, A., and Raychaudhuri, P. (1996). p21 Disrupts the interaction between cdk2 and the E2F-p130 complex. *Mol Cell Biol*, 16: 737-744.

Simon, H. G., Nelson, C., Goff, D., Laufer, E., Morgan, B. A., and Tabin, C. (1995). Differential expression of myogenic regulatory genes and Msx-1 during dedifferentiation and redifferentiation of regenerating amphibian limbs. *Dev Dyn*, 202: 1-12.

Singer, M. (1949). The invasion of the epidermis of the regenerating forlimb of the urodele, Triturus, by nerve fibers. *J Exp Zool*, 111: 189-209.

Singer, M. (1952). The influence of the nerve in regeneration of the amphibian extremity. *Quart Rev Biol*, 27: 169-200.

Singer, M., and Caston, J. D. (1972). Neurotrophic dependence of macromolecular synthesis in the early limb regenerate of the newt, Triturus. *J Embryol Exp Morphol*, 28: 1-11.

Singer, M., and Craven, L. (1948). The growth and morphogenesis of the regenerating forelimb of adult Truturus following denervation at various stages of development. *J Exp Zool*, 108: 279-308.

Singer, M., Rzehak, K., and Maier, C. S. (1967). The relation between the caliber of the axon and the trophic activity of nerves in limb regeneration. *J Exp Zool*, 166: 89-97.

Singer, M., and Salpeter, M. M. (1961). Regeneration in vertebrates: the role of the wound epithelium. *In* "Growth in living systems" (M. Zarrow, Ed.), pp. 277-311. Basic Books, New York.

Singh, P., Coe, J., and Hong, W. (1995). A role for retinoblastoma protein in potentiating transcriptional activation by the glucocorticoid receptor. *Nature*, 374: 562-565.

Skapek, S. X., Rhee, J., Kim, P. S., Novitch, B. G., and Lassar, A. B. (1996). Cyclin-mediated inhibition of muscle gene expression via a mechanism that is independent of pRB hyperphosphorylation. *Mol Cell Biol*, 16: 7043-7053.

Skapek, S. X., Rhee, J., Spicer, D. B., and Lassar, A. B. (1995). Inhibition of myogenic differentiation in proliferating myoblasts by cyclin D1-dependent kinase [see comments]. *Science*, 267: 1022-1024.

Sluder, G., Hinchcliffe, E. D., and Rieder, C. L. (1999). Mitosis: the culmination of the cell cycle. *In* "The molecular basis of cell cycle control" (G. Stein, R. Baserga, A. Giordano, and D. T. Denhardt, Eds.). Wiley Liss, New York.

Sluder, G., and Hinchcliffe, E. H. (1999). Control of centrosome reproduction: the right number at the right time. *Biol Cell*, 91: 413-427.

Smith, T. H., Kachinsky, A. M., and Miller, J. B. (1994). Somite subdomains, muscle cell origins, and the four muscle regulatory factor proteins. *J Cell Biol*, 127: 95-105.

Soddu, S., Blandino, G., Scardigli, R., Coen, S., Marchetti, A., Rizzo, M. G., Bossi, G., Cimino, L., Crescenzi, M., and Sacchi, A. (1996). Interference with p53 protein inhibits hematopoietic and muscle differentiation. *J Cell Biol*, 134: 193-204.

Sohal, G. S. (1995). Sixth Annual Stuart Reiner Memorial Lecture: embryonic development of nerve and muscle. *Muscle Nerve*, 18: 2-14.

Solari, F., Domenget, C., Gire, V., Woods, C., Lazarides, E., Rousset, B., and Jurdic, P. (1995). Multinucleated cells can continuously generate mononucleated cells in the absence of mitosis: a study of cells of the avian osteoclast lineage. *J Cell Sci*, 108: 3233-3241.

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

Spallanzani, L. (1768). Prodromo di un opera da imprimersi sopra la riproduzione animale. Giovanni Montanari, Modena.

Stark, D. R., Gates, P. B., Brockes, J. P., and Ferretti, P. (1998). Hedgehog family member is expressed throughout regenerating and developing limbs. *Dev Dyn*, 212: 352-363.

Steen, T. P. (1968). Stability of chondrocyte differentiation and contribution of muscle to cartilage during limb regeneration in the axolotl (Siredon mexicanum). *J Exp Zool*, 167: 49-78.

Steinmann, K. E., Belinsky, G. S., Lee, D., and Schlegel, R. (1991). Chemically induced premature mitosis: differential response in rodent and human cells and the relationship to cyclin B synthesis and p34cdc2/cyclin B complex formation. *Proc Natl Acad Sci U S A*, 88: 6843-6847.

Stewart, N., Hicks, G. G., Paraskevas, F., and Mowat, M. (1995). Evidence for a second cell cycle block at G2/M by p53. *Oncogene*, 10: 109-115.

Stockdale, F. E. (1992). Myogenic cell lineages. *Dev Biol*, 154: 284-298.

Stockdale, F. E., and Miller, J. B. (1987). The cellular basis of myosin heavy chain isoform expression during development of avian skeletal muscles. *Dev Biol*, 123: 1-9.

Stocum, D. L. (1968). The urodele limb regeneration blastema: a self-organizing system. I. Morphogenesis and differentiation of autografted whole and fractional blastemas. *Dev Biol*, 18: 457-480.

Stocum, D. L. (1995). "Wound repair, regeneration, and artificial tissues." R.G. Landes Co., Austin, Texas.

Strauch, A. R., and Reeser, J. C. (1989). Sequential expression of smooth muscle and sarcomeric alpha-actin isoforms during BC3H1 cell differentiation. *J Biol Chem*, 264: 8345-8355.

Strausfeld, U. P., Howell, M., Descombes, P., Chevalier, S., Rempel, R. E., Adamczewski, J., Maller, J. L., Hunt, T., and Blow, J. J. (1996). Both cyclin A and cyclin E have S-phase promoting (SPF) activity in Xenopus egg extracts. *J Cell Sci*, 109: 1555-1563.

Sun, X., Lewandoski, M., Meyers, E. N., Liu, Y. H., Maxson, R. E., Jr., and Martin, G. R. (2000). Conditional inactivation of Fgf4 reveals complexity of signalling during limb bud development. *Nat Genet*, 25: 83-86.

Tabin, C. H., and Cathieni, M. (1989). Second messengers in newt limb regeneration: cAMP adn cGMP levels and distirbution. *In* "Recent trends in regeneration research" (V. Kiortsis, S. Koussoulakos, and H. Wallace, Eds.). Plenum Press, New York.

Tajbakhsh, S., and Buckingham, M. E. (1995). Lineage restriction of the myogenic conversion factor myf-5 in the brain. *Development*, 121: 4077-4083.

Tajbakhsh, S., Bober, E., Babinet, C., Pournin, S., Arnold, H., and Buckingham, M. (1996a). Gene targeting the myf-5 locus with nlacZ reveals expression of this myogenic factor in mature skeletal muscle fibres as well as early embryonic muscle. *Dev Dyn*, 206: 291-300.

Tajbakhsh, S., and Buckingham, M. (2000). The birth of muscle progenitor cells in the mouse: spatiotemporal considerations. *Curr Top Dev Biol*, 48: 225-268.

Tajbakhsh, S., Rocancourt, D., and Buckingham, M. (1996b). Muscle progenitor cells failing to respond to positional cues adopt non- myogenic fates in myf-5 null mice. *Nature*, 384: 266-270.

Tajbakhsh, S., Rocancourt, D., Cossu, G., and Buckingham, M. (1997). Redefining the genetic hierarchies controlling skeletal myogenesis: Pax- 3 and Myf-5 act upstream of MyoD. *Cell*, 89: 127-138.

Tamir, Y., and Bengal, E. (1998). p53 protein is activated during muscle differentiation and participates with MyoD in the transcription of muscle creatine kinase gene. *Oncogene*, 17: 347-356.

Tanaka, E. M., Drechsel, D. N., and Brockes, J. P. (1999). Thrombin regulates S-phase re-entry by cultured newt myotubes [see comments]. *Curr Biol*, 9: 792-799.

Tanaka, E. M., Gann, A. A., Gates, P. B., and Brockes, J. P. (1997). Newt myotubes reenter the cell cycle by phosphorylation of the retinoblastoma protein. *J Cell Biol*, 136: 155-165.

Tapscott, S. J., Davis, R. L., Thayer, M. J., Cheng, P. F., Weintraub, H., and Lassar, A. B. (1988). MyoD1: a nuclear phosphoprotein requiring a Myc homology region to convert fibroblasts to myoblasts. *Science*, 242: 405-411.

Tapscott, S. J., Thayer, M. J., and Weintraub, H. (1993). Deficiency in rhabdomyosarcomas of a factor required for MyoD activity and myogenesis. *Science*, 259: 1450-1453.

Tassava, R. A., Goldhamer, D. J., and Tomlinson, B. L. (1987). Cell cycle controls and the role of nerves and the regenerate epithelium in urodele forelimb regeneration: possible modifications of basic concepts. *Biochem Cell Biol*, 65: 739-749.

Tassava, R. A., Johnson-Wint, B., and Gross, J. (1986). Regenerate epithelium and skin glands of the adult newt react to the same monoclonal antibody. *J Exp Zool*, 239: 229-240.

Tassava, R. A., and McCullough, W. D. (1978). Neural control of cell cycle events in regenerating salamander limbs. *Am Zool*, 18: 843-854.

Tassava, R. A., and Mescher, A. L. (1975). The roles of injury, nerves, and the wound epidermis during the initiation of amphibian limb regeneration. *Differentiation*, 4: 23-24.

Tate, J. M., Oberpriller, J. O., and Oberpriller, J. C. (1989). Analysis of DNA synthesis in cell cultures of the adult newt cardiac myocyte. *Tissue Cell*, 21: 335-342.

Taylor, W. R., Agarwal, M. L., Agarwal, A., Stacey, D. W., and Stark, G. R. (1999). p53 inhibits entry into mitosis when DNA synthesis is blocked. *Oncogene*, 18: 283-295.

Tevosian, S. G., Shih, H. H., Mendelson, K. G., Sheppard, K. A., Paulson, K. E., and Yee, A. S. (1997). HBP1: a HMG box transcriptional repressor that is targeted by the retinoblastoma family. *Genes Dev*, 11: 383-396.

Thoms, S. D., and Stocum, D. L. (1984). Retinoic acid-induced pattern duplication in regenerating urodele limbs. *Dev Biol*, 103: 319-328.

Thornton, C. S. (1956). The relation of epidermal innervation to limb regeneration in Ambystoma larvae. *J Exp Zool*, 133: 281-299.

Thornton, C. S. (1957). The effect of apical cap removal on limb regeneration in *Ambystoma* larvae. *J Exp Zool*, 134: 357-381.

Thornton, C. S. (1960). Influence of an eccesntric epidermal cap on lib regeneration in Ambystoma larvae. *Dev Biol*, 2: 551-569.

Thornton, C. S., and Thornton, M. T. (1970). Recuperation of regeneration in devervated limbs of *Ambystoma* larvae. *J Exp Zool*, 173: 293-301.

Thouveny, Y., and Tassava, R. A. (1998). Regeneration through phylogenesis. *In* "Cellular and molecular basis of regeneration." (P. Ferretti and J. Géraudie, Eds.). John Wiley & Sons, Chichester.

Tiainen, M., Pajalunga, D., Ferrantelli, F., Soddu, S., Salvatori, G., Sacchi, A., and Crescenzi, M. (1996). Terminally differentiated skeletal myotubes are not confined to G0 but can enter G1 upon growth factor stimulation. *Cell Growth Differ*, 7: 1039-1050.

Todd, T. J. (1823). On the process of reproduction of the memebrs of the acquatic salamander. *Quart Jour Sci, Lit Arts*, 16: 84-96.

Tomlinson, B. L., and Barger, P. M. (1987). A test of the punctuated-cycling hypothesis in Ambystoma forelimb regenerates: the roles of animal size, limb innervation, and the aneurogenic condition. *Differentiation*, 35: 6-15.

Tomoda, K., Kubota, Y., and Kato, J. (1999). Degradation of the cyclin-dependent-kinase inhibitor p27Kip1 is instigated by Jab1 [see comments]. *Nature*, 398: 160-165.

Tonegawa, S. (1983). Somatic generation of antibody diversity. *Nature*, 302: 575-581.

Torok, M. A., Gardiner, D. M., Shubin, N. H., and Bryant, S. V. (1998). Expression of HoxD genes in developing and regenerating axolotl limbs. *Dev Biol*, 200: 225-233.

Toyoshima, H., and Hunter, T. (1994). p27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to p21. *Cell*, 78: 67-74.

Tsai, K. Y., Hu, Y., Macleod, K. F., Crowley, D., Yamasaki, L., and Jacks, T. (1998). Mutation of E2f-1 suppresses apoptosis and inappropriate S phase entry and extends survival of Rb-deficient mouse embryos. *Mol Cell*, 2: 293-304.

Tsonis, P. A. (1996). "Limb regeneration." Cambridge University Press, Cambridge.

Tsonis, P. A. (2000). Regeneration in vertebrates. *Dev Biol*, 221: 273-284.

Tsonis, P. A., English, D., and Mescher, A. L. (1991). Increased content of inositol phosphates in amputated limbs of axolotl larvae and the effect of berillium. *J Exp Zool*, 259: 252-258.

Valdez, M. R., Richardson, J. A., Klein, W. H., and Olson, E. N. (2000). Failure of Myf5 to support myogenic differentiation without myogenin, MyoD, and MRF4. *Dev Biol*, 219: 287-298.

Viviano, C. M., and Brockes, J. P. (1996). Is retinoic acid an endogenous ligand during urodele limb regeneration? *Int J Dev Biol*, 40: 817-822

Wallace, H. (1981). "Vertebrate limb regeneration." Wiley and Sons, New York.

Wallace, H., and Maden, M. (1976). The cell cycle during amphibian limb regeneration. *J Cell Sci*, 20: 539-547.

Wang, J., Guo, K., Wills, K. N., and Walsh, K. (1997). Rb functions to inhibit apoptosis during myocyte differentiation. *Cancer Res*, 57: 351-354.

Wang, J., Helin, K., Jin, P., and Nadal-Ginard, B. (1995). Inhibition of in vitro myogenic differentiation by cellular transcription factor E2F1. *Cell Growth Differ*, 6: 1299-1306.

Wang, J., Huang, Q., Tang, W., and Nadal-Ginard, B. (1996a). E2F1 inhibition of transcription activation by myogenic basic helix- loop-helix regulators. *J Cell Biochem*, 62: 405-410.

Wang, J., and Walsh, K. (1996a). Inhibition of retinoblastoma protein phosphorylation by myogenesis- induced changes in the subunit composition of the cyclin-dependent kinase 4 complex. *Cell Growth Differ*, 7: 1471-1478.

Wang, J., and Walsh, K. (1996b). Resistance to apoptosis conferred by Cdk inhibitors during myocyte differentiation. *Science*, 273: 359-361.

Wang, L., Marchionni, M. A., and Tassava, R. A. (2000). Cloning and neuronal expression of a type III newt neuregulin and rescue of denervated, nerve-dependent newt limb blastemas by rhGGF2. *J Neurobiol*, 43: 150-158.

Wang, Y., and Jaenisch, R. (1997). Myogenin can substitute for Myf5 in promoting myogenesis but less efficiently. *Development*, 124: 2507-2513.

Wang, Y., Schnegelsberg, P. N., Dausman, J., and Jaenisch, R. (1996b). Functional redundancy of the muscle-specific transcription factors Myf5 and myogenin. *Nature*, 379: 823-825.

Wei, Y., Yu, L., Bowen, J., Gorovsky, M. A., and Allis, C. D. (1999). Phosphorylation of histone H3 is required for proper chromosome condensation and segregation. *Cell*, 97: 99-109.

Weinberg, R. A. (1995). The retinoblastoma protein and cell cycle control. *Cell*, 81: 323-330.

Weinmaster, G. (1997). The ins and outs of notch signaling. *Mol Cell Neurosci*, 9: 91-102.

Weintraub, H., Davis, R., Tapscott, S., Thayer, M., Krause, M., Benezra, R., Blackwell, T. K., Turner, D., Rupp, R., and Hollenberg, S. (1991). The myoD gene family: nodal point during specification of the muscle cell lineage. *Science*, 251: 761-766.

Weiss, C., and Rosenbaum, R. M. (1967). Histochemical studies on cell death and histolysis during regeneration. I. Distiribution of acid phosphomonoesterase activity in the normal, the regenerating and the resorbinf forelimb of the larval spotted salamander, *Ambystoma maculatum*. *J Morphol*, 122: 203-229.

Wheatley, S. P., Hinchcliffe, E. H., Glotzer, M., Hyman, A. A., Sluder, G., and Wang, Y. (1997). CDK1 inactivation regulates anaphase spindle dynamics and cytokinesis in vivo. *J Cell Biol*, 138: 385-393.

Whyte, P. (1995). The retinoblastoma protein and its relatives. *Semin Cancer Biol*, 6: 83-90.

Whyte, P., Williamson, N. M., and Harlow, E. (1989). Cellular targets for transformation by the adenovirus E1A proteins. *Cell*, 56: 67-75.

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

Wilmut, I., Schnieke, A. E., McWhir, J., Kind, A. J., and Campbell, K. H. (1997). Viable offspring derived from fetal and adult mammalian cells [see comments] [published erratum appears in Nature 1997 Mar 13;386(6621):200]. *Nature*, 385: 810-813.

Wittenberger, T., Steinbach, O. C., Authaler, A., Kopan, R., and Rupp, R. A. (1999). MyoD stimulates delta-1 transcription and triggers notch signaling in the Xenopus gastrula. *Embo J*, 18: 1915-1922.

Wolff, E., and Wey-Schué, M. (1952). Démonstration expérimentalede la migration des cellule de régénération dans la régénération des membres de *Triton cristatus*. *C R Soc Biol*, 146: 113-117.

Wolff, G. (1895). Entwicklungsphysiologische Studien. I. Die regeneration der urodelelinse. *Archiv für Mikroskopische Anatomie und entwicklugsmechanik der organismen.*, 1: 380-390.

Wright, W. E. (1984). Control of differentiation in heterokaryons and hybrids involving differentiation-defective myoblast variants. *J Cell Biol*, 98: 436-443.

Wright, W. E., and Lin, V. K. (1990). Uncoupling of muscle-specific protein expression in myocyte x myoblast heterokaryons. *Cell Differ Dev*, 29: 113-122.

Wright, W. E., Sassoon, D. A., and Lin, V. K. (1989). Myogenin, a factor regulating myogenesis, has a domain homologous to MyoD. *Cell*, 56: 607-617.

Xiong, Y., Hannon, G. J., Zhang, H., Casso, D., Kobayashi, R., and Beach, D. (1993a). p21 is a universal inhibitor of cyclin kinases [see comments]. *Nature*, 366: 701-704.

Xiong, Y., Zhang, H., and Beach, D. (1993b). Subunit rearrangement of the cyclin-dependent kinases is associated with cellular transformation. *Genes Dev*, 7: 1572-1583.

Yablonka-Reuveni, Z., and Rivera, A. J. (1994). Temporal expression of regulatory and structural muscle proteins during myogenesis of satellite cells on isolated adult rat fibers. *Dev Biol*, 164: 588-603.

Yablonka-Reuveni, Z., Rudnicki, M. A., Rivera, A. J., Primig, M., Anderson, J. E., and Natanson, P. (1999). The transition from proliferation to differentiation is delayed in satellite cells from mice lacking MyoD. *Dev Biol*, 210: 440-455.

Yaffe, D., and Saxel, O. (1977). Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. *Nature*, 270: 725-727.

Yamada, T. (1977). Control mechanisms in cell-type conversion in newt lens regeneration. *Monogr Dev Biol*, 13: 1-126.

Yamada, T., and McDevitt, D. S. (1974). Direct evidence for transformation of differentiated iris epithelial cells into lens cells. *Dev Biol*, 38: 104-118.

Yamashita, K., Yasuda, H., Pines, J., Yasumoto, K., Nishitani, H., Ohtsubo, M., Hunter, T., Sugimura, T., and Nishimoto, T. (1990). Okadaic acid, a potent inhibitor of type 1 and type 2A protein phosphatases, activates cdc2/H1 kinase and transiently induces a premature mitosis-like state in BHK21 cells. *Embo J*, 9: 4331-4338.

Yan, Z., DeGregori, J., Shohet, R., Leone, G., Stillman, B., Nevins, J. R., and Williams, R. S. (1998). Cdc6 is regulated by E2F and is essential for DNA replication in mammalian cells. *Proc Natl Acad Sci U S A*, 95: 3603-3608.

Yancopoulos, G. D., and Alt, F. W. (1986). Regulation of the assembly and expression of variable-region genes. *Annu Rev Immunol*, 4: 339-368.

Yancopoulos, G. D., DePinho, R. A., Zimmerman, K. A., Lutzker, S. G., Rosenberg, N., and Alt, F. W. (1986). Secondary genomic rearrangement events in pre-B cells: VHDJH replacement by a LINE-1 sequence and directed class switching. *Embo J*, 5: 3259-3266.

Yang, E. V., and Bryant, S. V. (1994). Developmental regulation of a matrix metalloproteinase during regeneration of axolotl appendages. *Dev Biol*, 166: 696-703.

Yang, E. V., Gardiner, D. M., Carlson, M. R., Nugas, C. A., and Bryant, S. V. (1999). Expression of Mmp-9 and related matrix metalloproteinase genes during axolotl limb regeneration. *Dev Dyn*, 216: 2-9.

Yang, E. V., Shima, D. T., and Tassava, R. A. (1992). Monoclonal antibody ST1 identifies an antigen that is abundant in the axolotl and newt limb stump but is absent from the undifferentiated regenerate. *J Exp Zool*, 264: 337-350.

Yang, J., Bardes, E. S., Moore, J. D., Brennan, J., Powers, M. A., and Kornbluth, S. (1998). Control of cyclin B1 localization through regulated binding of the nuclear export factor CRM1. *Genes Dev*, 12: 2131-2143.

Yanze, N., Groger, H., Muller, P., and Schmid, V. (1999). Reversible inactivation of cell-type-specific regulatory and structural genes in migrating isolated striated muscle cells of jellyfish. *Dev Biol*, 213: 194-201.

Yntema, C. L. (1959). Regeneration in sparsely innervated and anurogenic forelimbs of Amblystoma larvae. *J Expe Zool*, 140: 101-123.

Yoon, J. K., Olson, E. N., Arnold, H. H., and Wold, B. J. (1997). Different MRF4 knockout alleles differentially disrupt Myf-5 expression: cis-regulatory interactions at the MRF4/Myf-5 locus. *Dev Biol*, 188: 349-362.

Yoshida, N., Yoshida, S., Koishi, K., Masuda, K., and Nabeshima, Y. (1998). Cell heterogeneity upon myogenic differentiation: down-regulation of MyoD and Myf-5 generates 'reserve cells'. *J Cell Sci*, 111: 769-779.

Zachariae, W., and Nasmyth, K. (1999). Whose end is destruction: cell division and the anaphase-promoting complex. *Genes Dev*, 13: 2039-2058.

Zacksenhaus, E., Jiang, Z., Chung, D., Marth, J. D., Phillips, R. A., and Gallie, B. L. (1996). pRb controls proliferation, differentiation, and death of skeletal muscle cells and other lineages during embryogenesis. *Genes Dev*, 10: 3051-3064.

Zenjari, C., Boilly, B., Hondermarck, H., and Boilly-Marer, Y. (1997). Nerve-blastema interactions induce fibroblast growth factor-1 release during limb regeneration in Pleurodeles waltl. *Dev Growth Differ*, 39: 15-22.

Zenjari, C., Boilly-Marer, Y., Desbiens, X., Oudghir, M., Hondermarck, H., and Boilly, B. (1996). Experimental evidence for FGF-1 control of blastema cell proliferation during limb regeneration of the amphibian Pleurodeles waltl. *Int J Dev Biol*, 40: 965-971.

Zerfass-Thome, K., Schulze, A., Zwerschke, W., Vogt, B., Helin, K., Bartek, J., Henglein, B., and Jansen-Durr, P. (1997). p27KIP1 blocks cyclin E-dependent transactivation of cyclin A gene expression. *Mol Cell Biol*, 17: 407-415.

Zeschnigk, M., Kozian, D., Kuch, C., Schmoll, M., and Starzinski-Powitz, A. (1995). Involvement of M-cadherin in terminal differentiation of skeletal muscle cells. *J Cell Sci*, 108: 2973-2981.

Zhang, H., Hannon, G. J., and Beach, D. (1994). p21-containing cyclin kinases exist in both active and inactive states. *Genes Dev*, 8: 1750-1758.

Zhang, H., Xiong, Y., and Beach, D. (1993). Proliferating cell nuclear antigen and p21 are components of multiple cell cycle kinase complexes. *Mol Biol Cell*, 4: 897-906.

Zhang, J. M., Wei, Q., Zhao, X., and Paterson, B. M. (1999a). Coupling of the cell cycle and myogenesis through the cyclin D1- dependent interaction of MyoD with cdk4. *Embo J*, 18: 926-933.

Zhang, P. (1999). The cell cycle and development: redundant roles of cell cycle regulators. *Curr Opin Cell Biol*, 11: 655-662.

Zhang, P., Wong, C., Liu, D., Finegold, M., Harper, J. W., and Elledge, S. J. (1999b). p21(CIP1) and p57(KIP2) control muscle differentiation at the myogenin step. *Genes Dev*, 13: 213-224.

Zweigerdt, R., Braun, T., and Arnold, H. H. (1997). Faithful expression of the Myf-5 gene during mouse myogenesis requires distant control regions: a transgene approach using yeast artificial chromosomes. *Dev Biol*, 192: 172-180.