GROWTH AND DIFFERENTIATION 0F ENCAPSULATED MYOBLAST

THE EFFECT OF GROWTH FACTORS AND EXTRACELLULAR MATRIX

MATERIALS ON THE GROWTH AND DIFFERENTIATION OF

MICROENCAPSULATED MYOBLASTS

By

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TITLE: Optimization of the Microcapsule Environment: The Effect of Growth Factors and Extracellular Matrix Materials on the Growth and Differentiation of Microencapsulated Myoblasts

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ABSTRACT

An alternative gene therapy method, non-autologous somatic gene therapy, is the use of a genetically modified universal cultured cell line that can be implanted into different allogeneic recipients. When used as recombinant cells in microcapsules, myoblasts possess several advantages over other cell types, namely their ability to terminally differentiate thus preventing overcrowding within the capsular space. However, encapsulated myoblasts demonstrate decreased proliferation and myogenic differentiation when compared to unencapsulated myoblasts due to the unnatural capsule environment. This study aims to improve the microcapsule environment by incorporating basic fibroblast growth factor (bFGF) and insulin-like growth factor-II (IGF-II) and the extracellular matrix materials, collagen, laminin-1 and merosin (laminin-2) within the microcapsules in an attempt to mimic the natural surrounding required for myoblast growth and differentiation.

While bFGF lead to significant increases in encapsulated myoblast proliferation, it did not appear to be an ideal choice for optimizing the microcapsule environment due to its inhibitory effect on differentiation and the relative cost in therapeutic delivery of proteins. Both merosin and the combination of laminin and merosin together provide a better alternative for increasing myoblast growth and survival within microcapsules since they have no apparent inhibitory effect on myogenic differentiation, and produce similar proliferative results seen when using

bFGF. In terms of differentiation, the addition of IGF-II to the microcapsules or the use of a myoblast cell line overexpressing IGF-II, aid in increasing the myogenic differentiation of encapsulated myoblasts, however, differentiation levels still do not approach those seen in unencapsulated myoblasts.

The positive results obtained with the growth factors and matrix materials employed in this study are important steps towards the optimization of microcapsules by improving both the proliferation and differentiation of encapsulated myoblasts. However, more study is needed to elucidate possible solutions to the continued problem of decreased differentiation of myoblasts within APA microcapsules in order to achieve myogenic differentiation that is comparable to what is seen in unencapsulated myoblasts.

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1.0 INTRODUCTION

1.1 Non-autologous Cell-mediated Gene Therapy

An alternative gene therapy method, non-autologous somatic gene therapy, is to use a genetically modified universal cultured cell line that is foreign to the host (thus non-autologous) which can be implanted into different allogeneic recipients (Chang, 1995). In this case, to prevent immune rejection, modified cells can be protected through encapsulation by permselective membranes, or immunoisolation devices, that allow diffusion of nutrients, oxygen, and therapeutic gene products, but are biocompatible with the recipient (Wong and Chang, 1991; Chang et al., 1993; Tai and Sun, 1993; Hughes et al., 1994). Due to the cost effectiveness of this method and the potential for constant delivery of therapeutic gene products, non-autologous somatic gene therapy is an attractive alternative treatment for many diseases (Chang, P. L., 1995).

1.1.1 Immunoisolation devices

Macrocapsules and microcapsules are two of the main categories that immunoisolation devices are typically divided into. The most commonly fabricated type of macrocapsule is a hollow fibre encapsulation device that consists of a tubular membrane with a large inner diameter. These capsules have high mechanical stability, can be retrieved from implantation sites with relative ease, but may impair diffusion and cell viability (Emerich et al., 1992). Compounds used to fabricate macrocapsules include poly-vinyl alcohol (Burczak et al., 1996), polypropylene (Takebe et al., 1996), thermoplastic polymers (Aebischer, et al., 1991a), and polyvinyl chloride acrylate (Aebischer et al., 1991b, Sagen et al., 1993).

Microcapsules are generally much smaller (approximately 500 μm) than macrocapsules and tend to be spherical or tubular in shape. While this type of immunoisolation device allows for better diffusion of nutrients and cell products, they are more fragile and are not easily removed after implantation (Emerich et al., 1992). A variety of compounds have been used in the fabrication of microcapsules such as agarose hydrogels (Prevost et al., 1997; Lanza et al., 1999), chitosan (Gupta et al., 1993), poly ethylene glycol (Cruise et al., 1998; Cruise et al., 1999), and sol-gels (Peterson et al., 1998).

1.1.2 Alginate Polylysine Microcapsules

The most commonly used substance for encapsulation of cell lines for use in non-autologous gene therapy is alginate. Alginate is a polysaccharide composed of 1,4-linked β-D-mannuronic and α-L-guluronic acid and possesses the ability to form gel spheres spontaneously when exposed to divalent ions such as Ca^{2+} (Smidsrød and Skjåk-Bræk, 1990). The introduction of a second polymer, poly-L-lysine, into the capsular structure allows for control of permeability of the alginate. Ionic bonds form between the positively charged poly-L-lysine and the negatively charged carboxyl groups of the alginate to produce a stable membrane (Ma et al., 1994). Microcapsules of this type are called alginate-polylysine-alginate (APA) microcapsules.

APA microcapsules have been used to encapsulate many recombinant cell types for systemic and central nervous system delivery of therapeutic gene products. Initial studies employing alginate microcapsules attempted to treat diabetes by encapsulating pancreatic islet cells. Such studies utilized encapsulated cells from mice (Soon-Shiong et al., 1993), rats (Fan et al., 1990; Lum et al., 1992; Wang et al., 1997), pig (Kulseng et al., 1999), fish (Yang et al., 1997) and human (Lanza et al., 1995; Siebers et al., 1997).

Fibroblasts are another cell type that has been frequently used in APA microcapsule studies. Fibroblasts expressing human growth hormone (hGH) as a marker protein, have been used to successfully demonstrate the feasibility of using microencapsulated cells to deliver therapeutic proteins *in vivo* (Tai and Sun, 1993; Chang et al., 1993). These studies also showed that proliferation, viability and sustained delivery could be obtained *in vivo* as well as *in vitro* (Chang et al., 1994). Additional studies using fibroblasts transfected with human factor IX (FIX) demonstrated the feasibility of using microencapsulated recombinant cells to deliver FIX and for the potential treatment of Hemophilia B (Liu et al., 1993). Despite successes in protein delivery, fibroblasts do have drawbacks when used in microcapsules. Continued proliferation of fibroblasts leads to over-crowding within the microcapsule space which results in limited exchange of nutrients and metabolic wastes throughout the capsule (Chang et al., 1994).

1.1.3 Microencapsulation of Recombinant Myoblasts

Myogenic cell lines possess several advantages over other cell types, such as fibroblasts, when used as recombinant gene delivery vehicles in alginate-polylysine-alginate microcapsules. Cultured myoblasts are susceptible to stable genetic modification, can be easily transfected *in vitro*, will synthesize

large amounts of recombinant gene products, and have the ability to terminally differentiate (Barr and Leiden, 1991; Blau and Baltimore, 1991). The capability of myoblasts to terminally withdraw from the cell cycle through differentiation into myotubes circumvents the problem of continued proliferation and overcrowding of cells within the microcapsules. This permits long term viability of myogenic cells *in vivo* and allows for continuous expression and secretion of the therapeutic gene products, thus enabling long term treatment of diseases (Chang, 1995).

Due to their beneficial attributes when used as recombinant cells in microcapsules, myoblasts have been used for the delivery of a variety of therapeutic gene products. Correction of growth defects in dwarf mice resulted from delivery of mouse growth hormone (mGH) using APA encapsulated myoblasts (Al-Hendy et al., 1995). In this study, delivery of mGH lead to increases in linear growth, organ weight, and overall body weight. Additionally, the microencapsulated myoblasts remained viable and delivered mGH *in vivo* for up to 6 months after implantation. Similar studies utilized APA encapsulated myoblasts engineered to secrete human factor IX (FIX) as a vector to deliver FIX to mice (Hortelano et al., 1996; Hortelano et al., 1999). Encapsulated recombinant myoblasts were viable and continued to secrete FIX for up to 213 days post-implantation, demonstrating a feasible alternative therapy for treatment of Hemophilia B.

Alternative encapsulation devices that have been employed for therapeutic delivery of proteins using recombinant myoblasts are poly-ether-sulfone capsules. These polymer capsules were first used to deliver ciliary neurotrophic factor (CNTF) to the brain of rats. In addition to successful protein delivery, this study showed that only 12% of myoblasts continued to divide four days after the induction of differentiation *in vitro*, indicating that most encapsulated myoblasts were withdrawing from the cell cycle. Further, the presence of the herpes simplex thymidine kinase gene present in the expression vector allowed for the elimination of dividing myoblasts by exposure to ganciclovir (Delgon et al., 1996).

Additional studies using polymer-encapsulated myoblasts have aimed to treat β-thalassemia by delivering erythropoietin (Epo). Sustained delivery of Epo was seen for up to 12 weeks and efficacy was demonstrated by a reduced amount of free α-hemoglobin chain, a globin chain imbalance normally seen in β-thalassemia. Additionally, recovered encapsulated myoblasts were well preserved with multinucleated myotubes present in some devices (Regulier et al., 1998; Dalle et al., 1999).

1.2 Regulation of Myoblast Differentiation

1.2.1 Myoblast Differentiation

In myogenic cells, proliferation and differentiation are mutually exclusive events controlled by a complex of opposing cellular and environmental signals. Although some aspects of cell cycle control within the myogenic lineage have been elucidated, many of the exact signals that induce myogenesis *in vivo* are still relatively unclear (Olson, 1992). It is known, however, that when grown in culture, myogenesis can be tightly controlled by exogenous peptide growth factors, namely fibroblast growth factor (FGF) and transforming growth factor-β (TGF-β), that prevent myogenic differentiation until their concentrations fall below a critical threshold in the media. Myogenic cells respond to growth factor withdrawal by activation of the myogenic differentiation pathway which involves fusion of myoblasts to form multinucleate myotubes, withdrawal from the cell cycle, and transcriptional activation of muscle specific genes such as myosin heavy chain and creatine phosphokinase (Andrés and Walsh, 1996).

1.2.2 Muscle-specific Gene Expression

Induction of differentiation in myoblasts involves transcriptional activation of various muscle-specific genes. The muscle-regulatory factors (MRFs), also referred to as the MyoD family of transcription factors, are expressed in the developing muscle of both vertebrates and invertebrates (Emerson, 1990; Weintraub et al., 1991). MRFs have dominant regulatory activities than are indicative of their important functions in muscle development and differentiation. MRFs consist of a family of four closely related genes, myoD, myf5, myogenin, and MRF4, that encode skeletal muscle-specific helix-loop-helix (HLH) transcription factors (Wright, 1992). The myogenic HLH transcription factors bind DNA weakly when by themselves, but when heterodimerized, form a composite DNA-binding domain that has high affinity for E box consensus sequences (Weintraub et al., 1991). Heterodimerization of MRFs ususally occurs with ubiquitous HLH proteins, referred to as E proteins, such as E12 and E47 (Lassar et al., 1991). Activation of muscle-specific transcription occurs when MRF and E protein heterodimers bind to the E boxes in the control regions of muscle-specific genes (Figure 1.1) (Buckingham, 1994). Additionally, MRFs appear to form a regulatory hierarchy, whereby there is sequential activation of individual muscle-regulatory factor genes. It appears

that myoD and myf5 act early in myogenesis, while myogenin and MRF4 function later in differentiation (Braun et al., 1992).

While it has been demonstrated that the muscle-regulatory factors have temporal differences in expression, studies have also shown that the MRFs are to some degree functionally equivalent. Some overlap exists in terms of gene expression activation between some MRFs. Additionally, each MRF is able to cross activate expression of one or more of the other MRFs (Blau and Baltimore, 1991). Studies using myf5 and myoD knock-out mice have demonstrated that inactivation of one gene results in upregulation and compensation by another MRF (Rudnicki et al., 1992; Braun et al., 1992), demonstrating functional redundancy with respect to muscle development.
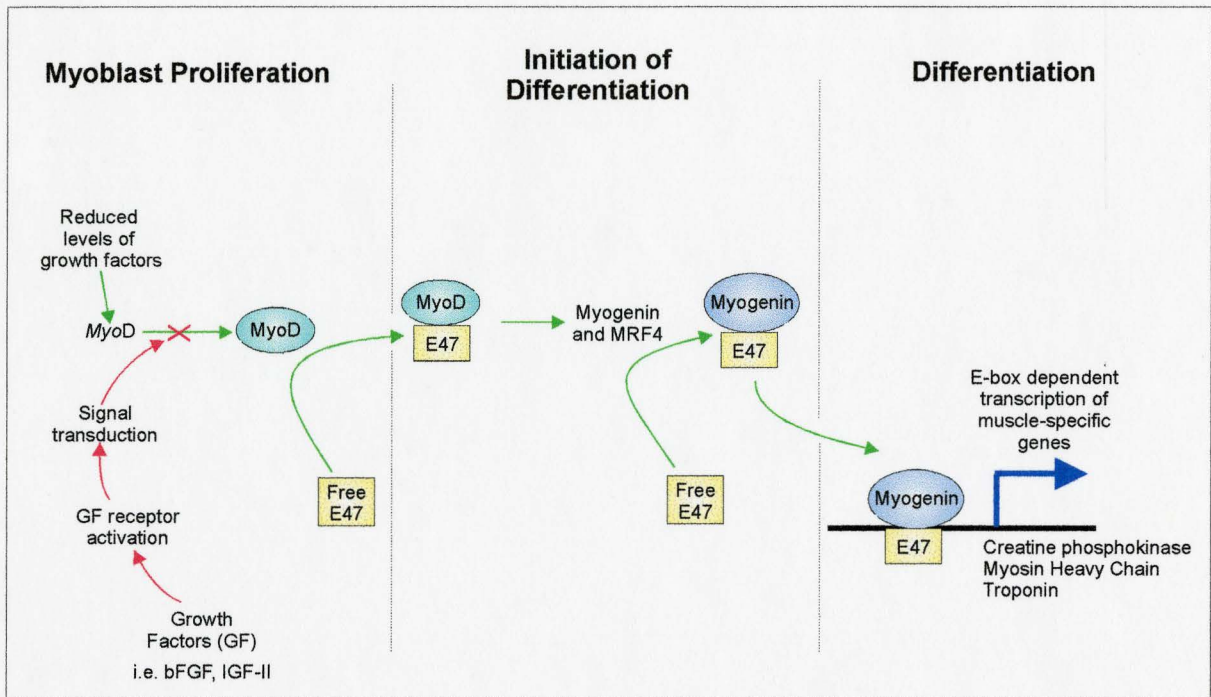
Figure I-1. Simplified pathway for the regulation of myoblast differentiation, proceeding from myoblast proliferation, through the initiation of differentiation to myogenic differentiation (adapted from Emerson, 1993).

1.3 Regulation of Myoblast Differentiation by Growth Factors

It has frequently been observed that myogenic differentiation in culture occurs upon a decrease in the level of growth factors in the medium and increasing evidence suggests that the binding of these growth factors to the extracellular matrix is the major mechanism regulating their activity (Taipale and Keski-Oja, 1997). Peptides which have been well characterized in terms of their inhibitory effect on differentiation include the fibroblast growth factors (FGFs), platelet-derived growth factors (PDGFs), hepatocyte growth factor (HGF) (Allen et al., 1995; Anastasi et al., 1997), and transforming growth factor-β (TGF-β) (Yoshiko et al., 1996). More recent studies have also shown that neuregulins (NRGs), a group of polypeptide growth factors that play roles in the development of the heart and central nervous system, also influence myoblast proliferation and differentiation (Kim et al., 1999).

Evidence from myoblast *in vitro* cell culture studies indicates that the initiation of myoblast differentiation occurs through growth factor signal transduction pathways and is regulated by myoD and myf5 (Clegg et al., 1987; Olson, 1992). Myoblasts exposed to growth factors proliferate and stably maintain expression of both myoD and/or myf5, which are functionally repressed. When growth factor concentrations drop, myoblasts withdraw from G_1 into the G_0 state (Crescenzi et al., 1990; Sorrentino et al., 1990). MyoD and

myf5 are no longer repressed and begin functioning to activate expression of myogenin, which in turn activates transcription of other myogenic protein genes (Buckingham, 1994).

1.3.1 Basic Fibroblast Growth Factor

Basic fibroblast growth factor (bFGF) belongs to a large family of growth factors that promote the proliferation of a wide variety of cells of mesodermal and neuroectodermal origin (Basilico and Moscatelli, 1992). It is an 18 kDa protein lacks a typical signal sequence for secretion and seems to be retained within the cell and the extracellular matrix (Vlodavsky et al., 1991). Mitogenic effects of bFGF are coincide with a block in the terminal myogenic differentiation pathway and half-maximal inhibition of differentiation occurs at a concentration of 0.05 ng of bFGF per ml. The inhibition of differentiation by bFGF is, however, transient and myoblasts will continue myogenic differentiation when removed from bFGF (Florini et al., 1991a).

1.3.1.1 Regulation of Myoblast Differentiation by bFGF

Initially bFGF was thought to exert its potent inhibitory effect on myogenic differentiation through a mechanism involving protein kinase C (Zhoe

et al., 1992). It has been proposed that bFGF stimulates protein kinase C which phosphorylates the conserved threonine in the basic region of MRFs and inhibits DNA binding and transcriptional activation of various muscle specific genes (Bengal et al., 1992; Li et al., 1992). bFGF has also been associated with the repression of muscle specific gene transcription by sustaining the upregulation of cyclin D1, a cyclin dependent kinase (Rao and Kohtz, 1995). Studies that demonstrate the significant reduction in MyoD and myogenin mRNA and protein in C2C12 mouse myoblasts exposed to bFGF support its inhibitory role in myoblast differentiation (Yoshida et al., 1996).

Additional studies on the mechanism of bFGF inhibition of myoblast differentiation have disagreed with the original theory of protein kinase C inhibitory pathway of MRFs. Inhibition of the activity of MRF4 by bFGF has now been shown to be independent of the phosphorylation status of the conserved threonine residue *in vivo*. This suggests that negative regulation of muscle regulatory factors by bFGF does not necessarily involve a direct modification by protein kinase C, but may indirectly act through modification of other regulatory factors such as c-Jun and c-Fos which also repress myogenesis (Hardy et al., 1993).

1.3.1.2 Receptor Binding of bFGF

bFGF and other members of the fibroblast growth factor family exert their effect on myoblasts through receptor tyrosine kinases (RTK). These receptors have both an extracellular ligand-binding domain and an intracellular effector function as a tyrosine kinase that delivers signals to the cells that are generated by the ligand binding (Lee et al., 1989; Ullrich and Schlessinger, 1990). Along with its receptor, bFGF also binds to heparan sulfate proteoglycans (HSPG) located at the cell surface and in the extracellular matrix (Neufelf and Gospodarowicz, 1986; Olwin and Hauschka, 1986). There is also substantial evidence that bFGF requires heparan sulfate binding for subsequent binding to the bFGF receptor (Ornitz et al., 1982; Rapraeger et al., 1991; Yayon et al., 1991), and that HSPG binding increases affinity of bFGF for its receptor (Prestrelsky et al., 1992).

1.3.2 Insulin-like Growth Factor-II

The traditional view that growth factors inhibit myogenic differentiation has been challenged by recent observations of the effects of the insulin-like growth factor (IGF) family (IGF-I and IGF-II) in promoting myoblast differentiation *in vitro* (Stewart and Rotwein, 1996). IGFs have also been shown

to promote C2C12 mouse myoblast proliferation *in vitro* and that the inhibitory and stimulatory effects on myogenesis depend on the concentration of IGFs added to the medium (Florini et al., 1986; Yoshiko et al., 1996). Myoblasts exposed to IGFs for 24-48 hours will proliferate (Ewton et al., 1987). However, longer exposure results in myogenin-mediated differentiation of myoblasts (Florini et al., 1991b).

Insulin-like growth factor-II (IGF-II), is a 67 amino acid polypeptide that is highly homologous with both IGF-I and insulin, and is the principal IGF expressed in differentiating myoblasts (Tollefsen et al., 1989; Rosenthal et al., 1991). IGF-II is expressed early during myogenic differentiation and appears to function as an autocrine/paracrine factor in both stimulating differentiation (Florini et al., 1991c) and down-regulating IGF-I receptor expression in muscle (Rosenthal et al, 1991).

Studies have suggested that IGF-II induced differentiation is mediated by the IGF-I receptor (Ewton et al., 1987) as well as the IGF-II /mannose 6-phosphate receptor (Rosenthal et al., 1994). It has been shown that IGF-II induced proliferation and differentiation of myoblasts is predominantly mediated by the IGF-I receptor and that the actions of IGF-II do not require activation of the IGF-II /mannose 6-phosphate receptor (Bach et al., 1995).

While initially it was not well understood how two opposing processes in myoblasts, proliferation and differentiation, could be mediated through the IGF-I

receptor, recent studies have begun to elucidate the IGF-II signaling pathways. One recent study has demonstrated that the MAP-kinase pathway plays a primary role in proliferative responses of myoblasts to IGF-II while activation of the phosphatidylinositol (PI) 3-kinase/p70 pathway is essential for IGF-II stimulated differentiation. These data support the theory that IGF-II signaling from the IGF-I receptor utilizes two distinct pathways leading to either proliferation or differentiation in myoblasts (Coolican et al., 1997).

One mechanism by which IGF-II activity is regulated is through interactions with insulin-like growth factor-binding proteins (IGFBPs). The IGFBPs are made up of a family of six secreted proteins, with IGFBP-6 having the highest affinity for IGF-II (Bach et al., 1994). The proposed mechanisms of action by which IGFBPs regulate IGF-II activity include (i) protection of IGFs from proteolytic degradation, (ii) tissue-specific targeting of IGFs, and (iii) regulation of availability of IGFs to their receptors by sequestration in the extracellular matrix (James et al., 1996).

1.4 Regulation of Myogenesis by Extracellular Matrix Materials

In vitro studies indicate that the composition and organization of the extracellular matrix is important in myoblast differentiation. When grown in

culture myoblasts preferentially adhere to type I collagen and fibronectin gels (Turner et al., 1983). However, mature myoblasts are usually more strongly associated with ECM components such as type IV collagen and laminin (Kuhl et al., 1986).

Several lines of evidence also indicate that ECM molecules can function as signaling mechanisms during myoblast differentiation (Buck and Horiwitz, 1987). Inhibitors of collagen synthesis in myoblasts have also been shown to inhibit myogenin expression, thus affecting myogenic differentiation (Nandan et al., 1990; Saitoh et al., 1992). Additionally, it has been shown that the synthesis of laminin (Olwin and Hall, 1985), and two proteoglycans, decorin (Brandan et al., 1991) and glypican (Campos et al., 1993), are upregulated in C2C12 mouse myoblasts during myogenesis, suggesting they have a role in differentiation.

Another mechanism through with the ECM affects myoblast differentiation is through the regulation of growth factor activity. An increasing number of growth factors, such as FGFs (Folkman et al., 1988; Benezra et al., 1993), PDGF (Raines et al., 1992), TGF-β (McCaffrey et al., 1992), and IGFs (Jones et al., 1993), have been found to associate with ECM proteins. ECM binding of growth factors may regulate activity by allowing local storage of large quantities of growth factors in a readily available form and protecting them from proteolytic degradation. In this manner, growth factors can be used to generate

rapid and highly localized signals (Taipale et al., 1995; Miyazawa et al., 1996). Furthermore, matrix bound growth factors may participate in cell-cell signal transmission and induction of anchorage-independent growth (Taipale and Keski-Oja, 1997).

1.4.1 Laminins

Laminins are extracellular glycoproteins that function in cell attachment, stimulation of growth and differentiation, and cell migration and morphology (Kleinman et al., 1985). They are composed of one heavy (α) chain of 400 kDa, and two similar (but not identical) light (β) chains of 200 kDa that combine to form a cross-shaped molecule (Engel, 1992) (Figure 1.2). Laminin-1 and merosin (laminin-2) are both synthesized by myoblasts and differ in the structure of their heavy chains, being composed of the $\alpha 1$ and $\alpha 2$ chains respectively (Ehrig et al., 1990).

Laminin-1 and merosin play similar and distinct roles at specific stages of myogenic differentiation. Myoblasts adhere equally well to both laminins, and both promote fusion of myoblasts into myotubes. However, they are also divergent in their function in that laminin-1 promotes myoblast proliferation while merosin promotes myotube stability (Vachon et al., 1996). Further evidence supporting the indispensable role of merosin in muscle development include

studies that demonstrate a murine muscular dystrophy caused by mutation of

the Lama2 gene which codes for merosin (Xu et al., 1994; Sundana et al.,

1995; Kuang et al., 1998).
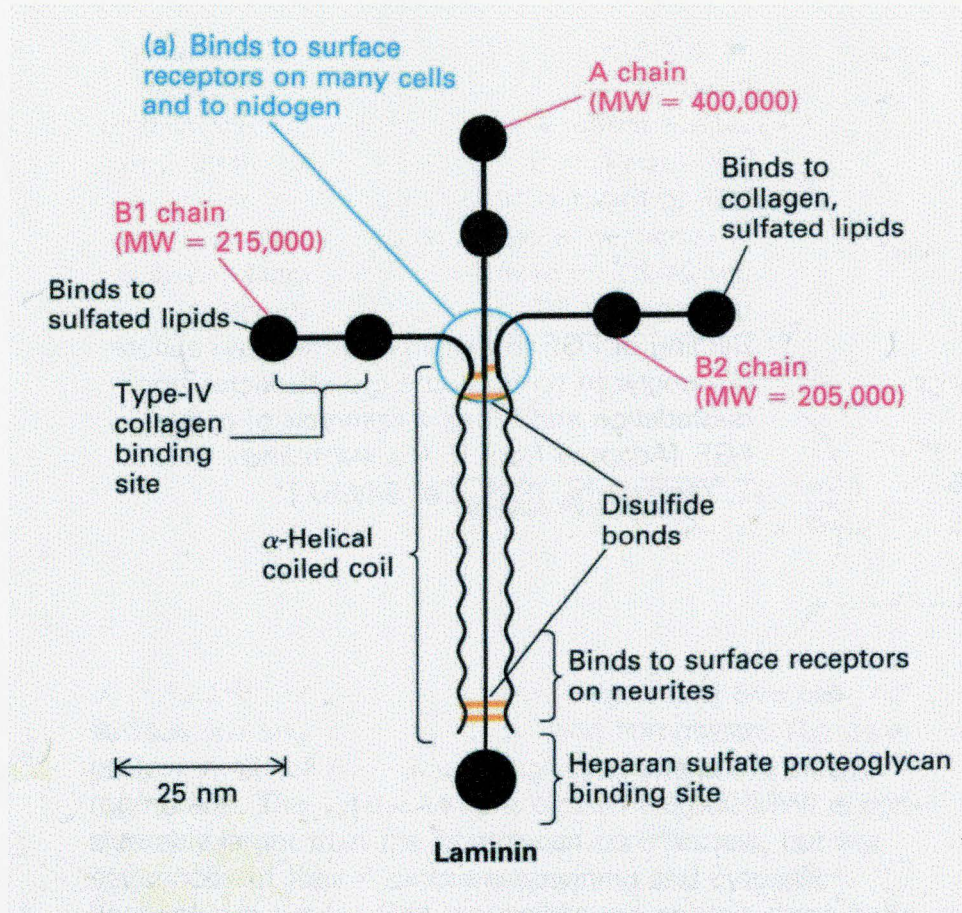
Figure I-2 Cross-shaped structure of laminin containing globular domain (black circles) and a coiled-coil region in which the three chains are linked by several disulfide bonds (yellow). From: MOLECULAR CELL BIOLOGY by Lodish et al. © 1986, 1990, 1996 by Scientific American Books, Inc. Used with permission by W.H. Freeman and Company.

1.4.2 Integrins

The mechanism through which laminin most commonly interacts with cell processes is through the integrin receptors (Menko and Boettiger, 1987). Myoblasts express several members of the integrin receptor family ($\alpha1\beta1$, $\alpha3\beta1$, $\alpha6\beta1$, and $\alpha7\beta1$) that recognize laminin and result in pronounced effects on myoblast behaviour (Crawley et al., 1997). While various integrins have the ability to recognize laminin, recent studies have shown that the major myoblast integrin that binds to laminin is $\alpha7\beta1$ and that several isoforms of this integrin are expressed during muscle development (Crawley et al., 1997). It has also been shown that interactions with integrins can regulate gene expression and thus provide a direct means for signals from the extracellular matrix to initiate terminal differentiation in myogenic cells (Menko and Boettiger, 1987).

1.5 Rationale and Goals for Thesis

While the employment of alginate-polylysine encapsulated recombinant myoblasts as vehicles for gene delivery seems to be an ideal system, it is not without difficulties. Recent studies in our laboratory have revealed two notable problems when dealing with encapsulated myoblasts.

Firstly, APA encapsulated myoblasts do not proliferate as well in culture as unencapsulated, or "naked", myoblasts. This loss of cell viability may be due to serum withdrawal, where the harsh changes in conditions lead to cell death, or simply due to the unnatural capsule environment. Secondly, encapsulated myoblasts have significantly decreased myogenic differentiation. This inability of encapsulated myoblasts to differentiate as a result of the suspension-type growth as well as the sparse distribution of cells within the capsules may contribute to the inhibition of myogenic differentiation.

These findings suggest that the microcapsule environment and the *in vitro* system used for myoblast culture must lack elements that promote myogenic proliferation and differentiation. This can conceivably be solved by two separate approaches. Firstly, an exogenous source of growth factors can be provided to the encapsulated cultured myoblasts to promote proliferation and differentiation. Secondly, an extracellular matrix or compound can be introduced within the microcapsular environment that will allow for myoblast adherence.

The objectives of this study are to enhance the proliferation and differentiation of alginate-poly-L-lysine alginate encapsulated C2C12 mouse myoblasts. Two growth factors known to affect myoblast proliferation and differentiation, bFGF and IGF-II, will be included within the microcapsules at various concentrations. A second C2C12 mouse myoblast cell line that

overexpresses IGF-II will also be evaluated in terms of its differentiation and proliferative capacity when grown within microcapsules. Additionally, collagen, laminin-1 and merosin will included as matrix materials within the microcapsules to assess any effect they may have on the proliferation and differentiation of C2C12 myoblasts.

2.0 METHODS

2.1 Cell Lines

All cell lines used were derived from the murine C2C12 myoblast cell line (ATCC, Rockville, Md; catalogue #CRL-1772). The pNMG3 myoblast line is transfected with human growth hormone (created by Kelly Bowie, 1997), while the C2IGF-II myoblast line was transfected and overexpressing insulin-like growth factor II (Stewart et al., 1996). The C2IGF-II cell line was a generous gift from Peter Rotwein in the Washington University School of Medicine.

The cells were maintained in 100 mm tissue culture dishes at 37°C in a 5% CO_2 water-jacketed incubator. Growth media consisted of Dulbecco's modified essential media supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Gibco). Differentiation media consisted of Dulbecco's modified essential media supplemented with 2% horse serum and 1% penicillin/streptomycin.

2.2 Microencapsulation of Cells

Cells were harvested at approximately 70% confluency from 100 mm plates using 0.5 ml of trypsin. Trypsinized cells were resuspended in 4 ml of growth media (DMEM 10% fbs, 1% P/S), transferred to a 50 ml conical tube, and spun at

1000 rpm fof 10 minutes at 4°C. The cell pellet was then resuspended in 5 ml of cold 1X Phosphate buffered saline (PBS) and cell number was determined using a Coulter Counter (Coulter Electronics, Hialeah, Florida; model Z1). After counting, the cell suspension was washed with an additional 35 ml of 1X PBS and was spun again at 1000 rpm for 10 minutes at 4°C. The cell pellet was subsequently resuspended in 0.5 ml of 0.9% NaCl and was mixed with 10 to 20 ml of 2.0% Kelmar potassium alginate (Kelco, Chicago, Ilinois) so that the concentration of cells was between 3 to 5 million cells per ml of alginate. The alginate solution had been previously sterilized using a 0.45 μm sterile syringe filter (Pall Gelman). The alginate/cell suspension was then transferred to a 20 ml syringe, placed into a delrin holder and attached to the microcapsule machine. The microcapsule machine consisted of an Orion Syringe pump (model M362) that was supported by a plexiglass-housing unit. The alginate/cell suspension was then extruded through a 27-gage 1 inch blunt end hypodermic needle (Vita Needle, MA) into a beaker containing 50 ml of cold 1.1% Calcium Chloride ($CaCl_2$) solution. As the alginate/cell suspension comes off the end of the needle and falls into the $CaCl_2$ it forms beads, or microcapsules, that contain the cells inside. The microcapsules were then transferred to a 50 ml conical tube and were subjected to a series of washes (Appendix B), where the capsules were mixed gently in the wash solution, allowed to settle, and the supernatant was removed. After completion of the washes, the microcapsules were transferred into growth media in a T150 tissue culture flask.

2.2.1 Microencapsulation of Cells with Growth Factors

Microencapsulation with growth factors was performed as described as above with one modification. Growth factors were added to the 0.5 ml final cell suspension before it was mixed with the alginate.

2.2.2 Microencapsulation of Cells with Matrix Materials

Microencapsulation with matrix materials was performed as described as above with several modifications. The matrix material being used was mixed with the sterile alginate immediately before being mixed with the final cell suspension. When collagen was used, it was adjusted to pH 7 using sterile 0.1M sodium hydroxide (NaOH) just prior to mixing with the alginate. All microcapsules made using matrix materials were extruded into a beaker containing 1.1% $CaCl_2$ that had been pre-warmed to 37°C in order to cause gelling of the matrix material. The concentration of matrix material used was 10 µg per ml of alginate. Both laminin-1 and merosin were obtained from Gibco BRL, while collagen-1 came from Collaborative Biomedical Products.

2.3 Isolation of encapsulated cells

Microcapsules were transferred into 50 ml conical tubes and were washed with 1X PBS until all media was washed away. 30 ml of 0.055 M Sodium Citrate was added and the tube was placed on ice on a rocking table for 10 minutes. Excess sodium citrate was removed and the microcapsules were drawn into a syringe and forced through a 26 ½ gage needle in order to break up the capsules. While being forced through the needle, the microcapsules were divided into separate 15 ml conical tubes in the following volumes: 1.0 ml in each of three tubes (CPK assay), 1.0 ml in one tube (MHC staining), and 4.0 ml in one tube (RNA isolation), for a total of 8.0 ml of capsules. The cells were then washed with 1X PBS and were spun at 1500 rpm at 4°C for 10 min. At this point a two-layer pellet existed; the top and bottom layers being composed of the capsule material and cell pellet respectively. The supernatant, along with the capsule material, was then drawn off and the cell pellet was washed and spun as before. If the cells were to be spun onto microscope slides, they were incubated at room temperature with 1.0 ml of trypsin until all cell clumps had broken up before the final wash and spin. Finally, the supernatant was drawn off and the cell pellet was suspended in 0.5 ml of 1X PBS.

2.4 Characterization of Encapsulated Cells

2.4.1 Determination of Viable Cell Number per Capsule

The number of viable cells per capsule was determined with the use of AlamarBlue reagent (AccuMed). AlamarBlue is a re-dox reagent that serves as an alternative electron acceptor. When reduced, it produces a detectable fluorometric change.

A volume of 100 µl of capsules was added to 500 µl of growth media in a 24-well sterile tissue culture plate. Naked cells seeded out at various known densities were also added to 500 µl of media in order to generate a standard curve. 50 µl of AlamarBlue (10% of the culture volume) was then added to each well, mixed, and the plate was incubated at 37°C for 4 hours. After the incubation period, a 100 µl sample of the media was transferred to a 96-well plate and was read on a fluorometer (excitation 530 nm, emission 590 nm). A standard curve was generated and was used to determine the total number of viable cells in the sample. The microcapsule samples were then recovered and counted (by eye), and the count was used to determine the viable cell number per capsule.

2.4.2 Determination of Total Cell Number per Capsule

Total cell numbers were determined via the DNA assay using propidium iodide (Appendix D). A 100 μl sample of microcapsules was counted (by eye), and was transferred to a 1.5 ml eppendorf tube to which 100 μl of 1X RNase solution was added. The sample was then frozen overnight to ensure all cells were dead. After thawing, 100 μl of the sample was loaded onto a 96-well plate (in duplicate) and 200 μl of propidium iodide (working concentration) was loaded into each sample well. The plate was then read with a fluorometer (excitation 530 nm, emission 645 nm) and fluorescence units were converted to total cell number per capsule using the standard curve.

2.5 Cytocentrifugation of Cells onto Slides

Cells were spun onto slides using a cytocentrifuge in the following manner. Assembly of slides in centrifuge was as follows: One aptex-coated microscope slide (Corning Glass Works) was slipped into the metal holder, on top of the slide was placed a cardboard filter that restricted the cells to a small circular area on the slide. The plastic loading chamber was then placed on top of the filter, and the metal holder was clamped over to hold everything in place. The slide assembly was than placed into the cytocentrifuge with the loading chamber

facing upwards. 70 µl of 1X PBS was loaded into the chamber and was spun onto the slides for 3 min at 500 rpm. 100 µl of cell suspension was then loaded and spun for 5 min at 800 rpm. After the cells were spun onto slides, they were fixed in ice cold 90% methanol for 5 min., washed in 1X PBS 3 times (at 5 min. each) and were stored at 4°C in 1X PBS (for up to 1 month) until they were stained for myosin heavy chain.

2.6 Determination of Myoblast Differentiation

2.6.1 Creatine Phosphokinase Activity

Creatine Phosphokinase (CPK) activity was analyzed using the CK-MPR 1 kit (Beohringher Mannheim). Briefly, intracellular proteins were released from an isolated myoblast suspension (~ 500 µl) by sonicating 3 times at 10 seconds each. 25 µl of the sonicated sample was then transferred to a 1.5ml microtube to which 625 µl of assay reagent was added. The sample was incubated at room temperature for 3 min. and was transferred to a quartz cuvette. Absorbance (at 340 nm) was read at 1 minute intervals (starting at t=0) for 3 minutes and the change in absorbance per minute was determined. This change was multiplied by 4217 in order to obtain the CPK activity (U/l), which was divided by the

amount of protein present in the original sample in order to express activity as U/l per μg of protein.

2.6.2 Myosin Heavy Chain Staining

Slides were first incubated at 37°C with 5% non-fat dry milk (NFDM) in 1X PBS for 30 min. by dropping enough liquid onto the slide to cover the cells. The liquid was then drained off and the slides were incubated with 1:10 dilution of MF20 monoclonal antibody (ATTC) in 5% NFDM for 60 min at room temperature. Slides were washed 3 times in 1X PBS for 5 min each, incubated with a 1:1000 dilution of HRP-conjugated goat anti-mouse IgG antiserum (BioRad) in 5% NFDM for 60 min. at room temperature, and were washed again as described. 6 mg of diaminobenzidine tetrahydrochloride (DAB) (Sigma) was then dissolved in 10 ml of 0.05M Tris buffer (pH 7.6). After addition of 0.1 ml of 3% hydrogen peroxide, the DAB solution was applied to the slides and the reaction was stopped by washing with water when the stain had developed.

2.6.3 Determination of Myogenic Index

Myogenic index was determined by scoring the number (percentage) of nuclei that existed in mulinucleate cells on slides that had been stained with MHC.

2.7 Analysis of RNA

2.7.1 Probe Preparation

Plasmid DNA (5 μg) was digested with the appropriate enzyme in order to release the insert DNA to be used as a probe. The restriction digest was set up with 1 μl of restriction enzyme, 2 μl of reaction buffer and was brought up to a total volume of 20 μl. The digest was then run for 1-2 h at 37 °C and samples were run on a 0.7% agarose gel at 80V for 1.5h. The desired DNA fragment was sliced out of the gel and was purified using the Gene Clean kit (BioRad). The clean probe was resuspended in 30 μl of TE buffer and concentration was determined using a spectrophotometer.

2.7.2 RNA Isolation

RNA was isolated from myoblasts with the use of TRIzol Reagent (Gibco) according to the instructions provided by Gibco. 3 mls of TRIzol reagent was added to isolated myoblasts. Lysed cells were pipetted up and down several times and transferred into a capped polypropylene round bottomed tube. Following a 5 minute incubation period, 600 μl of chloroform was added and the tube was vortexed for 15 seconds. The sample was then incubated at room temperature for 2-3 minutes and centrifuged at 10,000 rpm for 15 minutes at 5

°C. The aqueous phase (top layer) of the sample was then removed, transferred to a fresh tube, and 1.5 mls of isopropyl alcohol was added in order to precipitate the RNA (for 15 minutes). Precipitated RNA was centrifuged at 10, 000 rpm for 10 minutes at 5 °C following which the supernatant was removed. The pellet was then washed with 3 mls of 75% ethanol by vortexing and centrifuging at 8,000 rpm for 5 minutes at 5 °C. The washed pellet was allowed to air dry and was resuspended in RNase-free water. Samples were incubated at 55 °C for 5 minutes prior to storing at -70 °C.

2.7.3 Dot Blot

RNA (4-8 μg) was brought up to a volume of 25 μl in DEPC water. 500 μl formamide,162 μl 37% formaldehyde, and 100 μl of 10X MOPS was then added to each RNA sample. Samples were then incubated at 65°C for 5 min., immediately chilled on ice and 1 volume of cold 20X SSC was added. Hybond-N nylon membrane (Amersham) was then pre-wet in 10X SSC and the dot-blot apparatus as assembled. Prior to loading the samples, 200 μl of buffer solution (6 ml 20X SSC, 4 ml 37% formaldehyde, and 10 ml DEPC water) was rinsed through each well. RNA was then spotted onto membrane and allowed to sit for 30 min before applying the vacuum. Each well was then rinsed twice with buffer solution, and the membrane was removed, placed between 2 pieces of whatman paper and baked for 10 min at 80°C. The membrane was wrapped in plastic wrap

and RNA was fixed to the membrane by exposing to UV light for 3 min. The membrane was then placed in a 50 ml conical tube to which 5.0 ml of pre-hybridization buffer (with 50 μl of 10 mg/ml denatured sonicated herring sperm DNA) was added. The conical tube was placed inside a capped glass hybridization cylinder and incubated in rotating hybridization oven at 42 °C for 4 hours. The pre-hybridization buffer was removed and replaced with 5.0 mls of hybridization buffer (50 μl ^{32}P labelled probe, 50 μl of 10 mg/ml denatured sonicated herring sperm DNA) and was hybridized overnight (~ 16 h) at 42 °C in rotating oven.

Following hybridization, the membrane was washed with 2X SSC + 1% SDS at room temperature (2x at 15 min each) and 0.1 SSC + 0.5% SDS at 65 °C (2x at 30 min each). The membrane was then blotted with whatman paper, sealed in plastic wrap, exposed to a phosphor screen for ~ 2 h and read with a phosphorimager.

2.8 Statistical Analyses

All statistical analyses were determined using a t-test (Microsoft Excel, Microsoft Office 97) using a 95% confidence interval ($p < 0.05$).

3.0 RESULTS

3.1 Inclusion of Basic Fibroblast Growth Factor within Microcapsules

In order to study the effect of basic fibroblast growth factor (bFGF) on the growth and differentiation of encapsulated myoblasts, pNMG3-pooled myoblasts (a C2C12 mouse myoblast cell line transfected with human growth hormone by Kelly Bowie, 1997) were encapsulated with increasing concentrations of bFGF within the microcapsules. Additionally, rat collagen I (Collaborative Biomedical Products) was included in microcapsules containing bFGF to determine if an extracellular matrix material was necessary to hold the bFGF within the microcapsules.

pNMG3 myoblasts were encapsulated under the following conditions: (i) pNMG3 control (no bFGF, no collagen), (ii) pNMG3 with collagen, (iii) pNMG3 with collagen and 10 ng bFGF per ml alginate, (iv) pNMG3 with collagen and 100 ng bFGF per ml alginate, (v) pNMG3 with collagen and 1000 ng bFGF per ml alginate, and (vi) pNMG3 with 100 ng bFGF per ml of alginate (no collagen). Encapsulated myoblasts were cultured in growth media for 14 days and were sampled for proliferation on day 0 (day of encapsulation), day 7 and day 14. The microcapsules were then transferred to differentiation media and were sampled for growth and differentiation on day 0 (day microcapsules were switched into differentiation media), day 7 and day 14 of differentiation.

3.1.1 Effect of bFGF on Myoblast Growth and Survival

Figure 1 examines the effect of including bFGF within microcapsules on myoblast growth. Between day 0 and day 7 of growth (day 0g and day 7g), myoblasts encapsulated with 10 ng of bFGF per ml of alginate showed the greatest increase in viable cell number per capsule (Figure 1 A, B). These myoblasts showed a percentage growth increase of 146.6% ± 17.4 that was significantly higher (p < 0.05) than myoblasts encapsulated with no bFGF present (92.8% ± 35.6) . However, from day 7 to day 14 of growth, encapsulated myoblasts exposed to 10 ng/ml of bFGF had the smallest increase in growth, 30.0% ± 16.0, while myoblasts encapsulated with 1000 ng/ml bFGF had a significantly higher growth rate (p < 0.05) of 236.8% ± 6.3 when compared to the control value of 58.1% ± 25.1. When growth was examined over the two-week period, only myoblasts encapsulated with 1000 ng/ml of bFGF showed a significant increase (p < 0.05) in percentage growth of 333.2% ± 27.3 when compared to the control of 199.0% ± 14.1 (Figure 1C). Additionally, it appeared that when myoblasts were encapsulated with 100 ng/ml of bFGF, the presence of collagen has a small, but significant (p < 0.05) positive effect on myoblast growth (Figure 1C).

Figure 2 examines the effect of bFGF on the survival of differentiating myoblasts. During the first week of differentiation (day 0d to day 7d), all encapsulated myoblasts demonstrated sharp decreases in viable cell number,

regardless of the concentration of bFGF present in the microcapsules (Figure 2 A, B). The drop in cell numbers between day 7 and day 14 of differentiation for myoblasts encapsulated with 1000 ng/ml of bFGF + collagen, and 100 ng/ml bFGF with no collagen demonstrated values of 26.8% ± 7.7 and 25.2% ± 4.5 respectively. These values were significantly less ($p < 0.05$) than the 57.2% ± 4.4 percentage decrease in cell numbers experienced by control encapsulated myoblasts. There was no noted difference with respect to myoblast survival when comparing myoblasts encapsulated with 100 ng/ml of bFGF with and without collagen present (Figure 2B).

Figure 1

The effect of basic fibroblast growth factor (bFGF) on the growth of encapsulated pNMG3 myoblasts. (A) The number of viable cells per capsule sampled on day 0, day 7 and day 14 of growth (day 0g, day 7g and day 14g) as determined by the Alamar Blue assay. (B) Percentage growth increase (of number of viable cells) of myoblasts between day 0 and day 7 as well as day 7 and day 14 of growth. (C) Percentage growth increase of myoblasts between day 0 and day 14 of growth. Day 0 of growth corresponds to the day of encapsulation. Values given are the average of triplicate samples (± the standard deviation). Concentrations of bFGF are given in ng of bFGF per ml of alginate. Asterisks denote a significant difference ($p < 0.05$) compared to the control. Plus (+) sign denotes a significant ($p < 0.05$) difference between microcapsules containing 100 ng/ml of bFGF.
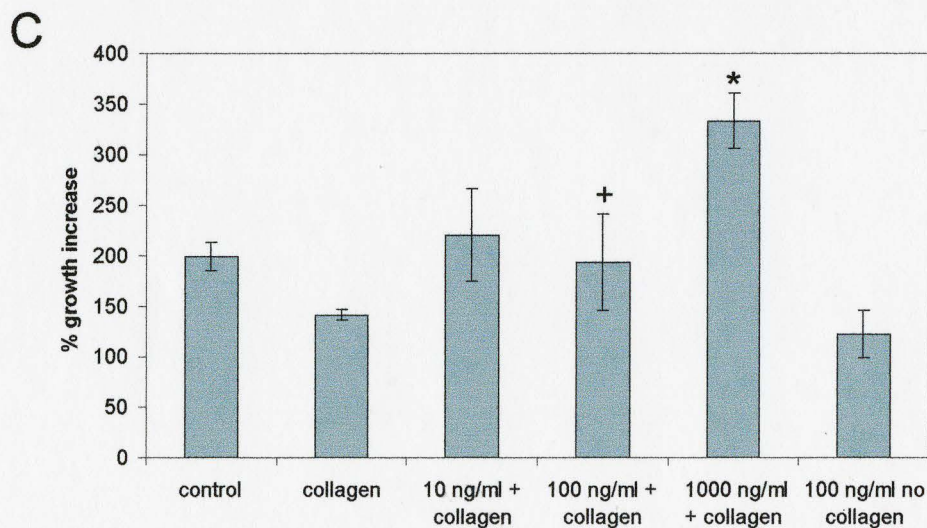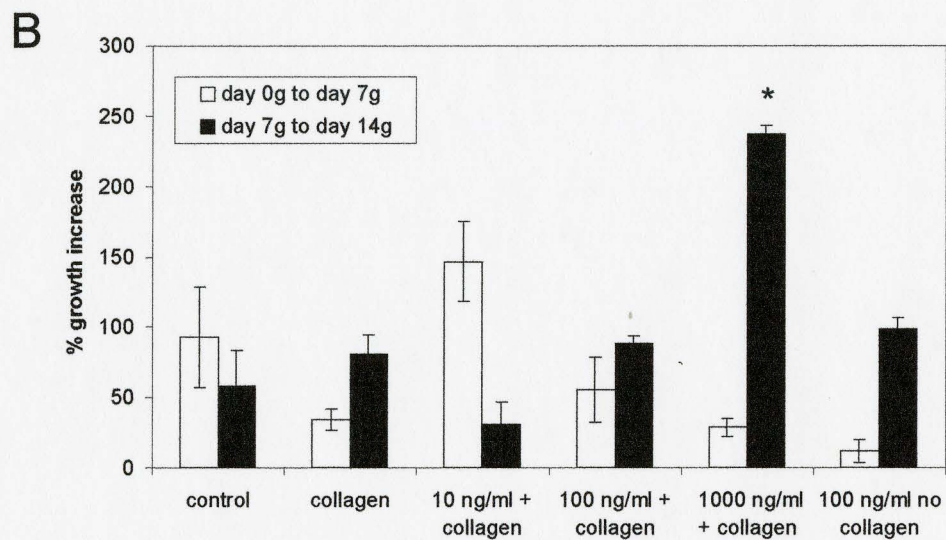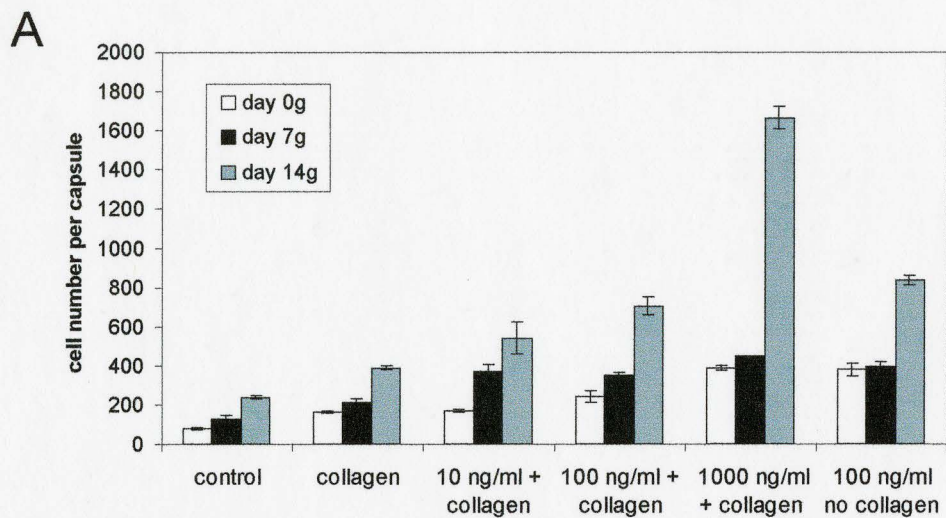
Figure 2

Effect of bFGF on survival of encapsulated pNMG3 myoblasts during differentiation. (A) The number of viable cells per capsule sampled on day 0, day 7 and day 14 of differentiation (day 0d, day 7d and day 14d) as determined by the Alamar Blue assay. (B) Percentage growth decrease (of number of viable cells) of myoblasts between day 0 and day 7 as well as day 7 and day 14 of differentiation. Day 0 of differentiation corresponds to the day encapsulated myoblasts were placed in differentiation media. Values given are the average of triplicate samples (± the standard deviation). Concentrations of bFGF are given in ng of bFGF per ml of alginate. Asterisks denote a significant difference ($p < 0.05$) compared to the control.

A
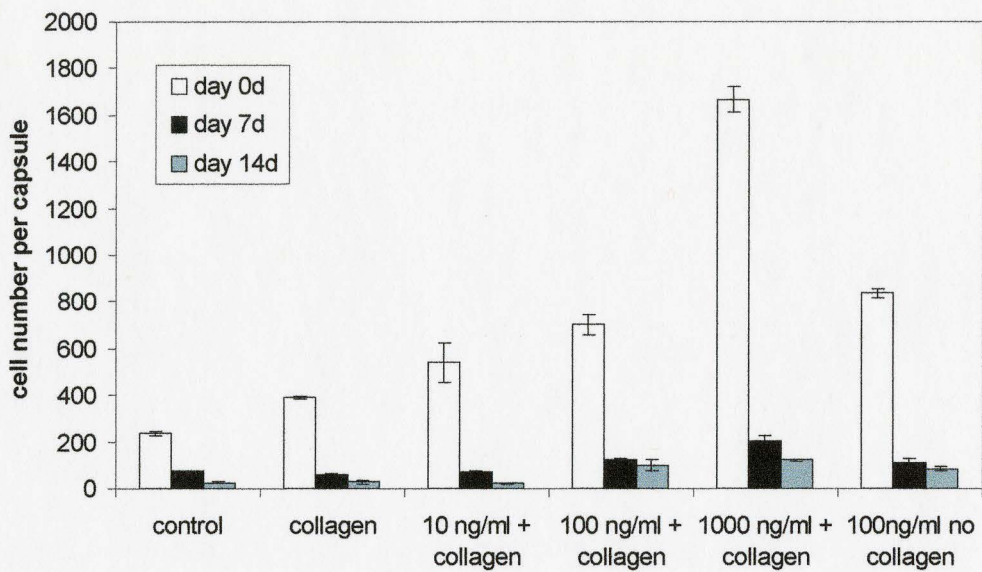
B
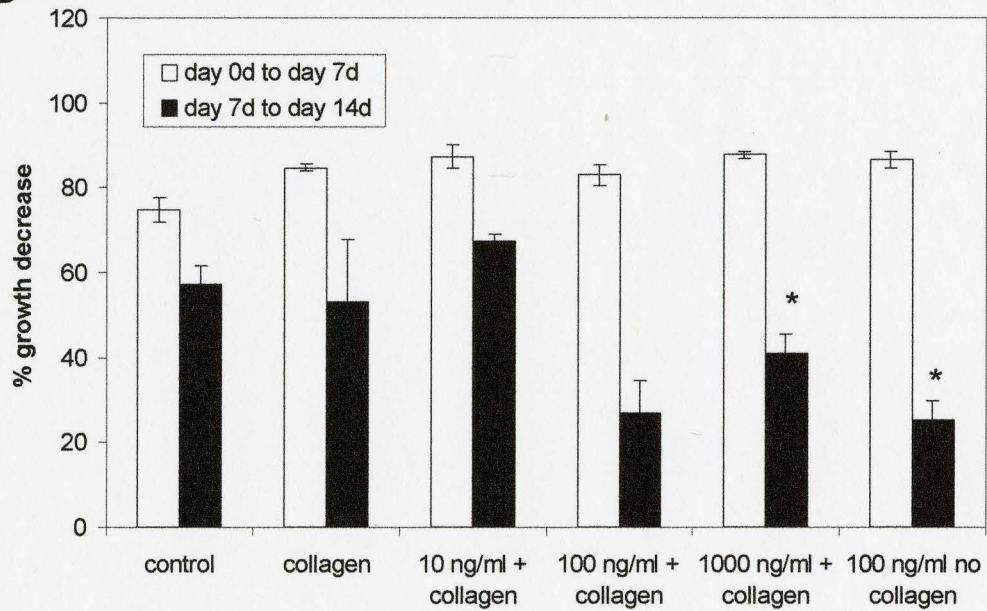
3.1.2 Qualitative Analysis of Myoblast Differentiation

In order to analyze myoblast differentiation, encapsulated cells were stained for the presence of myosin heavy chain (MHC), a protein that is upregulated prior to myoblast fusion during differentiation. While myoblasts grown on tissue culture plates showed a noted increase in MHC expression between day 0 and day 14 of differentiation, MHC protein expression in encapsulated myoblasts was greatly reduced (Figure 3). Additionally, there was no significant difference in MHC expression in any of the encapsulated myoblast, regardless of the concentration of bFGF that was present within the microcapsules (Figure 4).

In addition to MHC expression, the myogenic index (percentage of nuclei present in multinucleate cells) of encapsulated myoblasts was determined. Positive control myoblasts grown on tissue culture plates had begun to fuse by day 14 of differentiation (Figure 3B), while no fusion of encapsulated myoblasts was seen during the length of the differentiation period (Figure 3 D,E; Table 1).

Figure 3

Analysis of Myosin Heavy Chain expression of pNMG3 myoblasts encapsulated with bFGF. (A) Naked myoblasts (grown on tissue culture plates) on day 0 of differentiation. (B) Naked myoblasts on day 14 of differentiation (positive control). (C) Control encapsulated pNMG3 myoblasts on day 0 of differentiation. (D) Control encapsulated pNMG3 myoblasts on day 14 of differentiation. (E) pNMG3 myoblasts encapsulated with 1000 ng bFGF per ml of alginate on day 0 of differentiation. (F) pNMG3 myoblasts encapsulated with 1000 ng bFGF per ml of alginate on day 14 of differentiation. Arrowheads indicate cells that have stained positive for MHC. Small arrows indicate capsule material amongst the cells. Blue arrows indicate multinucleate cells and red arrows indicate examples of cells that are negative for MHC. Magnification is 400X.
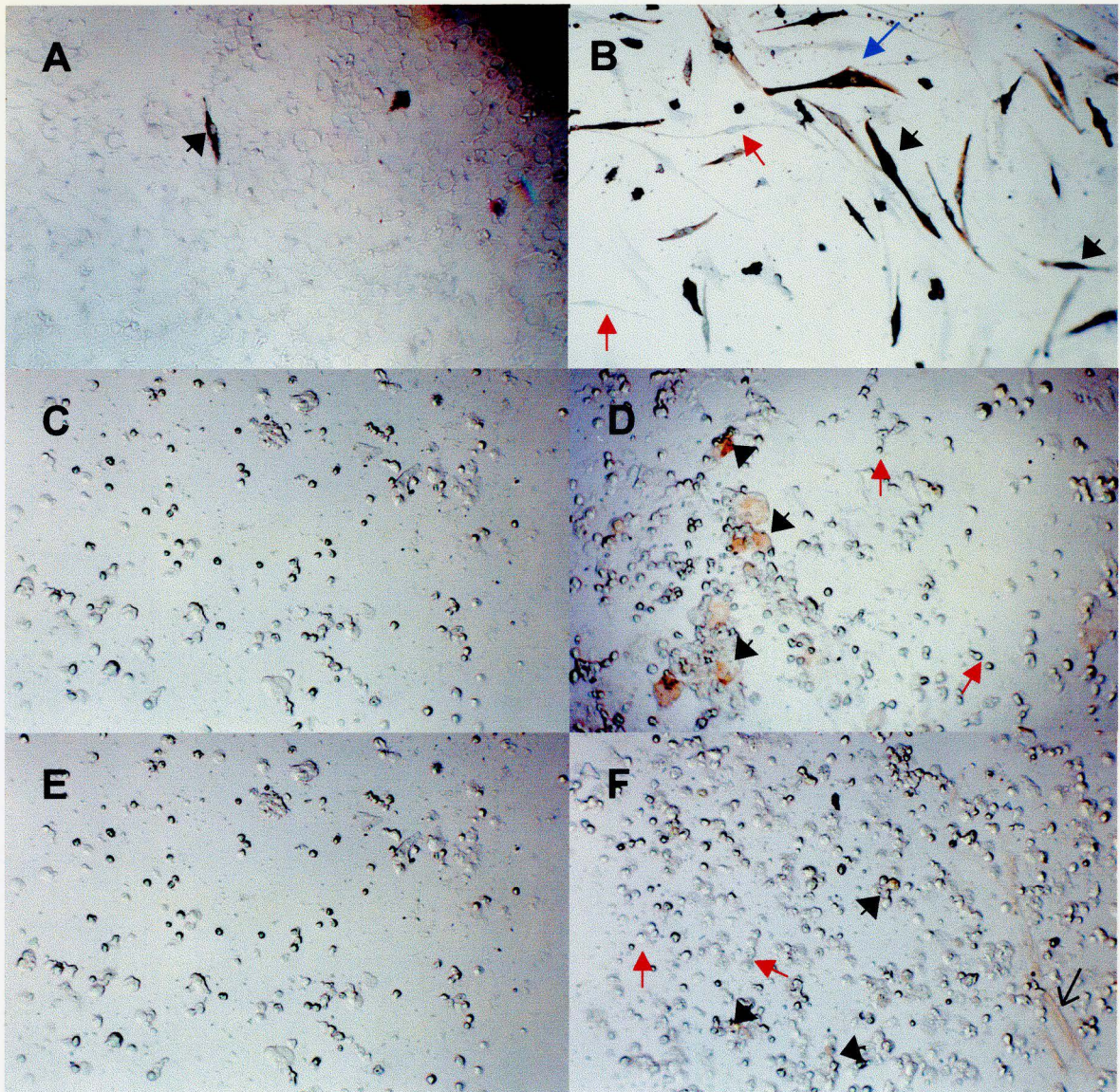
Figure 4

Qualitative analysis of myoblast differentiation: Effect of bFGF on MHC expression of encapsulated pNMG3 myoblasts. MHC expression of encapsulated myoblasts was determined on day 0, day 7 and day 14 of differentiation (day 0d, day 7d, and day 14d). Values given are the percentage of cells that stained positive for MHC and are the average of triplicate samples ($\pm$ the standard deviation). Sample values were determined by counting the number of cells positive for MHC in 5 fields at 400X magnification. Positive control values (unencapsulated cells) were 0% MHC-positive cells for day 0, and 31.4% $\pm$ 1.0 and 56.2% $\pm$ 2.3 for day 7 and day 14 of differentiation respectively. Values in brackets are the average number of cells counted for each sample.

% cells positive for MHC

Chart data (values in parentheses shown on bars):

- **control**
  - day 0d: (101 ± 16)
  - day 7d: (152 ± 39)
  - day 14d: (225 ± 21)
- **collagen**
  - day 0d: (119 ± 20)
  - day 7d: (125 ± 22)
  - day 14d: (245 ± 64)
- **10 ng/ml + collagen**
  - day 0d: (123 ± 19)
  - day 7d: (204 ± 101)
  - day 14d: (234 ± 74)
- **100 ng/ml + collagen**
  - day 0d: (114 ± 31)
  - day 7d: (137 ± 21)
  - day 14d: (308 ± 87)
- **1000 ng/ml + collagen**
  - day 0d: (152 ± 24)
  - day 7d: (251 ± 45)
  - day 14d: (338 ± 48)
- **100 ng/ml no collagen**
  - day 0d: (120 ± 23)
  - day 7d: (210 ± 66)
  - day 14d: (235 ± 79)

Legend: □ day 0d ■ day 7d ▨ day 14d

Table 1.

Qualitative analysis of myoblast differentiation: the effect of bFGF on the myogenic index of pNMG3 myoblasts. The myogenic index is expressed as the percentage of nuclei that were present in multinucleate cells. Values are a result of counting 5 fields of cells at 400X magnification and are the average of triplicate samples (± the standard deviation). Naked (unencapsulated) pNMG3 myoblasts were used as a positive control. Values in brackets are the average number of cells counted per sample.

Capsule type	Myogenic Index		
	Day 0	Day 7	Day 14
Control	0 (101 ± 16)	0 (157± 45)	0 (131 ± 21)
Collagen	0 (119 ± 20)	0 (129 ± 41)	0 (164 ± 39)
10 ng/ml + collagen	0 (123 ± 19)	0 (205 ± 22)	0 (186 ± 38)
100 ng/ml + collagen	0 (114 ± 31)	0 (134 ± 16)	0 (132 ± 22)
1000 ng/ml + collagen	0 (152 ± 24)	0 (186 ± 17)	0 (153 ± 25)
100 ng/ml no collagen	0 (121 ± 23)	0 (138 ± 22)	0 (119 ± 19)
naked cells	0 (98 ± 19)	24.8 ± 4.1 (141 ± 62)	41.3 ± 2.4 (217)

3.1.3 Quantitative Analysis of Myoblast Differentiation

As a quantitative measure of myogenic differentiation, RNA was isolated from encapsulated cells and was analyzed using dot blotting for expression of troponin I and myogenin mRNA, both of which are transcription factors upregulated during differentiation. Levels of Phosphoglycerate kinase (PGK) mRNA, which are consituitively expressed, were also analyzed and used as a positive control. Dot Blots were analyzed for the intensity of the signal using a phosphorimager (Figure 5) and muscle-specific transcripts were expressed by comparing the signal to the PGK sample where the level of PGK was set to a baseline of 1.0. Due to the large volume of microcapsules needed to isolate an adequate amount of mRNA, only one sample was analyzed for each sample day.

Since only one sample was used when analyzing mRNA levels, no conclusive results could be drawn from the data. RNA levels of both myogenin and troponin I were variable, but levels in all microcapsules types were in the same range for all sample days, with day 14 ranges for myogenin and troponin I being 0.029 to 0.048 and 1.71 to 2.51 respectively (Figure 6 A,B).

Additional quantitative data was obtained by analyzing the activity of creatine phosphokinase (CPK), an enzyme that is upregulated during myoblast differentiation (Figure 6 C). After 7 days of differentiation, there was no difference in CPK activity for all encapsulated myoblasts, regardless of the presence or concentration of bFGF. However, by day 14 of differentiation,

myoblasts encapsulated with 100 ng/ml and 1000 ng/ml of bFGF showed significantly less CPK activity ($p < 0.05$) with values of 299.4 ± 13.4 and 287.8 ± 15.4 U/l per μg of viable protein respectively, compared to the control activity of 400.7 ± 16.0 U/l. The presence of collagen in microcapsules containing 100 ng/ml of bFGF had no significant effect on CPK activity.

Figure 5

The effect of bFGF on the differentiation of encapsulated pNMG3 myoblasts: Dot Blots of PGK, myogenin, and troponin I RNA expression on day 0, day 7, and day 14 of differentiation. Images represent a computer-generated image using ImageQuant software (Molecular Dynamics). RNA isolated from naked pNMG3 myoblasts (grown on culture plates) 5 days after the induction of differentiation was used as a positive control.
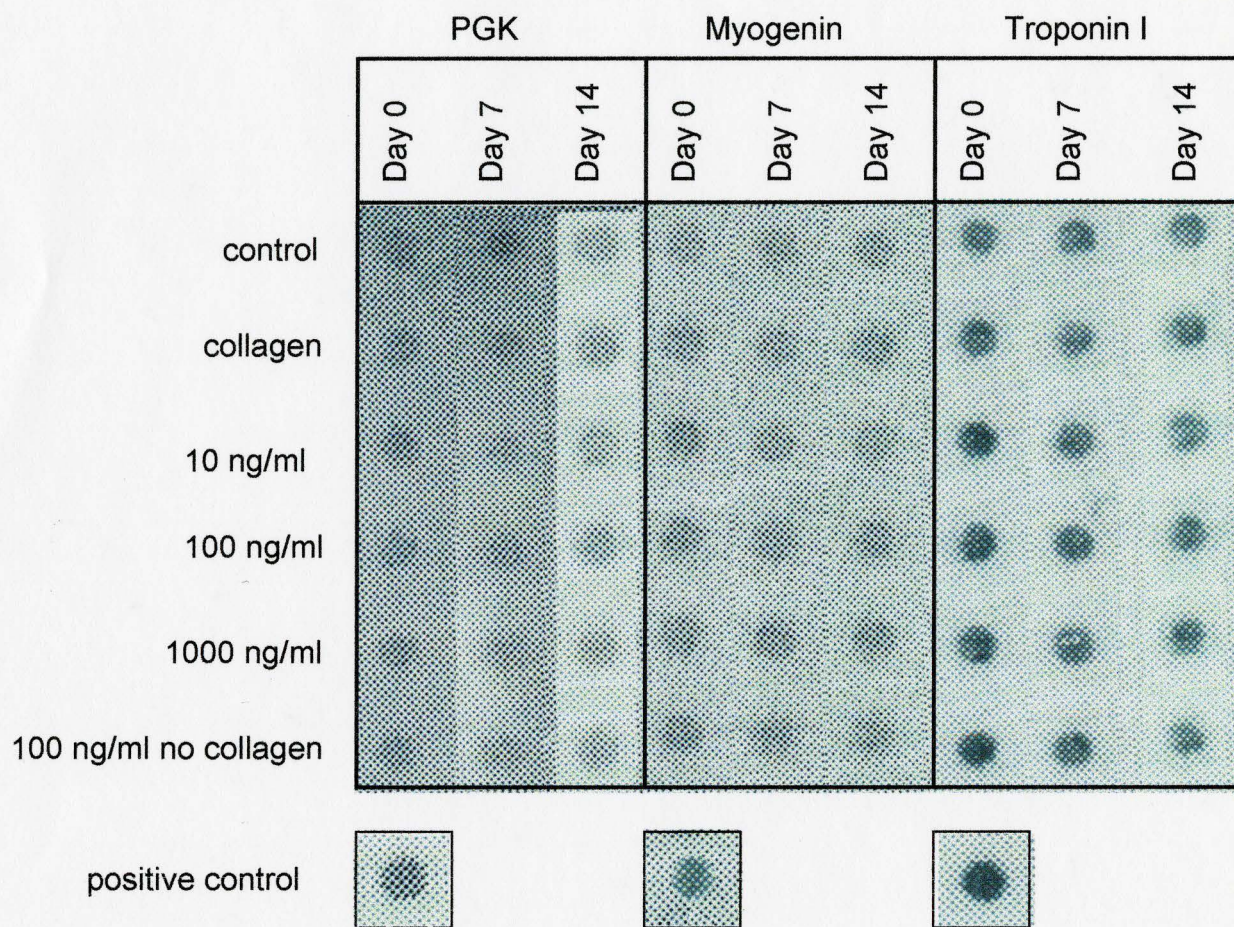
Figure 6

Quantitative analysis of myoblast differentiation: the effect of bFGF on the differentiation of encapsulated pNMG3 myoblasts. (A) Myogenin RNA levels (as determined by dot blot) of encapsulated pNMG3 myoblasts on day 0, day 7, and day 14 of differentiation. (B) Troponin I RNA levels (as determined by dot blot) of encapsulated pNMG3 myoblasts on day 0, day 7, and day 14 of differentiation. All RNA values were determined using ImageQuant software (Molecular Dynamics) which calculated the signal intensity for each sample and subtracted the local background signal present. The phosphorimager signal (PI signal) of each sample was then expressed relative to the corresponding PGK transcript level (set to a baseline of 1.0). (C) Creatine Phosphate Kinase (CPK) activity on day 0, day 7, and day 14 of differentiation (day 0d, day 7d, and day14d). Values are expressed as U/l per μg of protein and are the average of triplicate samples ($\pm$ the standard deviation). Asterisks denote a significant difference (p < 0.05) compared to the control.

3.2 The Effect of Insulin-like Growth Factor-II on Encapsulated Myoblasts

In order to study the effect of insulin-like growth factor-II (IGF-II) on the growth and differentiation of encapsulated myoblasts, pNMG3-pooled myoblasts (a C2C12 mouse myoblast cell line transfected with the gene for human growth hormone by Kelly Bowie, 1997) were encapsulated with increasing concentrations of IGF-II within the microcapsules. An additional C2C12 mouse myoblast cell line that overexpresses IGF-II (denoted C2IGF-II) was also encapsulated in order to assess the effect of IGF-II on encapsulated myoblasts. This cell line has been shown to increased rates of both growth and myogenic differentiation when grown in culture. Myoblasts were encapsulated under the following conditions: (i) pNMG3 control (no IGF-II), (ii) pNMG3 with 10 ng IGF-II per ml alginate, (iii) pNMG3 with 100 ng IGF-II per ml alginate, (iv) pNMG3 with 1000 ng IGF-II per ml alginate, and (vi) C2IGF-II myoblasts. All myoblasts were encapsulated with collagen and cultured in growth media for 14 days. The microcapsules were then transferred to differentiation media and were sampled for growth and differentiation on day 0 (day microcapsules were switched into differentiation media), day 7 and day 14 of differentiation. A separate (second) batch of microcapsules were used to determine the growth data on day 0, day 7, and day 14 of growth due to an incubator failure that took place in the first week of the initial experiment from which the differentiation data was obtained.

3.2.1 Effect of IGF-II on Myoblast Growth and Survival

Figure 7 examines the effect of IGF-II on the growth of encapsulated myoblasts. Over the two-week period of growth, all encapsulated myoblasts exhibited similar rates of growth, regardless of the presence or concentration of IGF-II (Figure 7 A,B,C). The range of percentage growth increases of viable cell number from day 0 to day 14 was 66.8% ± 1.8 in the control to 75.2% ± 5.0 in myoblasts encapsulated with 100 ng/ml of IGF-II (Figure 7 C). No differences between any of the encapsulation conditions produced any significant difference in myoblast growth.

The percentage growth decrease of viable cell number of myoblasts was examined during the differentiation period (Figure 8 A,B,C). The control encapsulated myoblasts exhibited growth decreases of 52.3% ± 2.6 and 65.6% ± 4.4 from day 0 to day 7 and day 7 to day 14 of differentiation respectively. In the first week of differentiation, the C2IGF-II myoblasts fared significantly better (p < 0.05) than the control myoblasts, with cell numbers dropping 44.5% ± 5.0. However, from day 7 to day 14 of differentiation, myoblasts encapsulated with 100 ng/ml, 1000 ng/ml and the C2IGF-II myoblasts showed significant increases in survival compared to the control with percentage decreases of 33.3% ± 0.9, 35%.0 ± 0.2, and 25%.0 ± 1.6 respectively (Figure 8 B). When percentage growth decrease was examined over the two-week period of differentiation, all encapsulated myoblasts showed significantly greater (p < 0.05) cell survival (10

ng/ml 75.5 ± 0.1, 100 ng/ml 77.2% ± 0.1, 1000 ng/ml 68.7 ± 0.4, and C2IGF-II

58.4 ± 2.9) than the control value of 83.7% ± 1.2 (Figure 8 C).

Figure 7

The effect of insulin-like growth factor-II (IGF-II) on the growth of encapsulated myoblasts. (A) The number of viable cells per capsule sampled on day 0, day 7 and day 14 of growth (day 0g, day 7g and day 14g) as determined by the Alamar Blue assay. (B) Percentage growth increase (of number of viable cells) of myoblasts between day 0 and day 7 as well as day 7 and day 14 of growth. (C) Percentage growth increase of myoblasts between day 0 and day 14 of growth. Day 0 of growth corresponds to the day of encapsulation. Values given are the average of triplicate samples (± the standard deviation). Concentrations of IGF-II are given in ng of IGF-II per ml of alginate. C2IGF-II refers to a C2C12 mouse myoblast cell line that over-expresses IGF-II. Asterisks denote a significant difference ($p < 0.05$) compared to the control.

A

B

C

Figure 8

Effect of IGF-II on the survival of encapsulated myoblasts during differentiation. (A) The number of viable cells per capsule sampled on day 0, day 7 and day 14 of differentiation (day 0d, day 7d and day 14d) as determined by the Alamar Blue assay. (B) Percentage growth decrease of myoblasts between day 0 and day 7 as well as day 7 and day 14 of differentiation. (C) Percentage growth decrease (of number of viable cells) between day 0 and day 14 of differentiation (day0d and day 14d). Day 0 of differentiation corresponds to the day encapsulated myoblasts were placed in differentiation media. Values given are the average of triplicate samples (± the standard deviation). Concentrations of IGF-II are given in ng of IGF-II per ml of alginate. C2IGF-II refers to a C2C12 mouse myoblast cell line that over-expresses IGF-II. Asterisks denote a significant difference (p < 0.05) compared to the control.
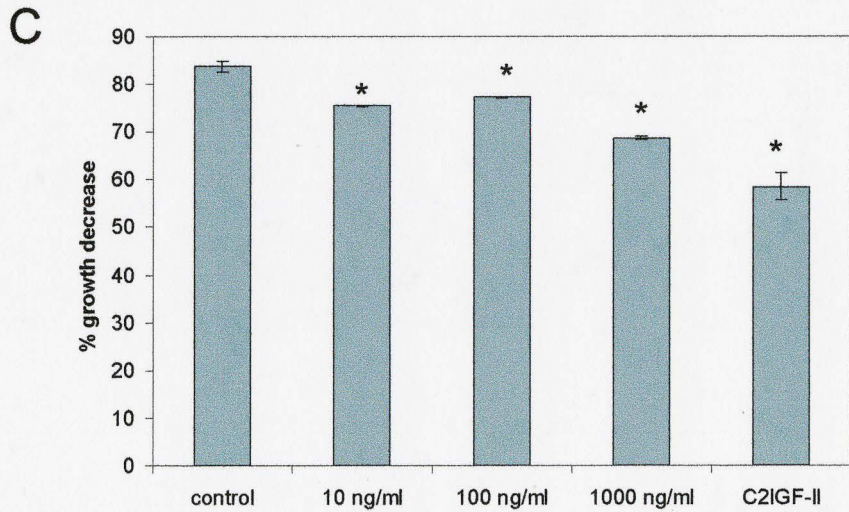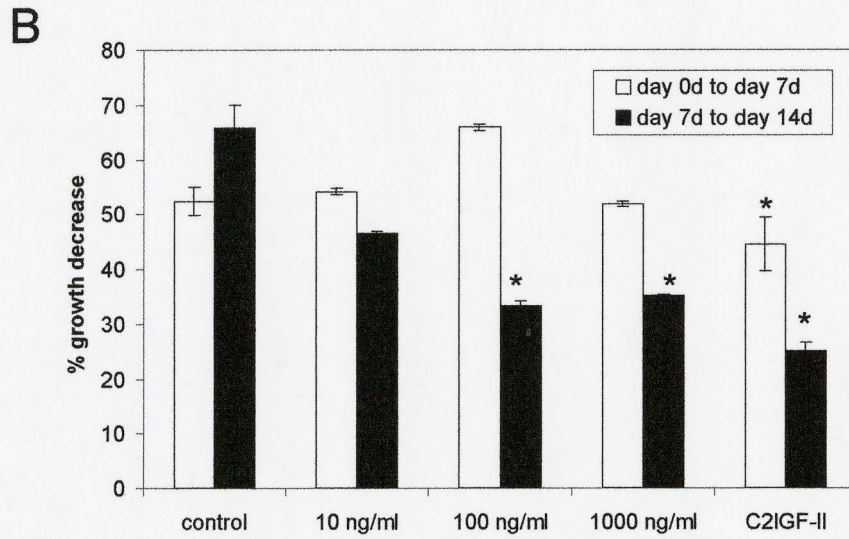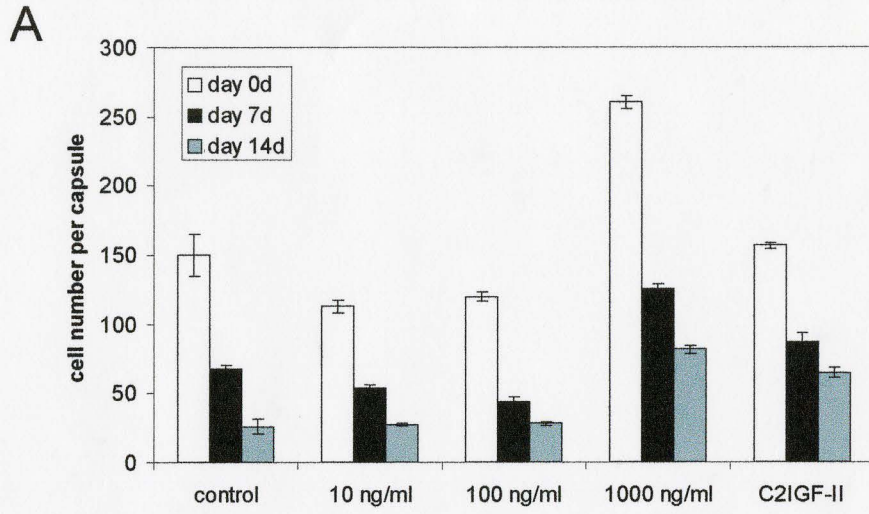
3.2.2 Qualitative Analysis of Myoblast Differentiation

As a qualitative measure of differentiation, encapsulated cells were stained for the presence of myosin heavy chain (MHC). While myoblasts grown on tissue culture plates showed a noted increase in MHC expression between day 0 and day 14 of differentiation, MHC expression in encapsulated myoblasts was greatly reduced (Figure 9). On day 7 of differentiation 100 ng/ml, 1000 ng/ml and C2IGF-II myoblasts had significantly higher percentages of MHC positive cells, with values of 5.2% ± 0.2, 8.1% ± 0.5, and 5.9% ± 0.5 respectively. These values were compared to the control value of 3.8% ± 0.2. By day 14 of differentiation, myoblasts encapsulated with 1000 ng/ml of IGF-II exhibited 13.0% ± 1.0 of cells that were MHC positive and were the only myoblasts that were significantly different ($p < 0.05$) than the control of 8.5% ± 0.4 (Figure 10).

In addition to MHC expression, the myogenic index (percentage of nuclei present in multinucleate cells) of encapsulated myoblasts was determined. Positive control myoblasts grown on tissue culture plates had begun to fuse by day 14 of differentiation (Figure 9 B), while no fusion of encapsulated myoblasts was seen during the length of the differentiation period (Figure 9 D,E; Table 2).

Figure 9

Analysis of Myosin Heavy Chain expression: the effect of IGF-II on the differentiation of encapsulated myoblasts (A) Naked myoblasts (grown on tissue culture plates) on day 0 of differentiation. (B) Naked myoblasts on day 14 of differentiation (control for positive staining). (C) Control encapsulated pNMG3 myoblasts on day 0 of differentiation. (D) Control encapsulated pNMG3 myoblasts on day 14 of differentiation. (E) pNMG3 myoblasts encapsulated with 1000 ng IGF-II per ml of alginate on day 0 of differentiation. (F) pNMG3 myoblasts encapsulated with 1000 ng of IGF-II per ml of alginate on day 14 of differentiation. Arrowheads indicate cells that have stained positive for MHC. Blue arrows indicate multinucleate cells and red arrows indicate examples of cells that are negative for MHC. Magnification is 400X.
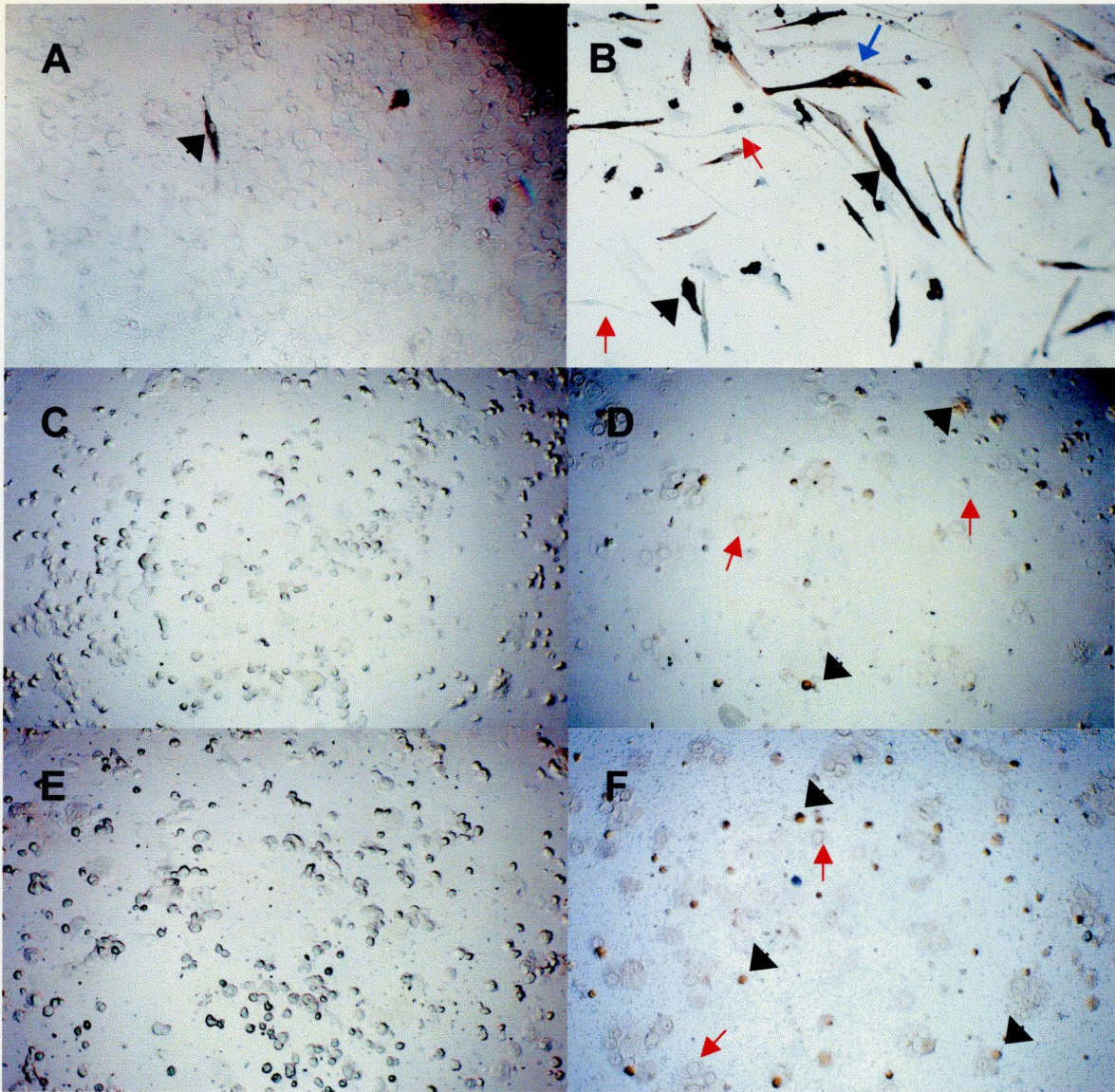
Figure 10

Qualitative analysis of myoblast differentiation: Effect of IGF-II on MHC expression of encapsulated myoblasts. MHC expression of encapsulated myoblasts was determined on day 0, day 7 and day 14 of differentiation (day 0d, day 7d, and day 14d). Values given are the percentage of cells that stained positive for MHC and are the average of triplicate samples (± the standard deviation). Sample values were determined by counting the number of cells positive for MHC in 5 fields at 400X magnification. Positive control values (unencapsulated cells) were 0% MHC-positive cells for day 0, and 29.1% ± 2.7 and 51.9% ± 4.2 for day 7 and day 14 of differentiation respectively. Values in brackets are the average number of cells counted for each sample. Asterisks denote a significant difference (p < 0.05) compared to the control.

% MHC positive cells

- □ day 0d
- ■ day 7d
- ▨ day 14d

control
(151 ± 35)
(239 ± 48)
(290 ± 30)

10 ng/ml
(117 ± 17)
(201 ± 28)
(278 ± 64)

100 ng/ml
(133 ± 51)
(213 ± 34) *
(176 ± 57)

1000 ng/ml
(127 ± 43)
(261 ± 71) *
(309 ± 65) *

C2IGF-II
(106 ± 22)
(229 ± 38) *
(334 ± 81)
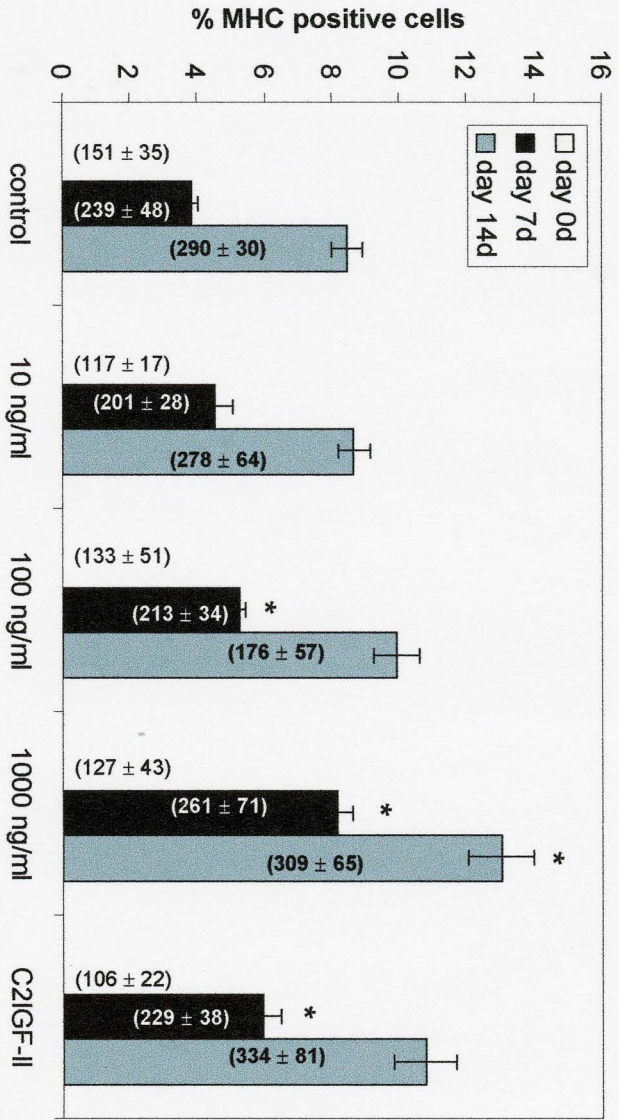
Table 2.

Qualitative analysis of myoblast differentiation: the effect of IGF-II on the myogenic index of encapsulated myoblasts. The myogenic index is expressed as the percentage of nuclei that were present in multinucleate cells. Values are a result of counting 5 fields of cells at 400X magnification and are the average of triplicate samples ($\pm$ the standard deviation). Naked (unencapsulated) pNMG3 myoblasts were used as a positive control. Values in brackets are the average number of cells counted per sample.

Capsule type	Myogenic Index		
	Day 0	Day 7	Day 14
Control	0 (152 $\pm$ 31)	0 (211 $\pm$ 34)	0 (128 $\pm$ 16)
10 ng/ml	0 (117 $\pm$ 26)	0 (234 $\pm$ 61)	0 (175 $\pm$ 34)
100 ng/ml	0 (133 $\pm$ 42)	0 (178 $\pm$ 23)	0 (161 $\pm$ 31)
1000 ng/ml	0 (127 $\pm$ 19)	0 (190 $\pm$33)	0 (169 $\pm$ 18)
C2IGF-II	0 (106 $\pm$ 21)	0 (186 $\pm$28)	0 (134 $\pm$ 25)
naked cells	0 (86 $\pm$10)	34.3 $\pm$ 2.4 (151 $\pm$ 27)	45.1 $\pm$ 3.4 (126)

3.2.3 Quantitative Analysis of Myoblast Differentiation

Levels of muscle-specific transcription factor mRNA were analyzed using dot blotting to quantitatively assess myoblasts differentiation. Dot Blots were analyzed for the intensity of the signal using a phosphorimager (Figure 11) and muscle-specific transcripts were expressed by comparing the signal to the PGK sample where the level of PGK was set to a baseline of 1.0. While myogenin mRNA levels were variable by day 14 of differentiation, all encapsulated myoblasts had similar levels on day 0. Myogenin levels were greater for C2IGF-II myoblasts on day 7 (0.104 volume/signal intensity) and on day 14 (0.087 volume/signal intensity) compared to the control values of 0.030 volume/signal intensity on day 7 and 0.019 volume/signal intensity on day 14 (Figure 12 A). Further, troponin I levels were higher on day 7 of differentiation in C2IGF-II myoblasts (5.034 volume/signal intensity) compared to the control value of 3.021 volume/signal intensity (Figure 12 B). While myogenin mRNA levels for myoblasts encapsulated with 1000 ng/ml IGF-II also appeared to be higher than control levels on day 0 and day 14 of differentiation (Figure 12 A), it is difficult to draw conclusions based on one sample. Only one sample was able to be analyzed due to the large volumes of microcapsules needed to isolate sufficient amounts of RNA.

Analysis of CPK activity demonstrated that, on day 7 of differentiation control myoblasts had a CPK activity of 44.8 ± 3.2 U/l per μg of viable protein.

Compared to this, 100 ng/ml, 1000 ng/ml and C2IGF-II myoblasts had significantly increased CPK activity ($p < 0.05$) at 56.9 ± 1.0, 69.1 ± 7.9, and 83.1 ± 5.2 U/l per μg of viable protein respectively. This pattern persisted on day 14 of differentiation, where CPK activity of 100 ng/ml, 1000 ng/ml and C2IGF-II myoblasts significantly ($p < 0.05$) exceeded the control (54.3 ± 2.7 U/l per μg of viable protein) with values of 96.0 ± 11.2, 145.4 ± 3.9, and 126.4 ± 5.4 U/l per μg of viable protein respectively (Figure 12 C).

Figure 11

The effect of IGF-II on the differentiation of encapsulated myoblasts: Dot Blots of PGK, myogenin, and troponin I RNA expression on day 0, day 7, and day 14 of differentiation. Images represent a computer-generated image using ImageQuant software (Molecular Dynamics). RNA isolated from naked pNMG3 myoblasts (grown on culture plates) 5 days after the induction of differentiation was used as a positive control.
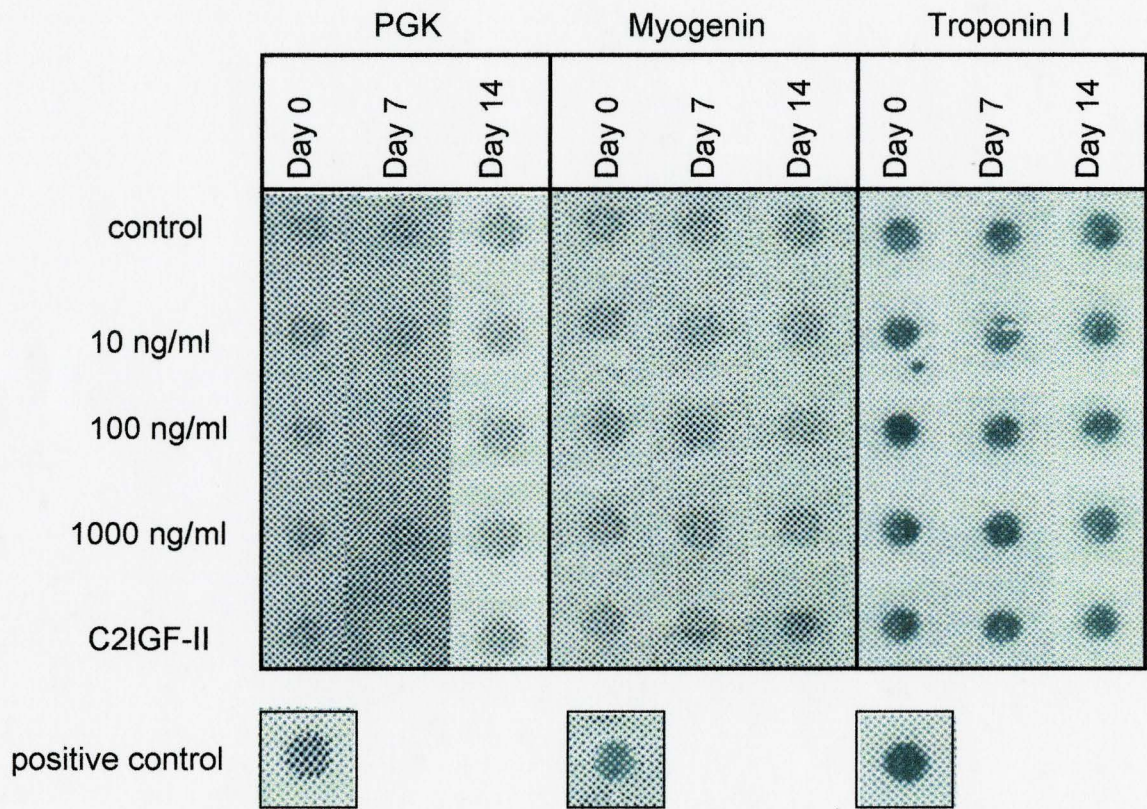
Figure 12

Quantitative analysis of myoblast differentiation: the effect of IGF-II on the differentiation of encapsulated myoblasts. (A) Myogenin RNA levels (as determined by dot blot) of encapsulated pNMG3 myoblasts on day 0, day 7, and day 14 of differentiation. (B) Troponin I RNA levels (as determined by dot blot) of encapsulated pNMG3 myoblasts on day 0, day 7, and day 14 of differentiation. All RNA values were determined using ImageQuant software (Molecular Dynamics) which calculated the signal intensity for each sample and subtracted the local background signal present. The phosphorimager signal (PI signal) for each sample was then expressed relative to the corresponding PGK transcript level (set to a baseline of 1.0). (C) Creatine Phosphate Kinase (CPK) activity on day 0, day 7, and day 14 of differentiation (day 0d, day 7d, and day14d). Values are expressed as U/l per μg of protein and are the average of triplicate samples (± the standard deviation). Asterisks denote a significant difference ($p < 0.05$) compared to the control.
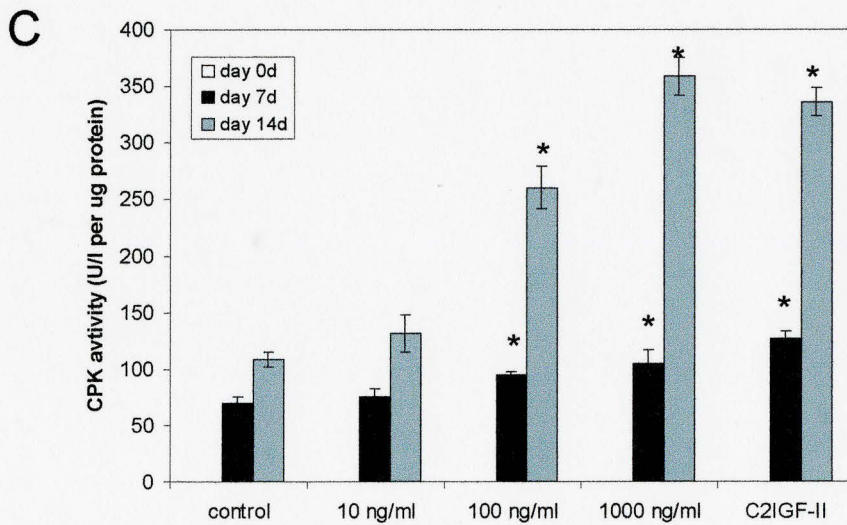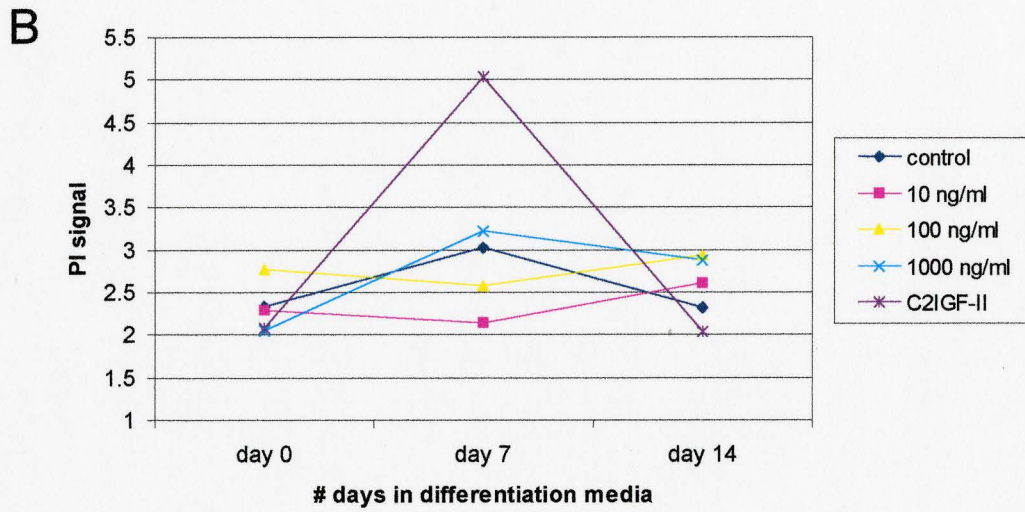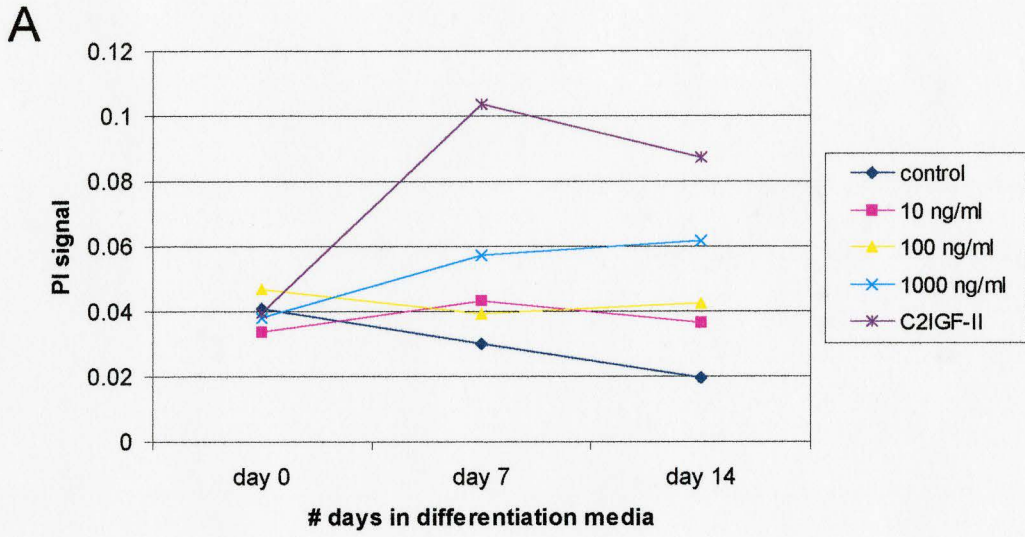
3.3 Inclusion of Extracellular Matrix Materials within Microcapsules

In order to study the effect of extracellular matrix materials (ECM) on the growth and differentiation of encapsulated myoblasts, pNMG3-pooled myoblasts (a C2C12 mouse myoblast cell line transfected with human growth hormone by Kelly Bowie, 1997) were encapsulated with rat collagen I (I (Collaborative Biomedical Products), mouse laminin-I (Gibco BRL), and human merosin (Gibco BRL), also know as laminin-2. For encapsulation, extracellular matrix materials were used at a total concentration of 10 µg per ml of alginate.

pNMG3 myoblasts were encapsulated under the following conditions: (i) pNMG3 control (no matrix material), (ii) pNMG3 with collagen, (iii) pNMG3 with laminin, (iv) pNMG3 with merosin, and (v) pNMG3 with laminin and merosin. Encapsulated myoblasts were cultured in growth media for 14 days and were sampled for proliferation on day 0 (day of encapsulation), day 7 and day 14. The microcapsules were then transferred to differentiation media and were sampled for growth and differentiation on day 0 (day microcapsules were switched into differentiation media), day 7 and day 14 of differentiation.

3.3.1 Effect of ECM on the Growth and Survival of Encapsulated Myoblasts

Figure 13 examines the effect of matrix materials on myoblast growth. Between day 0 and day 7 of growth, the presence of laminin and merosin together led to a 68.1% ± 7.6 increase in viable cell number, which was significantly greater ($p < 0.05$) than the 35.4% ± 15.4 increase experienced by the control (Figure 13 B). Additionally, in the second week of growth, the laminin/merosin combination produced significantly higher ($p < 0.05$) cell numbers with a 158.4% ± 14.4 growth increase compared to 57.5% ± 18.1 in the control myoblasts. When merosin was present alone, it also led to significant increases ($p < 0.05$) between day 7 and day 14 of growth with a percentage increase of 123.9% ± 4.8 (Figure 13 B). When percentage growth increase was examined between day 0 and day 14, both merosin and the laminin/merosin combination had significantly higher ($p < 0.05$) cell numbers, when compared to the control value of 112.1% ± 19.2, with values of 186.7% ± 12.0 and 333.8% ± 18.5 respectively (Figure 13 C).

After the onset of differentiation, all encapsulated myoblasts experienced a sharp drop in viable cell number between day 0 and day 7 of differentiation (Figure 14 A,B). While the control myoblasts showed a 74.7% ± 2.8 drop in cell number, myoblasts encapsulated with the laminin/merosin combination had a significantly greater decrease ($p < 0.05$) in percentage growth, with a value of 90.9% ± 0.7 (Figure 14 B). However, during the second week of differentiation,

merosin alone had a cell decrease of only 27% ± 2.8, significantly better than the 57.2% ± 6.8 decrease seen in control encapsulated myoblasts (Figure 14 B). When examined over the two-week period of differentiation, the control myoblasts experienced an 89.3% ± 1.3 decrease in cell numbers. Comparatively, the myoblasts encapsulated with merosin survived significantly better with 84.4% ± 0.7 decrease in cell number while the laminin/merosin combination had significantly lower survival with 93.8% ± 0.8 decrease in cell numbers (Figure 14 C).

Figure 13

The effect of extracellular matrix materials (ECM) on the growth of encapsulated pNMG3 myoblasts. (A) The number of viable cells per capsule sampled on day 0, day 7 and day 14 of growth (day 0g, day 7g and day 14g) as determined by the Alamar Blue assay. (B) Percentage growth increase (of number of viable cells) of myoblasts between day 0 and day 7 as well as day 7 and day 14 of growth. (C) Percentage growth increase of myoblasts between day 0 and day 14 of growth. Day 0 of growth corresponds to the day of encapsulation. Values given are the average of triplicate samples ($\pm$ the standard deviation). Concentrations of matrix materials used were 10 μg per ml of alginate. When laminin and merosin were used together, each was used at a concentration of 5 ug/ml to make a total ECM concentration of 10 ug/ml. Asterisks denote a significant difference ($p < 0.05$) compared to the control.

Figure 14

Effect of ECM materials on the survival of encapsulated pNMG3 myoblasts during differentiation. (A) The number of viable cells per capsule sampled on day 0, day 7 and day 14 of differentiation (day 0d, day 7d and day 14d) as determined by the Alamar Blue assay. (B) Percentage growth decrease (of number of viable cells) of myoblasts between day 0 and day 7 as well as day 7 and day 14 of differentiation. (C) Percentage growth decrease between day 0 and day 14 of differentiation (day0d and day 14d). Day 0 of differentiation corresponds to the day encapsulated myoblasts were placed in differentiation media. Values given are the average of triplicate samples (± the standard deviation). Concentrations of matrix materials used were 10 µg per ml of alginate. When laminin and merosin were used together, each was used at a concentration of 5 ug/ml to make a total ECM concentration of 10 ug/ml. Asterisks denote a significant difference (p < 0.05) compared to the control.
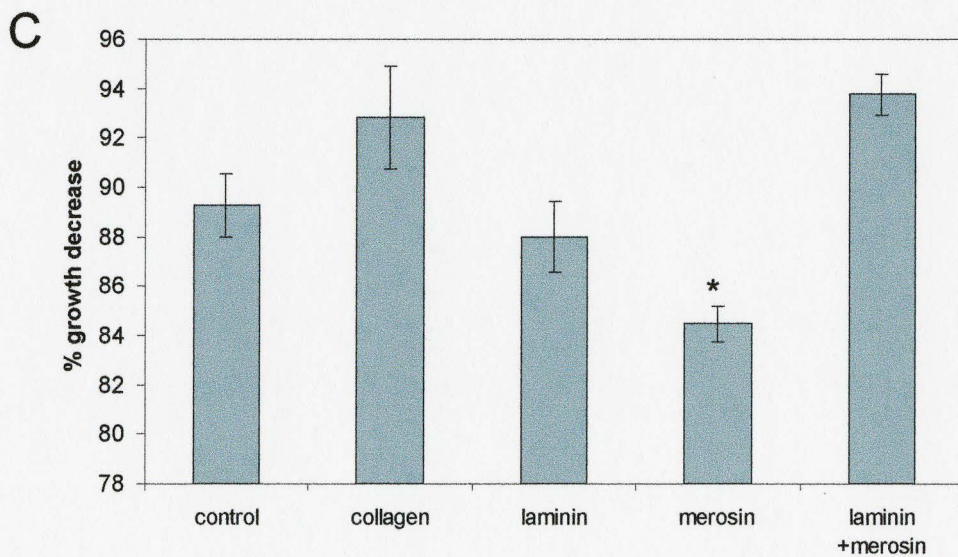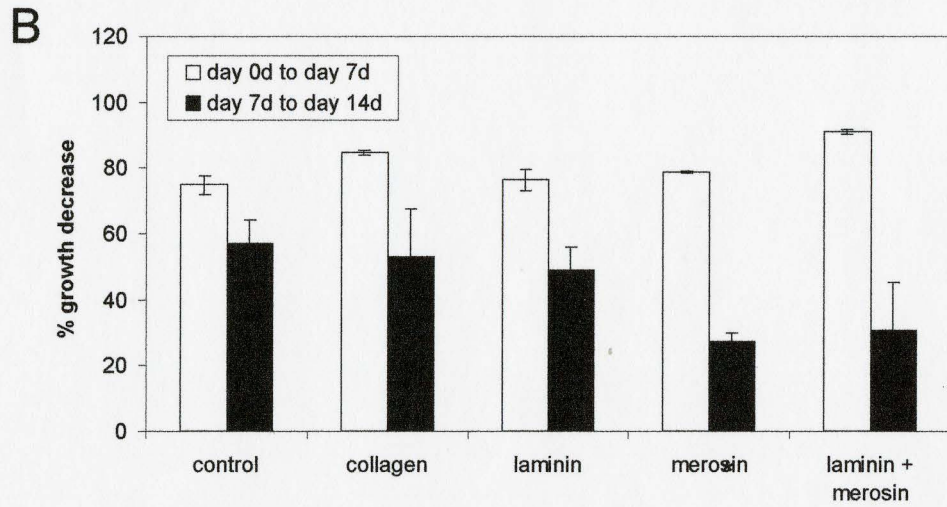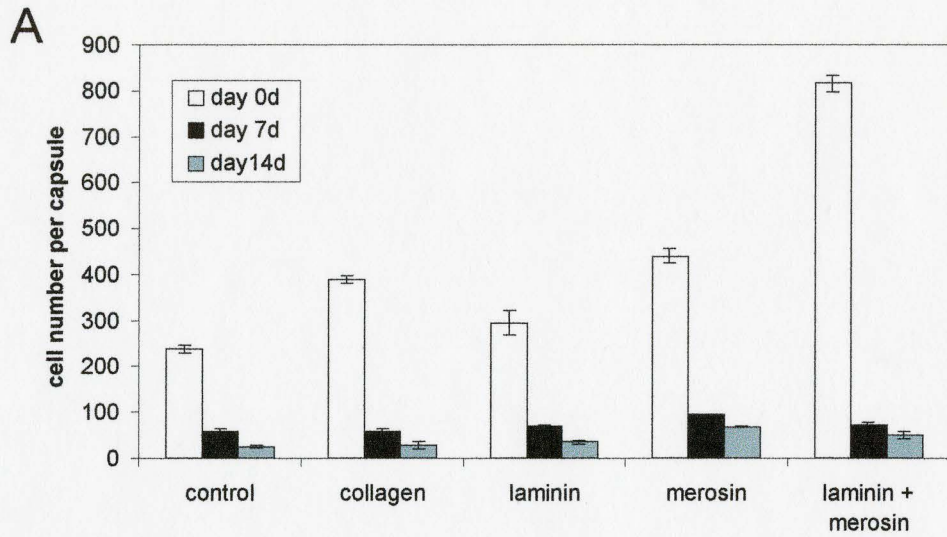
3.3.2 Qualitative Analysis of Myoblast Differentiation

Qualitative myoblast differentiation was analyzed by staining encapsulated myoblasts for the presence of myosin heavy chain (MHC). While myoblasts grown on tissue culture plates showed a noted increase in MHC expression between day 0 and day 14 of differentiation, MHC protein expression in encapsulated myoblasts was greatly reduced (Figure 15). Additionally, there was no significant difference in MHC expression in any of the encapsulated myoblast, regardless of the matrix material that was present within the microcapsules (Figure 16).

In addition to MHC expression, the myogenic index (percentage of nuclei present in multinucleate cells) of encapsulated myoblasts was determined. Positive control myoblasts grown on tissue culture plates had begun to fuse by day 14 of differentiation (Figure 15 B), while no fusion of encapsulated myoblasts was seen during the length of the differentiation period (Figure 15 D,E; Table 3).

Figure 15

Analysis of Myosin Heavy Chain expression of pNMG3 myoblasts encapsulated with ECM materials. (A) Naked myoblasts (grown on tissue culture plates) on day 0 of differentiation. (B) Naked myoblasts on day 14 of differentiation (positive control). (C) Control encapsulated pNMG3 myoblasts on day 0 of differentiation. (D) Control encapsulated pNMG3 myoblasts on day 14 of differentiation. (E) pNMG3 myoblasts encapsulated with 10μg of merosin per ml of alginate on day 0 of differentiation. (F) pNMG3 myoblasts encapsulated with 10 μg merosin per ml of alginate on day 14 of differentiation. Arrowheads indicate cells that have stained positive for MHC. Blue arrows indicate multinucleate cells and red arrows indicate examples of cells that are negative for MHC. Magnification is 400X.

Figure 16

Qualitative analysis of myoblast differentiation: Effect of ECM materials on MHC

expression of encapsulated pNMG3 myoblasts. MHC expression of encapsulated

myoblasts was determined on day 0, day 7 and day 14 of differentiation (day 0d,

day 7d, and day 14d). Values given are the percentage of cells that stained

positive for MHC and are the average of triplicate samples (± the standard

deviation). Positive control values (unencapsulated cells) were 0% MHC-positive

cells for day 0, and 31.4% ± 1.0 and 56.2% ± 2.3 for day 7 and day 14 of

differentiation respectively. Sample values were determined by counting the

number of cells positive for MHC in 5 fields at 400X magnification. Values in

brackets are the average number of cells counted for each sample.

% cells positive for MHC

day 0d
day 7d
day 14d

control
(101 ± 16)
(152 ± 39)
(225 ± 21)

collagen
(119 ± 20)
(125 ± 22)
(245 ± 64)

laminin
(127 ± 36)
(151 ± 12)
(326 ± 29)

merosin
(98 ± 27)
(148 ± 37)
(293 ± 34)

laminin + merosin
(108 ± 23)
(124 ± 35)
(247 ± 33)
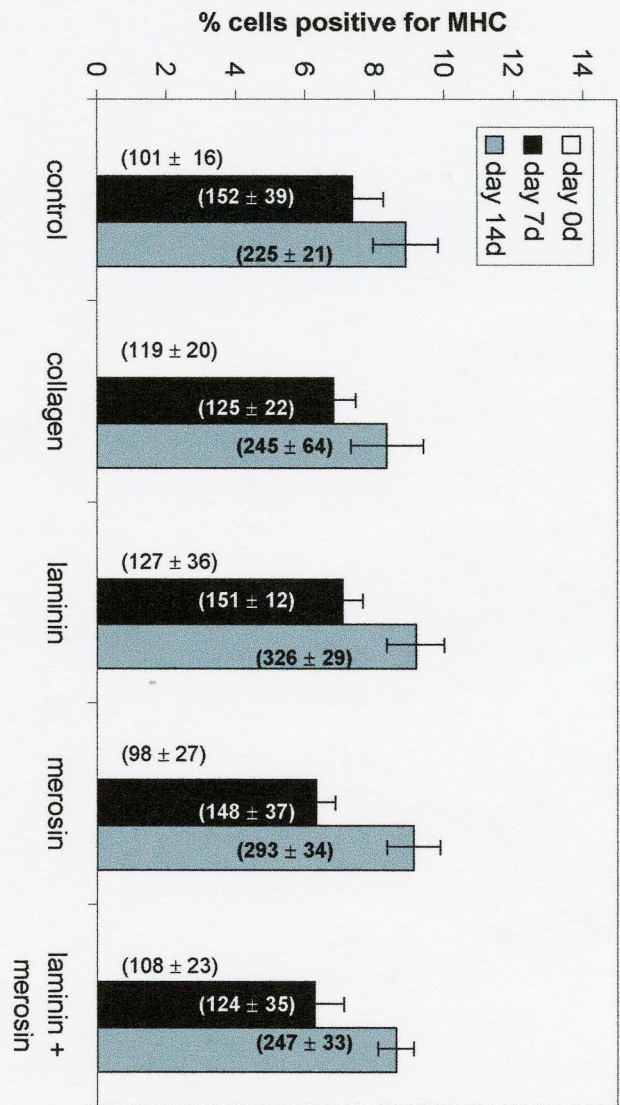
Table 3.

Qualitative analysis of myoblast differentiation: the effect of ECM materials on the myogenic index of pNMG3 myoblasts. The myogenic index is expressed as the percentage of nuclei that were present in multinucleate cells. Values are a result of counting 5 fields of cells at 400X magnification and are the average of triplicate samples (± the standard deviation). Naked (unencapsulated) pNMG3 myoblasts were used as a positive control. Values in brackets are the average number of cells counted per sample.

Capsule type	Myogenic Index		
	Day 0	Day 7	Day 14
Control	0 (101 ± 16)	0 (157± 45)	0 (131 ± 21)
Collagen	0 (119 ± 20)	0 (129 ± 41)	0 (164 ± 39)
Laminin	0 (127 ± 31)	0 (109 ± 10)	0 (288 ± 13)
Merosin	0 (98 ± 13)	0 (131 ± 14)	0 (230 ± 22)
Laminin + merosin	0 (108 ± 21)	0 (128 ± 36)	0 (192 ± 14)
naked cells	0 (98 ± 19)	24.8 ± 4.1 (141 ± 62)	41.3 ± 2.4 (217 ± 70)

3.3.3 Quantitative Analysis of Myoblast Differentiation

Levels of muscle-specific transcription factor mRNA were analyzed using dot blotting to quantitatively assess myoblasts differentiation. Dot Blots were analyzed for the intensity of the signal using a phosphorimager (Figure 17) and muscle-specific transcripts were expressed by comparing the signal to the PGK sample where the level of PGK was set to a baseline of 1.0. Myogenin mRNA levels (expressed as volume/signal intensity) for laminin, merosin, and the laminin/merosin combination were 0.045, 0.044, and 0.041 respectively on day 0 and were 0.043, 0.038, and 0.039, respectively on day 7 of differentiation (Figure 18 A). These values are slightly higher when compared to the control levels of 0.026 and 0.029 for day 0 and day 7 of differentiation respectively. However, these levels were variable, and by day 14 all myogenin mRNA levels appeared to be similar (Figure 18 A). Troponin 1 mRNA levels seemed to peak higher on day 7 of differentiation for both laminin (3.22 volume/signal intensity) and the laminin/merosin combination (2.70 volume/signal intensity) than the control (2.02 volume/signal intensity), however, all troponin I levels returned to within the same range by day 14 (Figure 18 B).

CPK activity was analyzed as a second quantitative measure of myoblast differentiation. When compared to the control, there were no significant differences in CPK activity for myoblasts encapsulated with matrix materials (Figure 18 C).

Figure 17

The effect of ECM materials on the differentiation of encapsulated⁻ pNMG3 myoblasts: Dot Blots of PGK, myogenin, and troponin I RNA expression on day 0, day 7, and day 14 of differentiation. Images represent a computer-generated image using ImageQuant software (Molecular Dynamics). RNA isolated from naked pNMG3 myoblasts (grown on culture plates) 5 days after the induction of differentiation was used as a positive control.
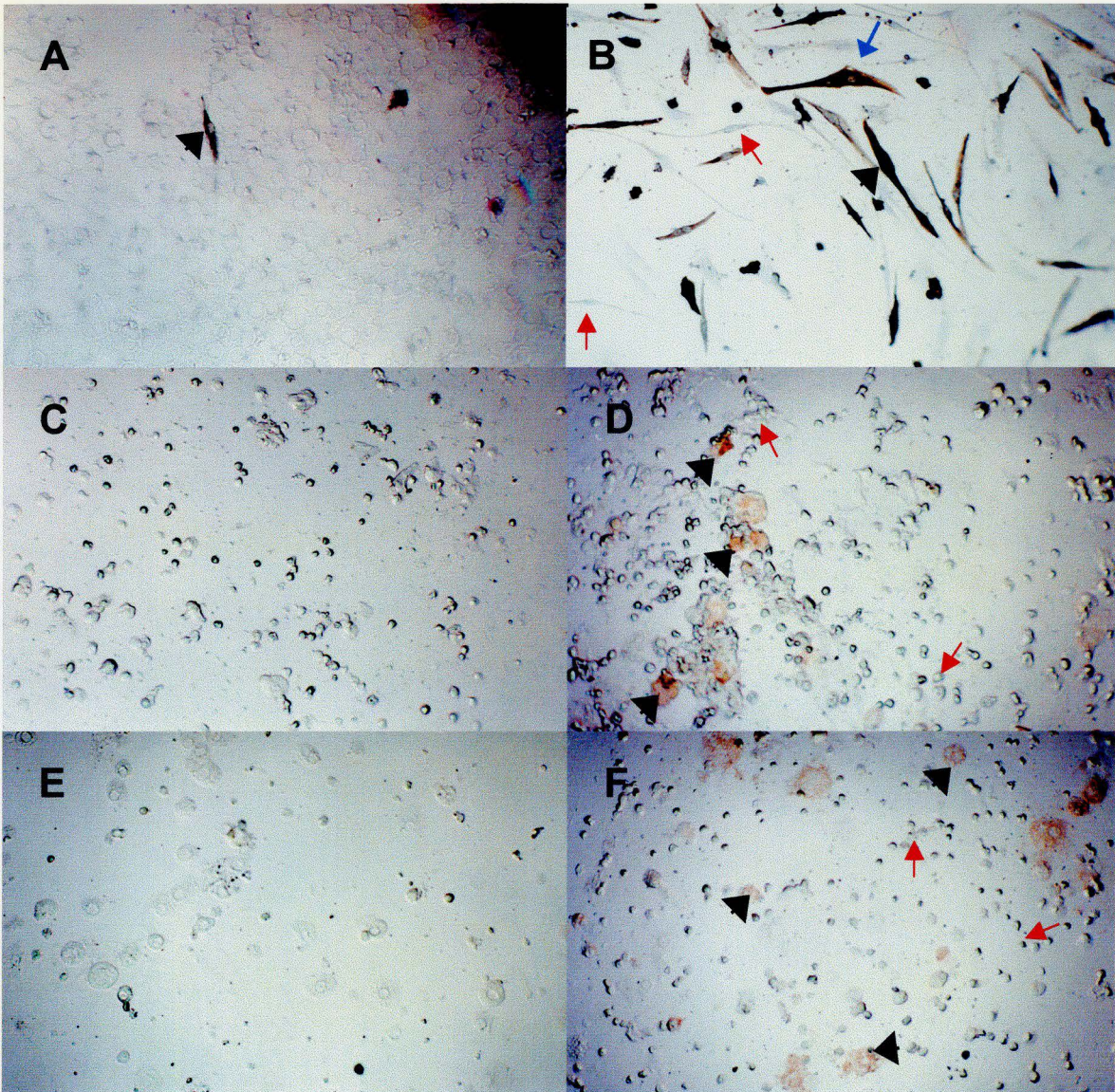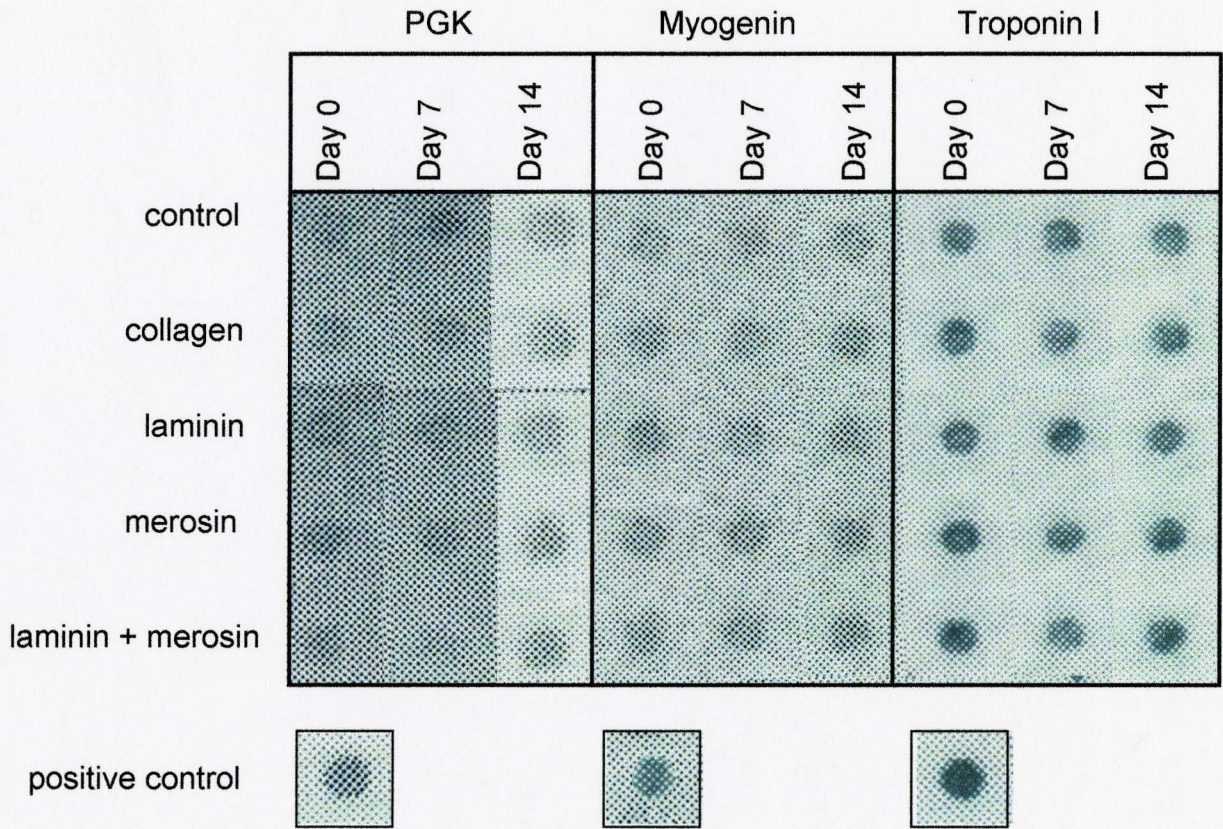
Figure 18

Quantitative analysis of myoblast differentiation: the effect of ECM materials on the differentiation of encapsulated pNMG3 myoblasts. (A) Myogenin RNA levels (as determined by dot blot) of encapsulated pNMG3 myoblasts on day 0, day 7, and day 14 of differentiation. (B) Troponin I RNA levels (as determined by dot blot) of encapsulated pNMG3 myoblasts on day 0, day 7, and day 14 of differentiation. All RNA values were determined using ImageQuant software (Molecular Dynamics) which calculated the signal intensity for each sample and subtracted the local background signal present. The phosphorimager signal (PI signal) of each sample was then expressed relative to the corresponding PGK transcript level (set to a baseline of 1.0). (C) Creatine Phosphate Kinase (CPK) activity on day 0, day 7, and day 14 of differentiation (day 0d, day 7d, and day14d). Values are expressed as U/l per μg of protein and are the average of triplicate samples (± the standard deviation).

4.0 DISCUSSION

4.1 Growth and Survival of Encapsulated Myoblasts

In an attempt to increase the growth and survival of encapsulated myoblasts, bFGF, IGF-II and the ECM materials, laminin-1 and merosin were enclosed within APA microcapsules. In each experiment, the viable cell number per capsule was monitored over a 4 week period (2 weeks growth, 2 weeks differentiation), in order to assess myoblast growth.

A marked increase in myoblast growth during the two weeks following encapsulation was observed when 1000 ng/ml of bFGF was included in the microcapsules, producing a significant percentage growth increase in viable cell number when compared to the control. However, while using a high concentration of bFGF seems to be beneficial to encapsulated myoblasts, cost and amount of the growth factor needed must be considered in terms of its practical use if it is to be adopted as part of a system for human gene therapeutics. Additionally, although higher concentrations of bFGF initially appeared to increase myoblast survival during differentiation, the subsequent data showed that myoblasts exposed to these high concentrations of bFGF were actually undergoing differentiation to a lesser degree than controls.

Therefore, it seems that bFGF is preventing myoblast death by restricting them from committing to the differentiation process. This agrees with bFGFs role as a mitogen and inhibitor of myogenic differentiation (Basilico and Moscatelli, 1992). Additionally, bFGF has been shown to protect several cell types from apoptosis (Kondo et al., 1996; Wang et al., 1999). Since the goals of this thesis were to increase both the proliferation and differentiation of encapsulated myoblasts, bFGF does not appear to be an appropriate choice for inclusion in microcapsules regardless of the beneficial results it produces in terms of growth.

The combination of laminin and merosin also produced significant increases in cell growth when used within microcapsules. This combination of matrix materials lead to a two-fold increase in growth compared to the control, and while merosin alone also had significantly increased cell growth, the combination produced the most striking results. This may be explained by evidence demonstrating that myoblasts express several members of the integrin family of cell surface receptors and each laminin probably binds to a different subset of these receptors that results in pronounced effects on myoblast behaviour (Crawley et al., 1997). This activation of different/more receptors may cause a synergistic effect in terms of growth compared to the effect seen when either matrix material is used alone, as was demonstrated

with the laminin/merosin combination. Synergism of this type has been observed with the syndecans, the major group of transmembrane heparan sulfate proteoglycans (Woods and Couchman, 1998). Additionally, synergism has been shown to exist between several of the integrin receptors (Vuori and Ruoslahti, 1994; Miyamoto et al., 1998; Arcangelis et al., 1999).

No effect on myoblast growth was seen when IGF-II was encapsulated with myoblasts, regardless of the concentration used. This is surprising considering the role of IGF-II as a stimulator of proliferation in myoblasts as well as differentiation. *In vitro* data have shown that a concentration of 20 ng/ml of IGF-II elicits a 50% increases in cell proliferation in cell based bioassays (product information, product # I2526, Sigma). It is not clear why encapsulated myoblasts do not respond to IGF-II in the same manner, however, it may be due to the unnatural capsule environment and suspension-type growth.

4.2 Differentiation of Encapsulated Myoblasts

Of the growth factors and matrix materials tested, IGF-II was the only substance that was able to increase the differentiation of myoblasts grown in microcapsules. CPK and MHC data showed that at concentrations of 100 and 1000 ng/ml of IGF-II, differentiation of encapsulated myoblasts was significantly

higher than the control. The responses of myoblasts to the higher concentrations of IGF-II (10 ng/ml being sub-optimal) agree with the effective published concentration of IGF-II (product information, Sigma). Levels of differentiation, however, were still greatly reduced when compared to what is seen in unencapsulated myoblasts (Bowie, 1997).

The overexpressing IGF-II cell line, C2IGF-II, also had higher CPK activity than controls and appeared to have higher levels of both myogenin and troponin I mRNA. This is supported by *in vitro* data where this cell line had increased CPK and muscle-specific mRNA compared to its parental (untransfected) cell line (Stewart et al., 1996). It should be noted, however, that compared to data from a previous study when C2IGF-II myoblasts were grown "naked" in culture (Stewart et al., 1996), the level of differentiation seen was still substantially reduced. Overexpression of IGF-II by an encapsulated myoblast cell line, however, may be a more feasible approach than exogenously added IGF-II for inducing myogenic differentiation when the cost of "scaling up" to human gene therapy with microcapsules is considered.

While the IGF-II had a pronounced effect on encapsulated myoblast differentiation, it was surprising that no effect on differentiation was seen when either laminin or merosin were used as a matrix material within the microcapsules. These data disagree with published results showing that both

laminin and merosin promote differentiation of myoblasts into myotubes (Ehrig et al., 1990; Vachon et al., 1996; Öcalan et al., 1998).

While laminin and merosin did not increase differentiation on the biochemical or morphological level, merosin did have an effect on myoblast survival during differentiation. During the second week after the induction of differentiation, encapsulated myoblasts had significantly higher rates of survival when merosin was present in the microcapsules compared to controls. Since it has been demonstrated that differentiating myoblasts have a specific requirement for merosin in terms of myotube survival and stability (Vachon et al., 1996), it is conceivable that encapsulated myoblasts benefit from the presence of merosin within the microcapsules as well.

Another interesting result obtained from matrix materials experiments was the significantly decreased survival of myoblasts that were encapsulated with both laminin and merosin when compared to the control. Matrix materials were used to a total concentration of 10 ug/ml of alginate and microcapsules containing both laminin and merosin had only half the concentration (5 ug/ml) of each material in order to keep the total concentration constant. The effective concentration of either material is 10 ug/ml for cell attachment (product information, Gibco). Considering the specific myoblast requirement for merosin and the results mentioned above, it is possible that the actual concentration of

this material (5 μg/ml) in the capsules containing both laminin and merosin was not sufficient to promote myoblast stability during differentiation. This however, only explains why the laminin/merosin combination had decreased cell survival compared to capsules containing only merosin. It is still unclear at this time why the laminin/merosin combination actually had decreased cell survival compared to the control.

While certain factors, such as IGF-II, were able to increase the myoblast differentiation within microcapsules, levels of differentiation were still not comparable to those seen in unencapsulated cells. Several studies using encapsulated differentiated myoblasts showed the presence of multinucleate cells in capsules retrieved from mice (Regulier et al., 1998; Dalle et al., 1999), indicating that myoblasts may be able to differentiate within polymer hollow-fiber capsules. These studies, however, lacked any biochemical analyses (i.e. CPK activity) of the retrieved myoblasts.

4.3 Binding of Growth Factors to the Extracellular Matrix

In order to determine if an extracellular matrix material was needed to hold growth factors within the microcapsules, collagen was included in capsules containing bFGF. Relative to the pore size of the microcapsules, bFGF is a

small protein of 18 kDa (Basilico and Moscatelli, 1992) that should easily be able to pass through the capsule membrane. Additionally, bFGF has a very short half-life determined to be about 9 hours *in vivo* (Sprugel, 1987). This suggests that the bFGF included within the microcapsules may escape through the pores or degrade very quickly.

Myoblast growth data indicated that there was a small, but significant difference in the behaviour of encapsulated myoblasts when both bFGF and collagen were present as compared to bFGF alone. The percentage growth increase was significantly higher for myoblasts encapsulated with collagen. Additionally, myoblasts encapsulated with 100 ng/ml of bFGF, but with no collagen did not differ from the control, indicating that collagen must be present in order for any beneficial effects of bFGF to be seen. Since the ECM can serve as a reservoir for bFGF (Folkman et al., 1988), it is possible that the collagen is in fact sequestering the growth factor within the microcapsules. It has been well characterized that bFGF is able to bind heparin-like molecules in the ECM and that it has a lower affinity for these molecules than it does for its own cell surface receptors (Moenner et al., 1986; Bashkin et al., 1990; Dionne et al., 1990). This allows the ECM to serve as a temporary storage site for bFGF, protecting it from proteolytic degradation (Sommer and Rifkin, 1989), and

allowing for controlled release of the growth factor (~2.8% released per day) over an extended period of time (Dinberg et al., 1996).

-

4.4 Optimization of the Microcapsule Environment:

Future Considerations

It is clear that while the growth and differentiation of APA encapsulated myoblasts can be improved using growth factors and extracellular matrix materials, the microcapsules still lack an environment that has the ability to cultivate myogenic differentiation *in vitro*. A previous study has reported the presence of differentiated myotubes in APA microcapsules that were retrieved from mice 6 months following implantation (Al-Hendy et al., 1995). This suggests that the *in vivo* environment may be able to provide differentiating-promoting elements that are lacking in the *in vitro* conditions. Evaluation of the use of both matrix materials and growth factors within the microcapsules *in vivo* must be attempted to test the actual benefits of using this approach to increase myoblast differentiation.

An additional parameter to be considered is the use of matrix materials and growth factors in different types of capsules. All in vitro work in this study

used hollow APA microcapsules, however, it has been shown that when implanted in vivo, solid capsules are more useful because they have greater mechanical strength and stability (Van Raamsdonk, 1999). Further improvements to the capsule structure have included using various molecular weight poly-lysine and poly-arginine (Van Raamsdonk, 1999), and substituting barium in place of calcium (Peirone et al., 1998) when cross-linking the alginate capsules. It would be interesting to determine whether the growth factors and matrix materials examined here will produce the same benefits in terms of myoblast growth and differentiation when using microcapsules that differ in their chemical and structural composition.

Another interesting consideration is how the inclusion of matrix materials within the microcapsules may affect recombinant transgene expression. A recent study examined the effect a foam matrix had on the secretion of a protein from recombinant encapsulated cells (Li et al., 1998). Cells that had been encapsulated with the foam matrix showed marked increases in protein secretion compared to the control. Therefore, the addition of matrix materials to encapsulated cells may offer a unique method for increasing secretion of recombinant proteins that are being delivered for gene therapy purposes.

It is suggested from the data presented in this thesis that the laminin/merosin combination provides the best conditions for growth of APA

encapsulated myoblasts, while the merosin and IGF-II encourage myoblast survival and differentiation respectively. It is evident that a variety of matrix materials and growth factors play distinct roles in myoblast proliferation and differentiation within microcapsules. In light of this, the next step in improving the microcapsule environment will be to determine combinations or "cocktails" of factors that will work together to mimic *in vivo* surroundings and will provide a stable environment for encapsulated myoblasts. Such cocktails have been used to increase the growth of neural stem cells encapsulated within a bioartificial organ (Schinstine et al., 1998). At this time it appears that the best combination for increasing encapsulated myoblast proliferation and differentiation would be to combine laminin and merosin (each at a concentration of 10 µg/ml of alginate) with 100-1000 ng/ml of IGF-II or a myoblast cell line overexpressing IGF-II. While this thesis examined the effect of several growth factors and extracellular matrix materials on the growth and differentiation of APA encapsulated myoblasts, it must be noted that these are but a small sample of factors that influence the behaviour of myoblasts.

4.5 Conclusions

While bFGF lead to significant increases in encapsulated myoblast proliferation, it does not appear to be highly beneficial for optimizing the microcapsule environment due to its inhibitory effect on differentiation and the relative cost of using it for therapeutic delivery of proteins using recombinant APA encapsulated myoblasts. Both merosin and the combination of laminin and merosin together seem to provide a better alternative for increasing myoblast growth and survival within microcapsules since they have no apparent inhibitory effect on myogenic differentiation, and produce similar proliferative results seen when using bFGF. In terms of differentiation, the addition of IGF-II to the microcapsules or the use of a myoblast cell line overexpressing IGF-II, aid in increasing the myogenic differentiation of encapsulated myoblasts, however, levels still do not approach those seen in unencapsulated myoblasts. While the use of growth factors and matrix materials have made important steps towards the optimization of microcapsules, more study is needed to elucidate possible solutions to the continued problem of decreased differentiation of myoblasts within APA microcapsules.

APPENDIX A

Solutions:

1.1% CaCl$_2$

11g CaCl$_2$
500 ml 0.9% NaCl
500 ml dH$_2$O
- autoclave

2% CHES stock

2g CHES (2-N-[cyclohexylamino]ethane-sulfonic acid)
100 ml 0.6% NaCl
- pH to 8.2 with NaOH

50X Denhardt's solution

1g Ficoll
1g polyvinylpyrrolidone
1g bovine serum albumin
up to 100ml dH$_2$O

Differentiation media

2% horse serum
1% penicillin/streptomycin

Growth media

10% fetal bovine serum
1% penicillin/streptomycin

Hoechst 33258 dye (stock)

1mg H33258 (Sigma)
1.0 ml dH$_2$O
- store foil wrapped at 4 °C for up to 6 months
- working concentration is 1 μg/ml, make up fresh, keep in dark

5X MOPS Buffer

10.46 g Morpholinopropanesulfonic acid (MOPS)
4.17 ml of 3M sodium acetate
2.5 ml 0.5M EDTA (pH 8.0)
- bring up to 200 ml with dH$_2$O
- adjust pH to 7.0 with 10N NaOH
- make up to 250 ml with dH$_2$O
- store at 4 °C

10X PBS

36g NaCl
0.9g KCl
5.175g Na$_2$HPO$_4$-7H$_2$O
0.382 KH$_2$PO$_4$
450 ml dH$_2$O
- pH to 7.4
- bring up to 500 ml with dH$_2$O

0.05% PLL

3 g poly-L-lysine (PLL)
500 ml 0.9% NaCl
- filter sterilize

2.0% alginate

2g of alginate
100 ml NaCl
- stir slowly overnight
- filter sterilize

Prehybridization buffer

5.0 ml deionized formamide
3.0 ml 20X SSC
1.0 ml 50X Denhardt's solution
0.3 ml dH$_2$O
0.5 ml 20% SDS
0.1 ml sonicated herring sperm DNA (10 mg/ml)

0.055 M Sodium Citrate

8.08 g sodium citrate dihydrate
500 ml 0.9% NaCl
- autoclave

20% SDS

20 g sodium laurylsulfate
90 ml dH$_2$O
- heat to 68 °C to dissolve
- adjust pH to 7.2 with HCl
- make up to 100 ml with dH$_2$O

20X SSC

87.66 g NaCl
44.12 g sodium citrate
to 500 ml with dH$_2$O
- adjust to pH 7.0 with glacial acetic acid
- autoclave

TE Buffer

1 ml 1 M Tris-Cl (pH 8.0)
0.2 ml 0.5 M EDTA (pH 8.0)
to 100 ml with dH$_2$O

0.125% Trypsin

10 ml 10X citrate saline
0.125 g trypsin
to 100 ml with dH$_2$O
-   filter sterilize

APPENDIX B

Order of microcapsule washes:

All washes were left long enough for the capsules to settle unless otherwise indicated.

1.1 % $CaCl_2$

0.55% $CaCl_2$

0.28% $CaCl_2$

0.1 % CHES – 3 minutes

1.1 % $CaCl_2$

0.05% PLL – 6 minutes

0.1 % CHES

1.1% $CaCl2$

0.9% NaCl

0.04% alginate – 4 minutes

0.9% NaCl

0.055 M Sodium Citrate – 6 minutes

0.9% NaCl

0.9% NaCl

Serum-free media

Serum-free media

Growth media

APPENDIX C

Plasmid Vectors for Use as Probes:

pCA-*pgk*3' – PGK

cDNA encodes mouse pgk-1 (Adra et al., 1987). The 700 bp insert (contains

sequence for ampicillin resistance) is released from the vector by digestion with

Pst1.

pEMSV – myogenin

cDNA encodes mouse myogenin (Wright et al., 1989). The mRNA size is

approximately 1500 bp. The insert size is 1600 bp (contains sequence for

ampicillin resistance) and is released from the vector by digestion with EcoRI.

CR165R – troponin I

cDNA clone homologous to rat troponin I (slow skeletal muscle isoform)

inserted into pEMBL18+ (Koppe et al., 1989). The mRNA size is

approximately 1200 bp. The insert size is 700 bp (contains sequence for

ampicillin resistance) and is released from the vector by digestion with

EcoRI

APPENDIX D:

Development of a DNA Assay for Determination of Total Cells within Microcapsules

D.1 Hoechst 33258

Hoechst 33258 (H33258) is a fluorescent dye which binds specifically to DNA in the A-T rich regions (Downs and Wilfinger, 1983; Stokke and Steen, 1985). Since cells actively pump the dye out they must be dead in order to stain the DNA. The idea behind this assay is that all mammalian cells contain the same amount of DNA, therefore, DNA fluorescence will be proportional to the total cell number in a given sample.

D.2 Use of H33258 to Quantitate Naked (unencapsulated) Cells

In order to assess the potential of using this dye to quantitate cell number, the assay was initially attempted using naked cells. Concentration of a cell suspension (in 1X PBS) was determined in triplicate using a Coulter Counter. Cells were then seeded out into a 96-well plate using increasing cell numbers. The volume of the sample was made up to 100 μl, and 200 μl of H33258 dye

(working concentration, Appendix A) was added to each well. The plate was then

read with a fluorometer (excitation 360nm, emission 460 nm), and cell number

was correlated with fluorescence units.
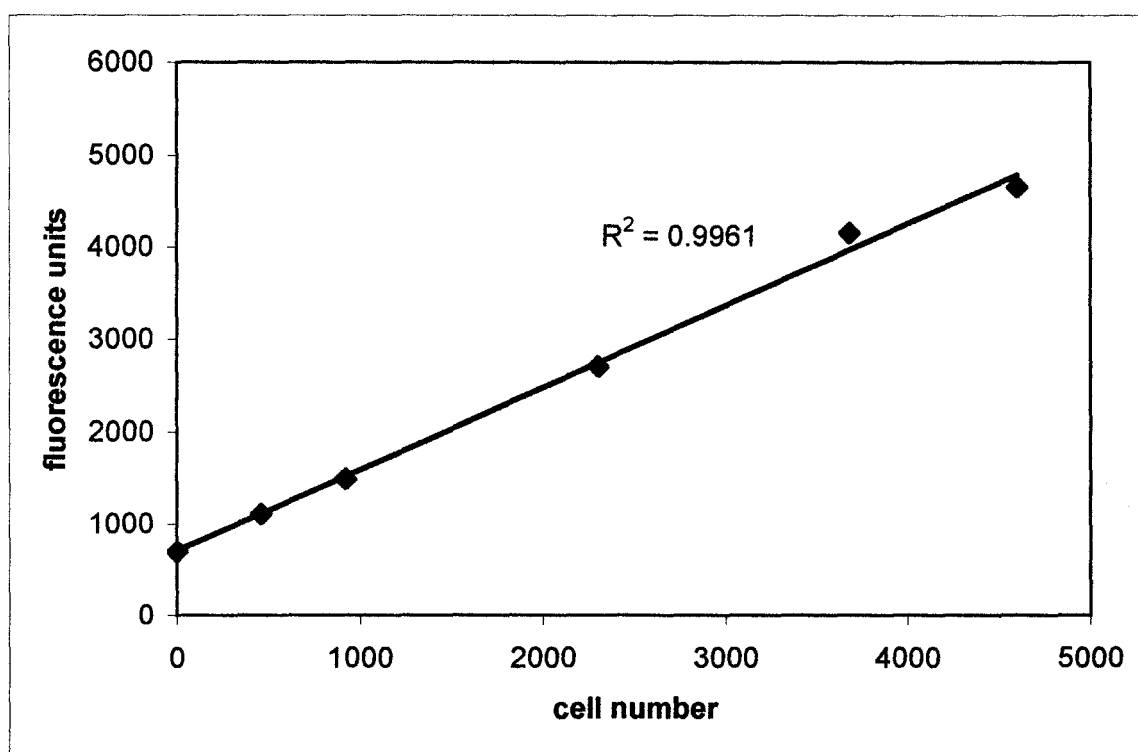
Figure D1. DNA assay (H33258) using naked (unencapsulated) cells as the

known standard. Cell number strongly correlates with fluorescence (R^2=0.9961).

D.3 Use of H33258 to Quantitate Microencapsulated Cells

Following the successful application of the H33258 dye to naked cells, the assay was used to attempt to quantitate encapsulated cells. Cell number per capsule of a "batch" of capsules was determined by crushing up a known number of capsules and determining the number of cells using a hemocytomoter. Known numbers of capsules were then placed in 1.5 ml eppendorf tubes, crushed, and loaded into the 96-well plate. The volume of the sample was made up to 100 μl, and 200 μl of H33258 dye (working concentration, Appendix A) was added to each well. The plate was then read with a fluorometer (excitation 360nm, emission 460 nm), and cell number was correlated with fluorescence units.

When encapsulated cells were used, there was no correlation between cell number and fluorescence units. Additionally, when empty capsule materials was used, fluorescence readings were much higher than those of the blank (1X PBS). Therefore, it appeared that the capsule material was contributing to the fluorescence. Attempts to dissolve the capsule material with EDTA, Triton X-100, and other detergents did not eliminate the background to acceptable levels.

D.4 Emission Spectra of H33258

Since the capsule material appeared to contribute to the fluorescence when H33258 was used as a dye for determining cell numbers, an emission spectra was performed to examine the nature of the background fluorescence.
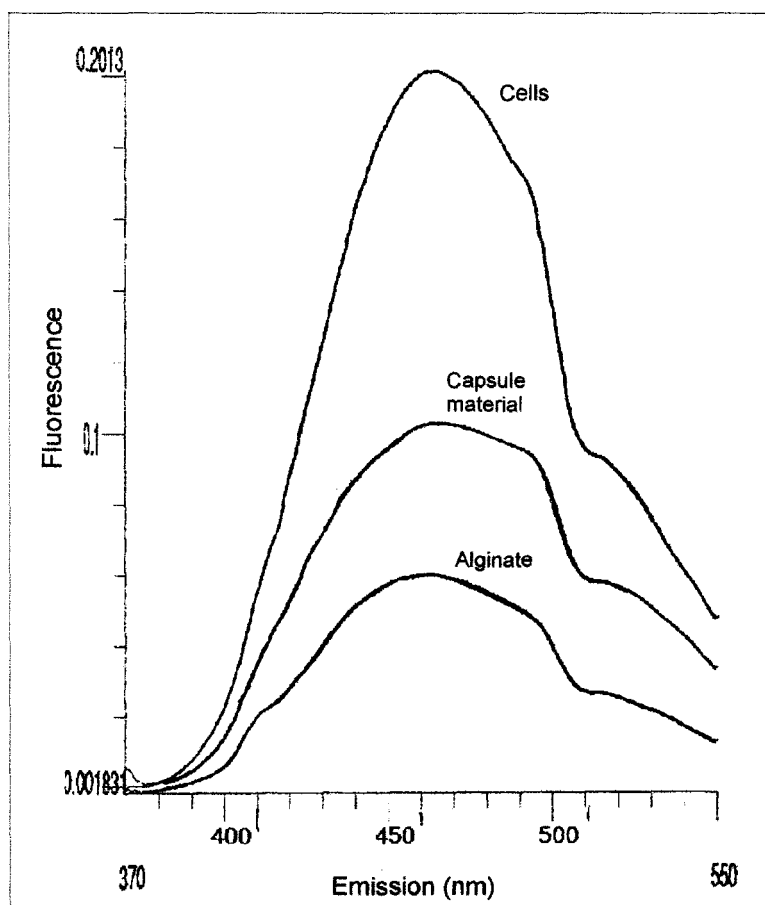
Figure D2. Emission spectra of naked cells (myoblasts), capsule material (alginate-poly-L-lysine-alginate), and alginate. Emission peaks were observed to be 455 nm, 455 nm, and 450 nm respectively. The emission spectra generated showed light scattering and were "smoothed" to provide a consistent curve.

Comparison of the emission spectra of the capsule material and alginate samples indicate that they are reacting with the H33258 dye in a manner that produces an emission peak that is very similar to that seen for naked cells. Therefore, it appears that the background produced by the capsule material limits the use of H33258 for quantitation of encapsulated cells.

D.5 Use of Propidium Iodide for Quantification of Encapsulated Cells

Due to the disappointing results obtained when using H33258 dye for quantitating encapsulated cells, an alternative fluorescent DNA-binding dye, propidium iodide (Sigma), was utilized. Encapsulated cell samples were prepared as described for the H33258 assay with the addition of an incubation step with RNase (see protocol, section D.6) in order to degrade any RNA that might contribute to the fluorescence. Fluorescence of the samples was determined using an excitation of 530 nm and an emission of 645 nm.
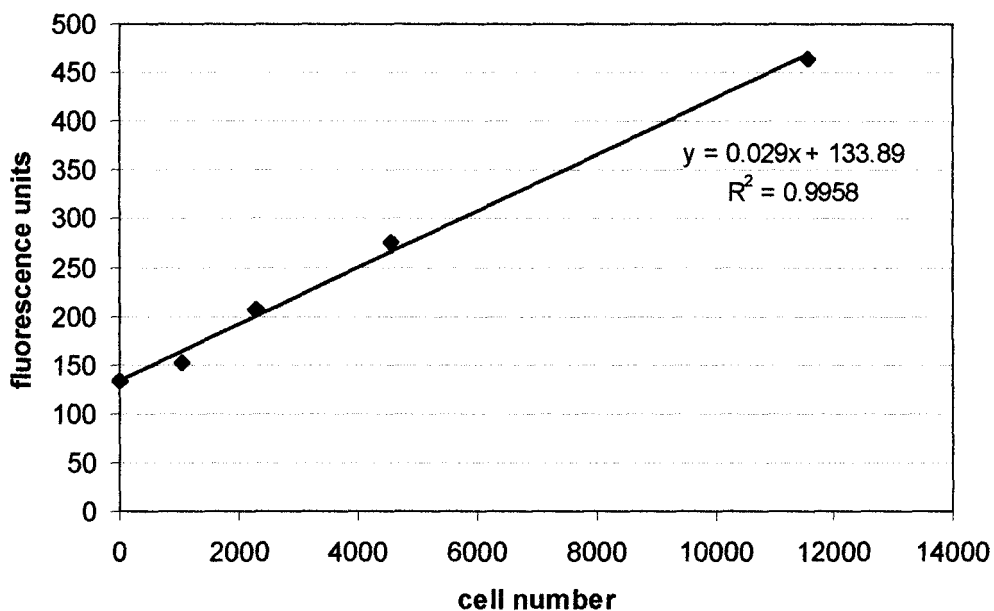
Figure D3. DNA assay (propidium iodide) using encapsulated cells.

When using propidium iodide to quantitate encapsulated cells, cell
numbers strongly correlate with fluorescence (R^2=0.9958). Additionally, the curve
generated when using encapsulated cells is the same as that generated with
naked cells, making propidium iodide a successful dye when quantitating both
naked and unencapsulated cells.

D.6 Protocol for Quantification of Encapsulated Cells using Propidium Iodide

-

RNase solution:

50 mM Tris-Cl, pH 8.0
10 mM EDTA, pH 8.0
100 μg/ml RNase A (Sigma)

Propidium Iodide

Stock: 1 mg/ml in 1X PBS (store foil-wrapped at 4 °C for up to 6 months)
Working: 1 μg/ml in 1X PBS (make up fresh, keep in dark)

Procedure:

1) Count the number of capsules in a 100 μl samlpe, transfer back to a microtube, and draw off liquid down to the 100 μl mark.
2) Freeze samples overnight to ensure all cells are dead.
3) Add 100 μl of RNase solution and incubate for 30 min at room temperature.
4) Mix the sample and load two 100 μl samples into a 96-well microtiter plate.
5) Add 200 μl of propidium iodide (concentration 1 μg/ml) to each sample well.
6) Read the fluorescence (excitation of 530 nm, emission of 645 nm)
7) Determine total cell number in your sample using a standard curve generated from unencapsulated cells. A standard cell suspension of known concentration can be store at −20 °C.

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