THE EFFECT OF TGF-β ISOFORMS ON PROGENITOR CELL RECRUITMENT

AND DIFFERENTIATION INTO CARDIAC AND SKELETAL MUSCLE

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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation
is my own original work and that I have not previously in its entirety or in part
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SUMMARY

Definition: Stem cells are unspecialised cells with the capacity for long-term self-renewal and the ability to differentiate into multiple cell-lineages.

The potential for the application of stem cells in clinical settings has had a profound effect on the future of regenerative medicine. However, to be of greater therapeutic use, selection of the most appropriate cell type, as well as optimisation of stem cell incorporation into the damaged tissue is required. In adult skeletal muscle, satellite cells are the primary stem cell population which mediate postnatal muscle growth. Following injury or in diseased conditions, these cells are activated and recruited for new muscle formation. In contrast, the potential of resident adult stem cell incorporation into the myocardium has been challenged and the response of cardiac tissue, especially to ischaemic injury, is scar formation.

Following muscle damage, various growth factors and cytokines are released in the afflicted area which influences the recruitment and incorporation of stem cells into the injured tissue. Transforming Growth Factor-β (TGF-β) is a member of the TGF-β-superfamily of cytokines and has at least three isoforms, TGF-β1, -β2, and -β3, which play essential roles in the regulation of cell growth and regeneration following activation and stimulation of receptor-signalling pathways. By improving the understanding of how TGF-β affects these processes, it is possible to gain insight into how the intercellular environment can be manipulated to improve stem cell-mediated repair following muscle injury. Therefore, the main aims of this thesis were to determine the effect of the three TGF-β isoforms on proliferation, differentiation, migration and fusion of muscle progenitor cells (skeletal and cardiac) and relate this to possible improved mechanisms for muscle repair.

The effect of short- and long-term treatment with all three TGF-β isoforms were investigated on muscle progenitor cell proliferation and differentiation using the C2C12 skeletal muscle satellite and P19 multipotent embryonal carcinoma cell-lineages as *in vitro* model systems. Cells were treated with 5 ng/mℓ TGF-β isoforms unless where stated otherwise. In C2C12 cells, proliferating cell nuclear antigen (PCNA) expression and localisation were analysed, and together with total nuclear counts, used to assess the effect of TGF-β on myoblast proliferation (Chapter 5). The myogenic regulatory factors MyoD and myogenin, and structural protein myosin heavy chain (MHC) were used as protein markers to assess early and terminal differentiation, respectively. To establish possible mechanisms by which TGF-β isoforms regulate differentiation, further analysis included determination of MyoD localisation and the rate of MyoD degradation in C2C12 cells.

To assess the effect of TGF-β isoforms on P19 cell differentiation, protein expression levels of connexin-43 and MHC were analysed, together with the determination of embryoid body numbers in differentiating P19 cells (Chapter 6). Furthermore, assays were developed to analyse the effect of TGF-β isoforms on both C2C12 and P19 cell migration (Chapter 7), as well as fusion of C2C12 cells (Chapter 8).

Whereas all three isoforms of TGF-β significantly increased proliferation of C2C12 cells, differentiation results, however, indicated that especially following long-term incubation, TGF-β isoforms delayed both early and terminal differentiation of C2C12 cells into myotubes. Similarly, myocyte migration and fusion were also negatively regulated following TGF-β treatment. In the P19 cell-lineage, results demonstrated that isoform-specific treatment with TGF-β1 could potentially enhance differentiation. Further research is however required in this area, especially since migration was greatly reduced in these cells.

Taken together, results demonstrated variable effects following TGF-β treatment depending on the cell type and the duration of TGF-β application. Circulating and/or treatment concentrations of this growth factor could therefore be manipulated depending on the area of injury to improve regenerative processes. Alternatively, when selecting appropriate stem or progenitor cells for therapeutic application, the effect of the immediate environment and subsequent interaction between the two should be taken into consideration for optimal beneficial results.

OPSOMMING

Definisie: Stamselle is ongespesialiseerde selle met die kapasiteit vir langtermyn selfvernuwing asook die vermoë om in veelsoortige seltipes te differensieer.

Die potensiaal wat die aanwending van stam- en voorloperselle vir kliniese behandeling geskep het, bied unieke moontlikhede vir die toekoms van herstellende genesing. Om egter van groter genesende waarde te wees, is daar 'n behoefte om die gebruik van die geskikste seltipe vir behandeling te bepaal, asook om meer doeltreffende stam- en voorlopersel insluiting in die beskadigde weefsel te bewerkstellig. In volwasse skeletspierweefsel dien satellietselle as die primêre stamselbron wat groei ná geboorte bemiddel. Hierdie selle word tydens siektetoestande of na beserings aktiveer om by te dra tot groei en herstel prosesse van die beskadigde weefsel. In teenstelling hiermee, is die natuurlike toepassing van reserwe volwasse stamselle in die hartspier minimaal, met die gevolg dat veral isgemiese beserings hoofsaaklik littekenweefsel in die hartspier vorm.

Verskeie groeifaktore en sitokiene word tydens siektetoestande of weens beserings in die aangetaste spier vrygestel wat die werwing en insluiting van stam- en voorloperselle in die beskadigde weefsel beïnvloed. Die Transformasie Groeifaktor-β (TGF-β) vorm 'n subklas van die TGF-β-superfamilie van sitokiene en het drie isovorme, TGF-β1, -β2, and -β3, wat noodsaaklike funksies verrig om die regulering van selgroei en regenerasie te beïnvloed. Beter kennis van die meganismes waardeur TGF-β hierdie prosesse reguleer kan help met die ontwikkeling van prosedures wat die intersellulêre omgewing tot so 'n mate sal manipuleer dat verbeterde genesing deur middel van stam- en voorloperselle sodoende bewerkstellig kan word. Die bepalende doelwitte van hierdie tesis was derhalwe om die effek van die drie TGF-β isovorme te ondersoek met betrekking tot proliferasie, differensiasie, migrasie en fusie, spesifiek ten opsigte van skelet- en hartspiervoorloperselle.

Die effek van beide kort- en langtermyn toediening van die drie TGF-β isovorme is ondersoek op proliferasie en differensiasie van C2C12 skeletspier voorloperselle en P19 multipotensiële embrioniese karsinoomselle wat as *in vitro* modelsisteme gedien het. Selle is behandel met 5 ng/mℓ TGF-β isovorme tensy anders vermeld. In C2C12 selkulture is die ekspressie en lokalisering van die proliferasie sel nukleêre antigeen analiseer, asook die selkerntotaal-tellings om sodoende die effek van TGF-β op proliferasie van dié seltipe te bepaal (Hoofstuk 5). Die miogeniese reguleringsfaktore MyoD en myogenien, asook die strukturele proteïen miosien swaar ketting (MSK) is gebruik as proteïenmerkers om onderskeidelik vroeë en finale differensiasie te analiseer.

Om vervolgens moontlike regulerende prosesse vas te stel waardeur die TGF-β isovorme hul uitwerking op C2C12 differensiasie bewerkstellig, is die lokalisering en afbrekingstempo van MyoD in hierdie selkultuur bepaal. Die effek van TGF-β isovorme op differensiasie in die P19 selkultuur is bepaal deur proteïen ekspressie vlakke van konneksien-43 en MSK te analiseer, asook om tellings van embrioniese-liggaampie vorming te bepaal (Hoofstuk 6). Protokolle is verder ontwikkel om die effek van TGF-β isovorme op migrasie van beide C2C12 en P19 miosiete te bepaal (Hoofstuk 7), asook om fusie in die C2C12 selkultuur te analiseer (Hoofstuk 8).

Al drie TGF-β isovorme het tot 'n beduidende toename in C2C12 miosiet proliferasie gelei. In die geval van differensiasie, het resultate egter daarop gedui dat veral langtermyn toediening van TGF-β beide die vroeë en finale differensiasie van dié selkultuur benadeel het en sodoende is verdere ontwikkeling van miosiete na miobuisies vertraag. Net so is die migrasie en fusie van C2C12 miosiete ook negatief beïnvloed deur die TGF-β isovorme. In die P19 selkultuur het resultate getoon dat TGF-β1 'n moontlike isovorm-spesifieke effek demonstreer wat potensieël differensiasie sou kon bevorder. Verdere navorsing is egter nodig om hiedie effek te bevestig, veral met inagneming dat P19 miosietmigrasie, in teenstelling, hoogs onderdruk was deur alle TGF-β isovorme.

In samevatting dui resultate op die veranderlike effek van behandeling met TGF-β isovorme wat grotendeels beïnvloed word deur die spesifieke selkultuur, asook die duur van TGF-β-toediening. Deur sirkulerende vlakke en/of terapeutiese konsentrasies van hierdie groeifaktor te manipuleer na gelang van die weefseltipe wat beskadig of aangetas is, kan regenererende behandeling meer doeltreffend toegepas word. Vervolgens, wanneer geskikte stam- of voorloperselle klinies of terapeuties aangewend word, sal dit noodsaaklik wees om die invloed van die omliggende mikro-omgewing in ag te neem wat grotendeels die doeltreffendheid van die behandeling sal bepaal.

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ACKNOWLEDGEMENTS

LIST OF CONFERENCE POSTER CONTRIBUTIONS AND ABSTRACTS

International

‣ **Schabort E.J.**, Van der Merwe M., Myburgh K.H., Niesler C.U. *Isoform-specific effect of TGF-β1 on muscle stem cell differentiation.* Presented at the FASEB Summer Research Conference, Tucson, Arizona, USA; 11-16 June 2005.

National

‣ **Schabort E.J.**, Moore F., Myburgh K.H., Niesler C.U. *Analysis of the effect of TGF-β isoforms on satellite cell differentiation using known and novel markers.* Presented at the 32nd Annual Congress of the Physiology Society of Southern Africa, Coffee Bay, South Africa; 12-15 September 2004.

‣ **Schabort E.J.**, Van der Merwe M., Myburgh K.H., Niesler C.U. *Effect of TGF-β isoforms on muscle satellite cell differentiation*. Presented at the 33rd Annual Congress of the Physiology Society of Southern Africa, Cape Town, South Africa; 7-9 September 2005.

‣ Van der Merwe M., **Schabort E.J.**, Niesler C.U. *Analysis of migration of myogenic progenitor cells.* Presented at the 33rd Annual Congress of the Physiology Society of Southern Africa, Cape Town, South Africa; 7-9 September 2005.

ABBREVIATIONS

ANF   atrial natriuretic factor

AQP   acute quadriplegic myopathy

ASC(s)   adult stem cell(s)

bHLH   *basic* helix-loop-helix (transcription factors)

BLSC(s)   blastomere-like stem cell(s)

BMESL   bone marrow-derived embryonic stem-like cells

BMP   bone morphogenetic proteins

bmSP   bone marrow side-population

BrdU   5-bromo2-deoxy-uridine

BSA   bovine serum albumin

CBE SC(s)   cord blood-derived embryonic-like stem cell(s)

CHX   cycloheximide

DMSO   dimethyl sulfoxide

EASC(s)   embryonic-like adult stem cell(s)

ECC   early committed cells

ECM   extracellular matrix

EGF   epidermal growth factor

EPC   endothelial progenitor cells

ESC(s)   embryonic stem cell(s)

ETS1   external transcribed spacer 1

FBS   foetal bovine serum

FGF   fibroblast growth factor

FSC(s)   foetal stem cell(s)

FSSC(s)   foetal somatic stem cell(s)

hBMSC(s)   human bone marrow-derived multipotent stem cell(s)

hESC-lines   human embryonic stem cell-lines

HGF   hepatocyte growth factor

HSC(s)   haematopoietic stem cell(s)

ICM	inner cell mass
IFNγ	interferon-gamma
IGF-1	insulin-like growth factor-I
IL	interleukins
LAP	latency-associated peptide
LIF	leukaemia inhibitory factor
Lin⁻	lineage commitment
LLC	large latent complex
LTBP	latent TGF-β binding protein
L-TGF-β	latent TGF-β form
MAPC	multipotent adult progenitor cells
MAPK	mitogen-activated protein kinase
MDR1	multi-drug resistance protein 1
MDSC(s)	muscle-derived stem cell(s)
MEF-2C	myocyte enhancer factor-2C
MHC	myosin heavy chain
MIAMI	marrow-isolated adult multilineage inducible cells
MNF	myocyte nuclear factor
mpc(s)	myogenic precursor cell(s)
MRF(s)	myogenic regulatory (also transcription) factor(s)
MSC(s)	mesenchymal stem cell(s)
mSP	muscle side-population
MTT	myoblast transfer therapy
N-CAM	neural cell adhesion molecule
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PDGF	platelet-derived growth factor
PGC	primordial germ cells
PKC	protein kinase C
PSC(s)	pluripotent stem cell(s)
PVDF	polyvinylidene difluoride

RGD	Arg-Gly-Asp specific amino acid sequence
RIPA	radio-immuno precipitation assay
ROCK	Rho-associated protein kinase
ROS	reactive oxygen species
SC(s)	stem cell(s)
Sca-1	stem cell antigen-1
SCF	stem cell factor
SDF-1	stromal cell-derived factor-1
SDS	sodium dodecyl sulphate
SFM	serum free medium
SKP	skin-derived precursors
SLC	small latent complex
Smad	"Mothers against decapentaplegic homolog"
SSC	somatic stem cells or self-renewing satellite cells
TAK1	TGF-β-activated kinase 1
TBS	tris-buffered saline
TCSC(s)	tissue-committed stem cell(s)
TGF-β RI-III	TGF-β receptors I-III
TGF-β	transforming growth factor-β
TNC	total nuclear count
TNF-α	tumour necrosis factor-α
USSC(s)	unrestricted somatic stem cell(s)
UTF1	undifferentiated embryonic cell transcription factor 1
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
VSEL SC(s)	very small embryonic-like stem cell(s)
γ3-AMPK	γ3-isoform of AMP-activated protein kinase

CHAPTER 1

INTRODUCTION

Stem cells are primitive, unspecialised cells, capable of dividing and generating multiple cell types of most tissues in the body depending on the developmental stage of the stem cell. This ability of stem cells to differentiate into mature, more specialised cell types, as well as to self-renew, have made them attractive potential agents for use in enhanced tissue repair and regenerative medicine of diseases and disorders for which no, or only partially effective treatments are currently available. The broad spectrum of potential therapeutic applications in which stem cells can be applied, has resulted in the rapid advancement of research in the hope of finding treatment for these genetic and degenerative diseases, as well as for improving the regenerative capacity of diseased and injured tissue.

Essential to the successful use and manipulation of stem cells, is understanding the importance of the *niche* in which stem cell populations are established. Such stem cell *niches* are anatomic locations that regulate the participation of stem cells in processes of regeneration, maintenance and repair, and constitute a basic unit of micro-environmental cells which co-ordinate tissue homeostasis and integrate inter- and intracellular signals to mediate a balanced response depending on the need of the organism. Importantly, the *niche*-environment protects stem cells from apoptotic stimuli, excessive stem cell production and other stimuli that would challenge stem cell reserves.

Interaction between stem cells and their *niche* creates a system necessary to maintain the balance between stem cell quiescence and activity and is therefore an essential attribute of a functional environment. Knowledge of this interaction is also required for the design of stem cell therapeutics. Elements of the local environment that participate in the regulation of stem cell activity in their *niche* include physical interaction of cell membranes with tethering molecules on neighbouring cells or surfaces, interaction with the extracellular matrix, signalling interactions between stem cells and several other cells in the immediate micro-environment, paracrine or endocrine signals from distant sources, neural input and metabolic products of tissue activity.

It is well known that adult *skeletal* muscle contains a population of resident stem cell-like cells called satellite cells which mediate postnatal muscle growth and regeneration. Following injury, satellite cells are activated and recruited for new muscle formation. Unlike skeletal muscle which is capable of essentially scar-free regeneration by means of these satellite cells, the response of *cardiac* tissue to especially ischaemic injury is scar formation. However, although the myocardium has long been regarded as a post-mitotic organ, a series of recent studies have indicated that autologous adult stem cells can be activated to promote at least partial reconstruction (and decrease scar formation) of the myocardium following an ischaemic insult.

The Transforming Growth Factor-β (TGF-β)-superfamily of cytokines plays a role in the regulation of cell proliferation, differentiation, migration and apoptosis by means of receptor-signalling pathways and can either promote or inhibit these processes depending on the local conditions and/or individual cytokine released. Specifically, the three isoforms of TGF-β have been shown to regulate growth and regeneration processes in both skeletal and cardiac muscle. Few studies, however, have characterised the isoform-specific effects of TGF-β on muscle stem and progenitor cell recruitment and differentiation.

The healing of impaired function of the human system is the goal of regenerative medicine, requiring knowledge and integration of diverse disciplines. The need is not only to replace that which is malfunctioning, but also to provide the elements required for *in vivo* repair and to devise replacements that interact with the living body without rejection. Mechanisms to stimulate the body's intrinsic capacity for regeneration, together with cell replacement therapy, have become elemental in regenerative medicine. An increased understanding of both the extrinsic and intrinsic signals recruiting and directing stem and progenitor cells *in vitro* and *in vivo*, as well as the identification of tissue-specific factors and signalling components that are required to generate and manipulate the stem cell progeny into the relevant tissue, are therefore essential for therapeutic applications to be successful.

In the following chapters, processes of proliferation, differentiation, migration and fusion are discussed, specifically analysing the effect of the three TGF-β isoforms on skeletal and cardiac progenitor cell growth and development. By improving the understanding of how the TGF-β isoforms affect these processes, it could become possible to gain insight into how the micro-environmental conditions can be manipulated to improve stem cell-mediated repair following muscle injury.

CHAPTER 2

BACKGROUND AND LITERATURE REVIEW

2.1   PROPERTIES OF STEM CELLS

A stem cell (SC) can be defined by three main criteria: **(A)** long-term proliferation and self-renewal while remaining totally unspecialised; **(B)** the ability to differentiate into multiple mature, functionally specialised cell types when stimulated under particular physiological or experimental conditions; *and* **(C)** the ability to reconstruct a given tissue *in vivo* (Lakshmipathy and Verfaillie, 2005). Of particular interest, especially regarding their therapeutic applicability, are the activation-mechanisms and signalling pathways required to induce SCs to develop into specific cell types *in vivo*. A true SC is capable of asymmetric division, dividing into one daughter cell which remains a true SC while the other becomes specialised and forms a progenitor cell capable of further differentiation along a particular cell-lineage depending on the environmental stimuli.

SCs can be found at various stages of embryogenesis, from the inner cell mass (ICM) of the embryo, through to various foetal and adult tissues, with a corresponding decline in differentiation potential as these cells become more specialised. As such, SCs can be classed as embryonic, (originating from the embryo; embryonic stem cells, ESCs), foetal (originating from foetal blood and haematopoietic organs; foetal stem cells, FSCs) or adult (originating from the umbilical cord or adult tissues; adult stem cells, ASCs).

At the top of the SC hierarchy is the *totipotent* fertilized egg (zygote), as well as the morula, which constitutes eight cells or less. Each cell of the zygote or morula has the ability to generate an entire organism (i.e. can generate both embryonic and supportive extra-embryonic tissue). Subsequent cell differentiation results in the formation of the blastocyst, composed of outer trophoblast cells and undifferentiated inner cells, referred to as the ICM (Figure 2.1).

Figure 2.1. Stem cell hierarchy.

[Adapted with modifications from Price et al., 2006; Wobus and Boheler, 2005]

TOTIPOTENT

TOTIPOTENT

MULTIPOTENT

UNIPOTENT

ZYGOTE

generates cells of the endoderm, mesoderm and ectoderm layers, PGC and supporting trophoblast layer

BLASTOMERES

MORULA

BLASTOCYST

cells isolated from ICM, generate primary germ layers, the endoderm, mesoderm and ectoderm, and PGC: Embryonic Stem Cells

cells isolated from PGC: Embryonic Germ Cells

ADULT STEM CELLS

stem cells isolated from adult tissues and organs, self-renew and differentiate into multiple organ-specific cell types

PROGENITOR or PRECURSOR CELLS from the -

ectoderm   *mesoderm*   *endoderm*

- neural lineages
- epithelial lineage/skin

- skeletal, smooth, cardiac muscle
- bone
- cartilage
- haematopoietic lineages
- connective tissue
- fat

- hepatocytes
- pancreatic cells
- tissues of the respiratory and digestive tracts

ESCs are derived from the undifferentiated ICM of the blastocyst stage of the embryo (Evans and Kaufman, 1981; Martin, 1981) and although both ESC and cells from the ICM are no longer totipotent and cannot form extra-embryonic tissues, they retain the capacity to give rise to cells of all three primary germ layers of the embryo: ectoderm, mesoderm and endoderm, as well as the primordial germ cells (PGC). The *pluripotent* ESCs are immortal and seemingly capable of unlimited self-renewal and proliferation *in vitro*, maintaining a non-committed state until stimulated to differentiate into a particular cell type (Lakshmipathy and Verfaillie, 2005; Thomson *et al.*, 1998; Wobus and Boheler, 2005). In this respect, ESCs differ from the ICM cells: whereas both cell types have similar differentiation capacities, ICM cells *within the embryo* do not exhibit prolonged self-renewal abilities.

SCs isolated from various adult organs are *multipotent* and have the potential to self-renew and differentiate into multiple organ-specific cell types. Cells committed to a particular cell-lineage with limited or no self-renewal ability, are termed progenitor or precursor cells (Lakshmipathy and Verfaillie, 2005) (Table 2.1).

Embryonic and adult stem cells have demonstrated great potential for generating tissues of therapeutic value. The characteristics of these cells reveal the benefits, as well as deficiencies associated with each and can be applied to establish the best strategy for clinical use. It remains to be determined whether embryonic and adult SCs will be equivalent in their capacity to produce large numbers of specific cell types for transplantation purposes, as well as retain their function over long periods, thereby optimising their therapeutic potential (Passier and Mummery, 2003).

Table 2.1. Stem and progenitor cell definitions.

DIFFERENTIATION	The process by which a cell, in response to stimuli, becomes more specialised.
TRANSDIFFERENTIATION	The ability of a cell of one tissue, organ or system, to differentiate into a cell type of another tissue, organ or system, with the concomitant loss of the tissue-specific markers and function of the original cell type.
DE-DIFFERENTIATION	The regression of a normally specialised cell to a less specialised cell.
PLASTICITY	The potential to differentiate into other cell types not originally thought to be within the differentiation spectrum of that cell; *OR* The capacity to adapt or change.
TOTIPOTENCY	Ability to differentiate into all cell types, both embryonic and extra-embryonic. Totipotent cells can create a complete organism.
PLURIPOTENCY	Ability to grow into any cell type except for totipotent stem cells. Pluripotent stem cells are therefore able to differentiate into stem cells of all three germ layers and are only unable to form a complete organism.
MULTIPOTENCY	Ability to produce cells of a subset of cell-lineages; *OR* Cells that are committed to producing cells that have a particular function, e.g. blood stem cells are multipotent: they can produce red blood cells, white blood cells and platelets.
OLIGOPOTENT	Ability to give rise to a more restricted subset of cell-lineages than multipotent stem cells, e.g. lymphoid progenitors can give rise to B- and T-lymphocytes.
UNIPOTENT	Ability to contribute only one mature cell type.
STEM CELL	Capable of self-renewal, differentiation into at least one cell type and functional reconstitution of the tissue of origin.
EMBRYONIC STEM CELL	Pluripotent stem cells derived from the ICM of the blastocyst, capable of self-renewal and differentiation into all somatic cell types, germ cells and progenitors of all three germ layers.
ADULT STEM CELL	An unspecialised cell derived from adult tissue which can greatly and efficiently be expanded in culture and is capable of self-renewal and differentiation into specialised mature cells.
HAEMATOPOIETIC STEM CELL	A stem cell which can proliferate and differentiate into all mature blood cells.
MESENCHYMAL STEM CELL	A stem cell which can proliferate and differentiate into mesenchymal tissues such as bone, cartilage and muscle.
MESODERMAL PROGENITOR CELL	An unspecialised cell capable of yielding mesodermal tissue such as muscle; progenitor cells are not capable of self-renewal.
HEMANGIOBLAST	Earliest mesodermal precursor of both blood and vascular endothelial cells.
ANGIOBLAST	An endothelial progenitor cell.

2.1.1   Embryonic Stem Cells and their Characteristics

As mentioned above, human ESCs are pluripotent cells derived from the ICM of *in vitro* fertilised human blastocysts. When cultured *in vitro* or injected into a host, ESCs spontaneously differentiate and form embryoid bodies composed of the three embryonic germ layers (Itskovitz-Eldor *et al.*, 2000). Despite the versatility of ESCs to differentiate into all tissues of the adult body, their direct use in cell therapy is currently restricted because of issues such as immune rejection (except in the central nervous system), tumour formation and ethical objections. In addition, because ESCs can differentiate into any cell type, they need to be directed down a particular cell-lineage prior to use *in vivo*. *In vitro*, this can be achieved by maintaining culture conditions with specific growth factors, however *in vivo*, precise mechanisms directing ESCs down the desired cell-lineage remains to be fully determined.

Techniques developed to establish murine embryonic stem cell-lines have been critical in the generation of human embryonic stem cell-lines (hESC-lines). However, many of these hESC-lines are inappropriate for therapeutic applications due to retroviral infections and xenogenic contamination (often from the culture medium using animal products). In addition, due to the variability among hESC-lines (growth characteristics, directing differentiation potential, culturing techniques), reliable molecular- and cellular markers need to be established to distinguish undifferentiated pluripotent SCs from the differentiated state. Although such cell surface and molecular markers have been identified critical for the identification of undifferentiated mouse and human ESCs, many are still inadequate to characterise the specific stages of differentiation (Wobus and Boheler, 2005). Such markers defining these cells' pluripotentiality include the transcription factors *Oct-3/4, Sox2* and *Nanog*, and the transcriptional co-activator *UTF1* (Wei *et al.*, 2005). The expression of selected SC markers is further outlined in Chapter 4.

An additional source of ESCs is genetically matched pluripotent ESCs generated from nuclear transfer or parthenogenesis (Kim *et al.*, 2001). Parthenogenesis involves the development of an embryo directly from an oocyte without fertilisation. Together with pluripotent SCs produced from fertilised embryos or embryos created by somatic-cell nuclear transfer (Rideout *et al.*, 2002), parthenogenesis provides a method for creating pluripotent SCs that could potentially serve as a source of tissue for transplantation with less risk of tissue rejection (Taylor *et al.*, 2005).

2.1.2   Foetal Stem Cells and their Characteristics

In addition to being isolated from foetal blood and haematopoietic organs during early pregnancy, FSCs can also be isolated from a variety of foetal somatic organs (liver, lung, bone marrow, pancreas, skeletal muscle, and kidney), and amniotic fluid and the placenta throughout gestation. Foetal blood is a rich source of haematopoietic SCs which proliferate more rapidly than those found in cord blood or adult bone marrow, as well as mesenchymal SCs, which also appear to be more primitive and with greater multipotentiality than the mesenchymal SCs found in adult tissue (Guillot *et al.*, 2006).

Where the use of ESCs in therapeutic applications have resulted in ethical and safety concerns, and with ASCs having a more limited regeneration capacity, FSCs may represent an intermediate cell type and prove to be advantageous in cell-based therapy, taking into consideration the advantages these cells have over ASCs.

2.1.3   Adult Stem Cells and their Characteristics

ASCs (also referred to as somatic SCs because they can additionally be located in foetuses, the umbilical cord and infants) reside in most mammalian tissues and have been found in all three embryonic germ layers (Mays *et al.*, 2007; Serafini and Verfaillie, 2006). Under non-stimulated conditions they are considered to be quiescent, however, similar to ESCs, ASCs are capable of self-renewal and differentiation when stimulated *in vitro*, or when influenced by their immediate environment *in vivo* (Lin, 1997). ASCs are however more rare than ESCs and methods of growing them in culture also more complicated, limiting their use when large cell numbers are needed for SC therapies.

Unlike ESCs which are totally unspecialised, the differentiation potential of ASCs is more limited. ASCs are generally regarded as being multipotent, but committed to a particular cell fate and only able to produce cells from the tissue of origin and not cross tissue or germ layer boundaries to generate cell types of different lineages (Lakshmipathy and Verfaillie, 2005; Moraleda *et al.*, 2006; Wagers and Weissman, 2004). Recent studies have however demonstrated that ASCs can, when stimulated under certain micro-environmental conditions, give rise to cell types *different* to those in the tissue of origin. Such *transdifferentiation* (Table 2.1) would potentially result in cells being able to contribute to a much wider field of differentiated tissues, and as such, greater use for clinical application. The suggestion that ASCs may transdifferentiate has given rise to the concept of tissue *plasticity*, which holds that the lineage-determination of ASCs is flexible and allows them to direct their differentiation depending on the environmental conditions (Blau *et al.*, 2001).

Adult bone marrow, brain, skeletal muscle, liver, pancreas, fat and skin have all shown to possess stem or progenitor cells with the capacity to differentiate or transdifferentiate into cell types other than their tissue of origin (Table 2.2). Of these tissues, bone marrow has shown the greatest potential for multi-lineage differentiation. The majority of studies presented have been performed both *in vitro* and *in vivo* in rodents. Importantly, most reports on ASC plasticity are only based on the expression of genetic markers and therefore, the tissue-specific functionality of possible transdifferentiated cell types still remain to be substantiated to determine their potential for clinical use. It also needs to be taken into consideration that a number of studies have reported a failure to detect transdifferentiation between cell-lineages (Choi *et al.*, 2003; Ono *et al.*, 2003; Vallieres and Sawchenko, 2003; Wagers *et al.*, 2002; Wagers and Weissman, 2004). Inconsistent results could be due to differences in injury models, cell types analysed, culture conditions, as well as purification and identification strategies or protein markers applied. As an example, with regards to circulating haematopoietic SCs, it is possible that these cells can be located in many non-haematopoietic tissues, and therefore may confound interpretation of results (Asakura *et al.*, 2002).

Figure 2.2 illustrates possible mechanisms for plasticity (Lakshmipathy and Verfaillie, 2005; Wagers and Weissman, 2004) which could involve: **(A)** cell *transdifferentiation* where SCs potentially contribute to cell types of different lineages; **(B)** cell *fusion* of transplanted and local cells (Terada *et al.*, 2002; Ying *et al.*, 2002); **(C)** the use of heterogeneous cell populations where infusion of a non-purified population could result in co-infusion of *multiple* different SCs; **(D)** *de-differentiation* of a tissue-specific cell to a more primitive cell type with subsequent re-differentiation along a new lineage [in this instance, nuclei from the transplanted cell undergoes re-programming during which the existing genetic information is removed and replaced by newly expressed genes and proteins consistent with the new cell-lineage (Wilmut *et al.*, 1997)]; *and* **(E)** a single, rare, *pluripotent* SC present in bone marrow or other tissues could possibly co-purify in protocols designed to enrich for tissue-specific SCs.

Figure 2.2. Potential mechanisms for ASC plasticity. Tissue-specific stem cells are represented by red or green ovals, pluripotent stem cells by blue ovals and differentiated cells by yellow ovals and green pentagons. *[Adapted with modifications from Wagers and Weissman, 2004]*

(A) Transdifferentiation   (B) Fusion   (C) Multiple stem cells   (D) De-differentiation   (E) Pluripotent stem cells
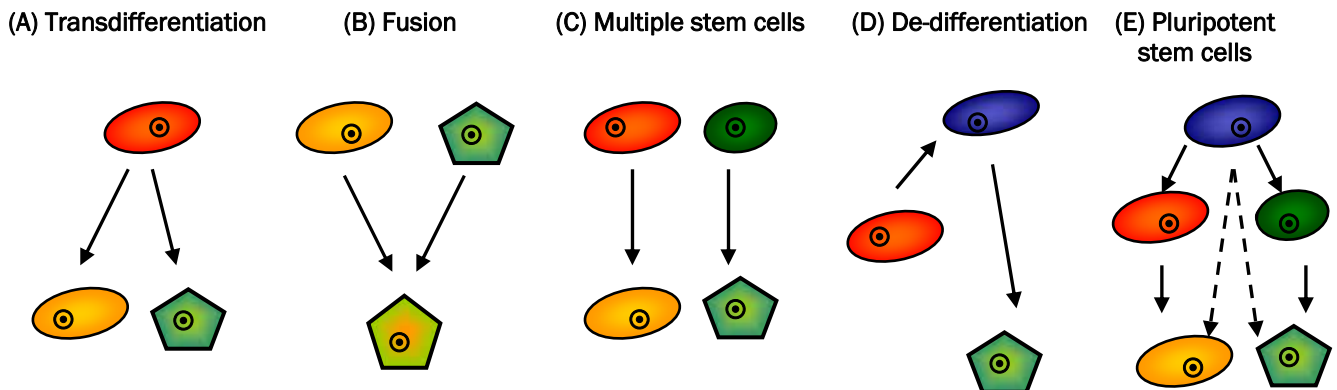
Transdifferentiation-studies have been contested by several research groups who have questioned the concept of plasticity since it defies developmental principles of lineage restriction being imparted during morphogenesis (Goodell, 2003; Hawley and Sobieski, 2002; Holden and Vogel, 2002; Lemischka, 2002; Verfaillie *et al.*, 2002). In addition, most studies have not shown that the apparent lineage deviation is derived from the same cell that differentiates into the expected cell type (Lakshmipathy and Verfaillie, 2005). With regards to myocardial repair strategies, while some models claim transdifferentiation of adult bone marrow cells results in functional repair (Leri *et al.*, 2005; Orlic *et al.*, 2001b), other studies have failed to demonstrate such effects (Murry *et al.*, 2004; Nygren *et al.*, 2004).

Nevertheless, the ability of ASCs to possibly adapt and change depending on external signals, could potentially add to tissue regeneration strategies once the concept of plasticity is better characterised. This illustrates the importance of understanding the micro-environmental effects on cell fate before any *in vivo* therapeutic SC applications can be applied.

Table 2.2. The ability of selected adult stem cells to change by processes of *differentiation* or *transdifferentiation*.

TISSUE OF ORIGIN	NEWLY FORMED TISSUE	REFERENCES (*et al.*)
Resident connective tissue cells Mesenchymal committed progenitors and pluripotent stem cells	skeletal muscle	Young 1995, 2001
Circulating bone marrow-derived ASC Unfractionated	brain	Mezey 2000; Brazelton 2000
	kidney	Poulsom 2001; Imasawa 2001
	skeletal muscle	Ferrari 1998
Bone marrow stromal cells or Mesenchymal stem cells	bone	Owen and Friedenstein 1988
	fat, haematopoietic stem or progenitor cells	Umezawa 1992
	skeletal, cardiac and smooth muscle, neovascularisation	Grounds 2002; Toma 2002; Devine 2003
	cartilage and tendon	Ashton 1980
	brain	Azizi 1998; Kopen 1999
	ectoderm- and mesoderm-derived tissue	Pittenger and Martin 2004
Haematopoietic stem cells	platelets, all lineages of mature blood cells	Morrison 1995; Kondo 2003
	liver	Petersen 1999; Theise 2000
	epithelium of lung, skin, kidney, GI-tract	Krause 2001; Kale 2003
	endothelial cells	Jackson 2001
	skeletal and cardiac muscle	Jackson 2001; Brazelton 2003
	brain, pancreas	Priller 2001; Ianus 2003
Endothelial progenitor cells	vasculogenesis	Asahara 1999; Murohara 2000
MAPC	brain, retina, lung, skeletal and cardiac muscle, liver, intestine, kidney, spleen, bone marrow, blood and skin	Reyes and Verfaillie 2001; Jiang 2002; Schwartz 2002
TCSC	skeletal and cardiac muscle, neural, epidermal and hepatic tissue	Ratajczak 2004
Bone marrow-derived satellite cells or Bone marrow side-population cells	skeletal and cardiac muscle	LaBarge and Blau, 2002; Dreyfus 2004
BMESL	ectodermal, endodermal and mesodermal lineages	Terada 2002
Skeletal muscle Satellite cells	skeletal muscle	Cornelison and Wold 1997
	fat, bone, cartilage	Asakura 2001; Wada 2002
Skeletal muscle side-population	skeletal and cardiac muscle	Murry 1996; Ghostine 2002
	blood	Gussoni 1999; Seale 2001
	fat, bone	Asakura 2002
Central nervous system Neural stem cells	neural progenitors	Palmer 2001
	skeletal and cardiac muscle, kidney, stomach, intestine and liver	Clarke 2000; Condorelli 2001
	blood	Bjornson 1999; Shih 2001
Liver Liver stem cells	hepatocyte progenitors	Semino 2003
	bile duct, pancreas, cardiac muscle	Petersen 1998; Malouf 2001
Adipose tissue Adipose progenitors	adipocytes	Zuk 2001
	pancreas, chondrogenic and osteogenic differentiation	Zuk 2001
	skeletal and cardiac muscle	Mizuno 2002; Di Rocco 2006
Vascular system Vascular endothelial stem cells; Mesangioblasts	blood vessels	Liu 2007
	skeletal and cardiac muscle	Condorelli 2001; Sampaolesi 2003
Skin Dermal stem cells (skin-derived precursors)	ectodermal progeny	Toma 2005
	bone, brain, fat, skeletal and smooth muscle	Toma 2001; Musina 2005
Dermal fibroblasts	skeletal muscle	Gibson 1995
Pancreas Pancreatic progenitors (pancrea-derived multipotent precursors)	pancreatic tissue	Seaberg 2004
	fat, brain, muscle, liver	Dabeva 1997

BMESL, bone marrow-derived embryonic stem-like cells; **MAPC**, multipotent adult progenitor cells;

TCSC, tissue-committed stem cells. Red font indicates *differentiation* of *multipotent* stem cells and blue font

postulates *transdifferentiation* of *pluripotent* stem cells.

ASCs can be isolated from various sources and as such be divided into several sub-populations. These are discussed below and summarised in Figure 2.3. It is important to consider, however, that within a tissue there may be micro-environments where closely related or identical cells express different markers, and also, that cells isolated directly from the tissue may differ in surface molecule expression after a period of being cultured *in vitro* (Pittenger and Martin, 2004).

2.1.3.1 Bone marrow-derived adult stem cells
2.1.3.1 (A) Mesenchymal stem cells

Bone marrow contains different ASCs, one of the most important populations being the *mesenchymal stem cells* (MSCs) that give rise to various mesodermal tissues. Despite being present as a very rare population (0.001% to 0.01% of the nucleated cells), MSCs can readily be grown in culture (Pittenger and Martin, 2004). These MSCs can also be isolated from stroma of the spleen and thymus, cartilage, trabecular bone, periosteum, synovial membrane and fluid, dermis, blood vessels, muscle, tendon, foetal lung, adipose tissue (Deans and Moseley, 2000; Zuk *et al.*, 2002) and cord blood (Bieback *et al.*, 2004). Under appropriate conditions, MSCs have multi-lineage differentiation potential and depending on the tissue in which they reside, they can be stimulated to differentiate into adipocytes, neural cells, myocytes, chondrocytes, hepatocytes, osteoblasts, marrow stromal cells, fibroblasts or tendon cells (Jiang *et al.*, 2002a; Tuan *et al.*, 2003), as well as skeletal and smooth muscle cells (Devine *et al.*, 2003) (Table 2.2). MSCs have shown potential for therapeutic use in the cardiovascular system where improved recovery has been observed following injection of MSCs either directly into the infarct, or via the intracoronary artery (Pittenger and Martin, 2004). A major advantage of these MSCs is the option of autologous usage and thereby full immune tolerance.

Sub-populations of MSCs have been characterised. *Marrow-derived stromal cells* also found in bone marrow should not be confused with MSCs, but rather be classified as an early differentiated progeny of MSCs (Tuan *et al.*, 2003). A population of rapidly dividing cells, termed *recycling stem cells*, has also been characterised as a sub-population, although only in culture.

2.1.3.1 (B) Haematopoietic stem cells

In addition to the bone marrow, *haematopoietic stem cells* (HSCs) can also be isolated from peripheral and cord blood (Broxmeyer *et al.*, 1989) and are stromal cells that can differentiate into all blood cell types, as well as megakaryocytes. Despite the successful use of these cells to treat haematopoietic disorders via autologous bone marrow or allogenic umbilical cord blood, they are unfortunately rare. HSCs may also differentiate into other major cell types from the endoderm, ectoderm and mesoderm (Table 2.2).

2.1.3.1 (C) Multipotent Adult Progenitor Cells

A further sub-population of bone marrow cells that has been described, is the *multipotent adult progenitor cells* (MAPC). These cells, isolated from postnatal bone marrow, can be expanded *in vitro* for extended periods, and differentiate into mesodermal, neuro-ectodermal and endodermal cells *in vitro* and into all embryonic lineages *in vivo* (Jiang *et al.*, 2002b; Reyes and Verfaillie, 2001). When injected into the early blastocyst, MAPC have shown to contribute to most somatic cell types. Despite their versatility, the long growth delay of MAPC in bone marrow cultures has suggested the possibility that these cells may represent a tissue culture-specific cell with no source *in vivo* (Passier and Mummery, 2003).

2.1.3.1 (D) Endothelial progenitor cells

Endothelial progenitor cells (EPC), which have also been identified in adult peripheral and umbilical cord blood, can be expanded for long periods *in vitro* and engraft into areas of injury where they contribute to postnatal vasculogenesis (Asahara *et al.*, 1999; Murohara *et al.*, 2000). There is evidence, at present only in mice, that a precursor for EPC, the hemangioblast, may exist in the bone marrow. These hemangioblasts have shown to give rise to HSCs, EPC and smooth muscle cells (Bailey and Fleming, 2003; Forrai and Robb, 2003; Pelosi *et al.*, 2002; Pelton *et al.*, 1991).

2.1.3.1 (E) Bone marrow side-population cells

Similar to the skeletal muscle side-population cells [*section 2.1.3.2 (B)*], are the bone marrow-derived multipotent SCs termed *bone marrow side-population cells* (bmSP), or *bone marrow-derived satellite cells*. This population, which can be incorporated into both skeletal and cardiac muscle, also contains HSCs (Gussoni *et al.*, 1999). Although it has been demonstrated that bmSP can contribute to both regenerating myofibers, as well as to the muscle satellite cell pool (Dreyfus *et al.*, 2004; LaBarge and Blau, 2002), this contribution seems to be below functional significance (Wernig *et al.*, 2005).

2.1.3.1 (F) Bone marrow-derived embryonic stem like cells

Co-culture of bone marrow cells with ESCs have produced colonies with an ESC-morphology (Terada *et al.*, 2002). These ASCs, which have been termed *bone marrow-derived embryonic stem-like cells* (BMESL), differentiate *in vitro* into endodermal-, ectodermal- and mesodermal-lineages.

2.1.3.1 (G) Tissue-committed stem cells

The bone marrow also contains sub-populations of non-haematopoietic cells capable of differentiating into neural, epidermal and hepatic tissue, as well as skeletal and cardiac muscle, termed *tissue-committed stem cells* (TCSCs) and perhaps even more primitive, *pluripotent stem cells* (PSCs) (Kucia *et al.*, 2005; Ratajczak *et al.*, 2004). These TCSCs and PSCs, when released from the bone marrow, circulate at low levels in the blood and accumulate in peripheral tissues under normal steady-state conditions to maintain a pool of SCs. Possibly, their circulating levels increase during periods of stress or tissue injury to allow them to take part in regeneration processes. *Cardiac TCSCs*, a sub-population of TCSCs expressing cardiac-specific markers, have recently been identified in both mice and humans (Kucia *et al.*, 2004). Taken together, due to their enhanced differentiation potential, the possibility exists that TCSCs can be expanded in culture to be utilised in multiple therapeutic applications (Dawn and Bolli, 2005a).

2.1.3.2 Skeletal muscle adult stem cells

Various SC populations which contribute to postnatal muscle growth, repair and regeneration, have been identified for *skeletal muscle*. Such stem and precursor cells include both resident muscle SCs, as well as non-muscle SCs.

2.1.3.2 (A) Satellite cells

These resident cells, located on the surface of the myofiber beneath the basal lamina, are capable of self-renewal and myogenic differentiation in response to physiological and pathological stimuli. *Satellite cells* are the main source of myoblasts for postnatal skeletal muscle regeneration (*section 2.2.1.1*).

2.1.3.2 (B) Muscle side-population cells

A population of multipotent ASCs, termed *muscle side-population* (mSP) *cells* isolated from skeletal muscle, has shown to commit to myogenic conversion *in vivo*, give rise to satellite cells, as well as reconstitute the haematopoietic system (Asakura *et al.*, 2002; Gussoni *et al.*, 1999).

A sub-type of mSP cells, Sk-34 cells, has been characterised as a population distinct from satellite cells, located in the interstitial spaces of skeletal muscle (Tamaki *et al.*, 2002). These Sk-34 cells are presumed myo-endothelial progenitor cells which possibly serve as a reservoir for satellite cells.

2.1.3.2 (C) Muscle-derived stem cells

Multipotential *muscle-derived stem cell*s (MDSCs) are highly proliferative, late adhering cells also with a high regenerative capacity which contribute to both the satellite cell pool and myonuclei, although only at a low frequency (Torrente *et al.*, 2001). Observations do however suggest that they are progenitors of satellite cells (Jankowski *et al.*, 2002; Qu-Petersen *et al.*, 2002). In addition, MDSCs represent a heterogeneous population and have shown to contain haematopoietic- (Asakura *et al.*, 2002), as well as neurogenic potential (Alessandri *et al.*, 2004).

2.1.3.2 (D) Somatic stem cells or self-renewing satellite cells

Somatic stem cells, also known as *self-renewing satellite cells* (SSC) (Baroffio *et al.*, 1996) are small, self-renewing myoblasts that do not divide or fuse unless they are induced to do so.

2.1.3.2 (E) Post-mitotic myonuclei

The efficient salvage of myonuclei from damaged myofibers could provide a large pool of nuclei for generation of new myoblasts during muscle repair. However, the extent to which such *post-mitotic myonuclei* within the sarcoplasm of damaged myofibers would contribute to the reversal of myonuclear fate, remains to be determined (Grounds *et al.*, 2002).

Non-muscle SCs which have demonstrated myogenic potential by means of potential transdifferentiation are indicated in Table 2.2 and include neural SCs, MSCs and various bone marrow-derived populations (Charge and Rudnicki, 2004; Grounds *et al.*, 2002). Such plasticity of SCs however still needs to be justified.

2.1.3.3   Cardiac muscle adult stem cells

Despite previous beliefs that the damaged myocardium can only be replaced by scar tissue, various cardiac stem and progenitor cell populations have been identified to show that potential for *in vivo* cardiac regeneration does exist. This has promoted a shift in paradigm of the heart from being a terminally differentiated, post-mitotic organ to one which is self-renewing (Anversa *et al.*, 2007).

The existence of *Lin⁻/c-kit⁺* (a known marker for HSCs), self-renewing, multipotent cells with SC properties have been reported in the myocardium. After *in vitro* treatment, these *early committed cells* (ECC) differentiate into cardiomyocytes, smooth muscle and endothelial cells (Beltrami *et al.*, 2003; Urbanek *et al.*, 2005), and when injected into an ischaemic heart, contribute to regeneration of the damaged myocardium (Dawn and Bolli, 2005b).

A small population of *adult heart-derived cardiac progenitor cells*, expressing the cell surface marker Sca-1⁺ (a cardiac and HSC maker), has also been isolated from the myocardium (postnatal mouse) (Oh *et al.*, 2004). Although these cells don't express cardiac structural genes or Nkx2.5, they have shown to differentiate *in vitro* into beating cardiomyocytes.

In both the embryonic and postnatal heart (from mouse, rat and human), another small population of *cardioblasts* has been identified on the basis of expressing a cardiac transcription factor, Isl1 (Laugwitz *et al.*, 2005). These myocardial-derived SCs can be isolated and transplanted into the damaged heart with evidence of functional improvement (Messina *et al.*, 2004).

Bone marrow-derived stromal cells with cardiac potential (Sca-1⁺) have been characterised which can give rise to cardiomyocytes after injection into the damaged myocardium (Bittner *et al.*, 1999; Jackson *et al.*, 2001). In addition, cardiomyocytes can also be formed from *bone marrow-derived HSCs, MSCs* and *endothelial SCs* (Jackson *et al.*, 2001; Toma *et al.*, 2002). Similar to skeletal muscle, the possibility of transdifferentiation of other non-resident SCs, such as neural and hepatocyte SCs into cardiomyocytes, can be debated (Table 2.2).

2.1.3.4   Cord blood-derived stem cells

Together with HSCs, MSCs and EPC, human cord blood contains an additional, essentially pluripotent SC population termed *unrestricted somatic stem cells* (USSCs) (Koblas *et al.*, 2005; Kogler *et al.*, 2004). *In vitro* cultures of these USSCs have shown differentiation into osteoblasts, chondroblasts, adipocytes, neural precursors and haematopoietic cells, whereas mesodermal and endodermal differentiation have been demonstrated *in vivo.*

A second small population, the *cord blood-derived embryonic-like* (CBE) *stem cells* has also been isolated from umbilical cord blood (McGuckin *et al.*, 2003; Zhao *et al.*, 2006). These cells display ESC characteristics such as a high potential for self-renewal and the expression of ESC-specific markers (e.g. Oct4 transcription factor). CBE SCs have shown *in vitro* differentiation into hepatocytes, haematopoietic and neuroglial progenitors (McGuckin *et al.*, 2004).

2.1.3.5 Very small embryonic-like stem cells

Similar to CBE SCs, a population of non-haematopoietic, *"very small embryonic-like"* (VSEL) *stem cells* has been characterised in murine bone marrow (Ratajczak *et al.*, 2006). These cells are rare, display features of primary ESCs (their nuclei are large, surrounded by a narrow rim of cytoplasm and contain open-type chromatin, all typical of ESCs) and immunohistochemical analysis revealed the presence of pluripotent SC markers (Kucia *et al.*, 2006a). It has been suggested that VSEL SCs are deposited into the bone marrow during stages of early development and could be a reserve population of embryonic-like, pluripotent SCs for tissue and organ regeneration. Their ability to differentiate and expand into cells from all three germ-cell layers when plated into cultures promoting tissue differentiation, potentially suggests a source for therapeutic intervention as an alternative to ESCs (Kucia *et al.*, 2006b).

2.1.3.6 Primitive embryonic-like adult stem cells or Blastomere-like stem cells

Scientists at a research company *(Moraga Biotechnology)* have recently discovered a very primitive SC in adult tissues with properties similar to that of ESCs. These primitive *"Embryonic-like Adult Stem Cells"* (EASCs) or *"Blastomere-Like Stem Cells"* (BLSCs) have shown to differentiate into most tissues and organs of the body, including spermatogonia. In contrast to most ASCs, these SCs normally reside in large numbers in peripheral blood and adult tissues, making them easy to isolate and purify for clinical use. Using very specific treatment conditions and reagents, the scientists were able to clone these ASCs into various cell-lines from a single cell. Without sufficient scientific data, these results remain to be confirmed and the cells' characteristics established.

2.1.3.7 Additional sources of adult stem cells

Various other populations of multipotent ASCs have been characterised, including *oval cells* (liver), *pancreatic SCs*, and cells isolated from the *central nervous system*, *intestine*, *lung*, and *skin*. Vessel-associated ASCs include *vascular-endothelial SCs* and multipotential *mesangioblasts* (Sampaolesi *et al.*, 2003). Fibroblastic MSCs isolated from *adipose tissue* can differentiate into mesenchymal lineages with similar characteristics and behaviour to bone marrow-derived MSCs and have been termed *adipose stromal*, *adipose progenitor* or *processed lipoaspirate cells* (Gronthos *et al.*, 2001; Zuk *et al.*, 2002). All these ASCs have shown the ability to regenerate cells from the tissue in which they reside, as well as in some instances, to potentially *transdifferentiate* into other cell-lineages following transplantation into the host-tissue (Passier and Mummery, 2003; Serafini and Verfaillie, 2006).

In addition to the *in vivo* ASC populations, more potent SC cultures have recently been developed *in vitro* (Serafini and Verfaillie, 2006). Multi- and pluripotent SC populations have been cultured from skin, bone marrow, muscle, umbilical cord blood and embryos. These purified SCs include *human bone marrow-derived multipotent stem cells* (hBMSCs) (Yoon *et al.*, 2005), *foetal somatic stem cells* (FSSCs) (Kues *et al.*, 2005), *marrow-isolated adult multilineage inducible* (MIAMI) *stem cells* (D'Ippolito *et al.*, 2004), and *skin-derived precursors* (SKP) (Toma *et al.*, 2001). These cells are all capable of differentiation into various cell types of different embryonic germ layers, and although they have been cultured by extensive manipulation and therefore might not exist *in vivo*, they could be of future use in clinical medicine.

Taken together, ASCs seem to possess a much greater capacity for differentiation than previously thought, and are directly influenced by the immediate environment and local signalling factors. This makes them good candidates for clinical transplantation. The various *in vitro* and *in vivo* sources of ASCs described above are summarised in Figure 2.3.

Figure 2.3. *In vivo* and *in vitro* sources of adult stem cells.

CORD BLOOD-DERIVED STEM CELLS
- Cord blood-derived embryonic-like stem cells
- Unrestricted somatic stem cells

SKELETAL MUSCLE
- Satellite cells
- Somatic stem cells *or* Self-renewing satellite cells
- Muscle-derived stem cells
- Post-mitotic nuclei
- Muscle side-population cells
  - Sk-34 cells

VASCULAR SYSTEM
- Vascular-endothelial stem cells
- Multipotential mesangioblasts

VERY SMALL EMBRYONIC-LIKE STEM CELLS

BONE MARROW
- Unfractionated population
- Haematopoietic stem cells
- Endothelial progenitors
- Pluripotent stem cells
- Bone marrow-derived satellite cells *or* Bone marrow side-population cells
- Multipotent adult progenitor cells
- Tissue-committed stem cells
  - Cardiac TCSC
- Bone marrow-derived embryonic stem-like cells
- Recycling stem cells
  - Marrow-derived stromal cells
- Mesenchymal stem cells

RESIDENT CONNECTIVE TISSUE
- Mesenchymal committed progenitors

CENTRAL NERVOUS SYSTEM
- Neural stem and progenitor cells

SKIN
- MSC *or* Dermal stem cells
- Dermal fibroblasts

ADIPOSE TISSUE
- Fibroblastic MSC
- Adipose stromal *or* progenitor cells

ADULT STEM CELL SOURCES

PANCREAS
- Pancreatic stem cells

LIVER
- Liver stem cells *or* Oval cells

CARDIAC MUSCLE
- Cardioblasts
- Multipotent cardiac stem cells *or* Early committed cells
- Adult heart-derived cardiac progenitor cells

OTHER ADULT STEM CELL SOURCES
- Lung
- Intestine

PRIMITIVE EMBRYONIC-LIKE ADULT STEM CELLS *or* BLASTOMERE-LIKE STEM CELLS

IN VITRO-DERIVED ADULT STEM CELL SOURCES
- Skin-derived precursors
- Foetal somatic stem cells
- Marrow-isolated adult multilineage inducible cells
- Human bone marrow-derived multipotent stem cells

2.2   MYOGENIC GROWTH, DIFFERENTIATION, REPAIR AND REGENERATION

It has always been assumed that both skeletal and cardiac muscle are composed of terminally differentiated myocytes, incapable of, or having very limited capacity for regeneration (Wollert and Drexler, 2005). As discussed in the above section, progress in the field of SC research has however confirmed the potential use of both embryonic and adult SCs in skeletal (Charge and Rudnicki, 2004; Ferrari *et al.*, 1998) and cardiac (Etzion *et al.*, 2001; Herreros *et al.*, 2003; Marin-Garcia *et al.*, 2006; Pittenger and Martin, 2004) repair strategies and regeneration.

Although various muscle precursor cells have been identified in skeletal muscle (*section 2.1.3.2*), regeneration has been found to be largely dependent on the satellite cell population (Hawke and Garry, 2001; Partridge, 2004). Similarly, in cardiac muscle (*section 2.1.3.3*), evidence have been presented that a fraction of resident cardiomyocytes may be activated to re-enter the cell cycle (Beltrami *et al.*, 2003; Urbanek *et al.*, 2003) and furthermore, that regeneration, even though limited, can occur through the recruitment of circulating extracardiac progenitor cells (Anversa and Nadal-Ginard, 2002; Jackson *et al.*, 2001; Kucia *et al.*, 2004; Orlic *et al.*, 2001c). Therefore, possible treatment options to restore muscle function during phases of regeneration, could include: **(A)** activation of local (stem) cells in the injured or diseased muscle; **(B)** recruitment and migration of endogenous myogenic precursor (stem) cells of a different cell-lineage; *or* **(C)** the exogenous application (cell grafting) of myoblasts or other myogenic precursor (stem) cells as a cellular transplantation mechanism.

2.2.1   Skeletal Muscle Regeneration and Repair in Disease and Injury

Postnatal muscle growth in terminally differentiated adult skeletal muscle fibers is made possible by various sources of skeletal muscle precursor cells, which include: **(A)** satellite cells, the population of reserve stem cell-like cells on the surface of mature myofibers and primary SC source in adult skeletal muscle; **(B)** other local myogenic precursor cells (mpcs) within the myofiber; **(C)** "post-mitotic" myonuclei within the sarcoplasm of damaged myofibers that may re-enter the cell cycle; *and* **(D)** cell types originating *beyond* the muscle. These sources of skeletal muscle stem and precursor cells are outlined in Figure 2.4.

Figure 2.4. Sources of skeletal muscle stem and progenitor cells.

VASCULAR SYSTEM

Mesangioblasts

Circulating bone marrow-derived stem cells

Vascular endothelial stem cells

AC133+

BLASTOCYST

Embryonic stem cells

IN VITRO-DIFFERENTIATION

Skeletal muscle, HSC. MSC. etc......

SKIN

Dermal fibroblasts

Dermal stem cells *or* Skin-derived progenitors

CENTRAL NERVOUS SYSTEM

Neural progenitors

LOCAL MUSCLE PROGENITOR CELLS

Satellite cells

Muscle-derived stem cells

Post-mitotic nuclei

Muscle side-population cells

Sk-34

Somatic stem cells *or* Self-renewing satellite cells

INTERSTITIAL CONNECTIVE TISSUE

Mesenchymal committed progenitors

ADIPOSE TISSUE

Adipose stromal *or* progenitor cells

Adipose tissue-derived MSC *or* Fibroblastic MSC

BONE MARROW

Tissue-committed stem cells

Multipotent adult progenitor cells

Haematopoietic stem cells

Bone marrow-derived satellite cells *or* Bone marrow side-population cells

Bone marrow stromal cells *or* MSC

2.2.1.1   Contribution of satellite cells to skeletal muscle repair and regeneration

Satellite cells supply myonuclei to growing myofibers before becoming mitotically quiescent in the muscle as it matures. They are then activated from this quiescent state when influenced by extrinsic signals such as exercise, muscle damage, or disease to function in routine maintenance, hypertrophy and repair of adult muscle (Grounds *et al.*, 2002; Sabourin *et al.*, 1999).

2.2.1.1 (A) Satellite cell origin and identification

During embryogenesis, the paraxial mesoderm gives rise to the somite from which all skeletal musculature is generated (Hawke and Garry, 2001; Mauro, 1961; Ordahl, 1999; Schultz and McCormick, 1994). Mononuclear satellite cells were already identified in 1961 (Mauro, 1961) based on their distinct location at the periphery of multinucleated myofibers, between the basal lamina and sarcolemma (Ordahl, 1999). Two paradigms exist for the developmental origin of satellite cells. Initial studies hypothesised that all muscle precursors, including the satellite cell population, originate from multipotential mesodermal cells of the somite (Schultz and McCormick, 1994). A more recent hypothesis suggests that satellite cells may be derived from endothelial cells or a precursor common to both satellite and endothelial cells (De Angelis *et al.*, 1999; Ordahl, 1999). Given the differences in myogenic programs between muscle of different embryological origin, it is possible that satellite cells in different muscle groups have different embryological origins themselves (Tajbakhsh, 2005) and display functional and phenotypic heterogeneity (Zammit *et al.*, 2006a).

Satellite cells are produced at ~day 17 of development and make up 2-7% of the nuclear fraction of a myofiber (Cossu *et al.*, 1985). This *proportion* varies with age (reduces with age, as well as in diseased muscle), species, muscle group and fiber-type, based on which they can be divided into further sub-classes (Rosenblatt *et al.*, 1996; Schultz and McCormick, 1994). The *number* of satellite cells, however, remains relatively constant due to their ability to self-renew following repeated bouts of muscle damage which would otherwise deplete the satellite cell-reserve. One postulated mechanism for such self-renewal involves asymmetric cell division where a daughter cell differentiates, while the other continues to proliferate, or else returns to quiescence (Moss and Leblond, 1971). A further mechanism is dependent on expression of specific myogenic regulatory (also transcription) factors and involves possible de-differentiation of committed mpcs (Seale and Rudnicki, 2000). Following activation, satellite cells can therefore adopt different fates depending on the micro-environmental conditions and either continue proliferation, initiate a programme of differentiation, or return to quiescence (Zammit *et al.*, 2004).

Although these cells have long been regarded as being monopotential and only able to give rise to cells of the myogenic lineage (Bischoff and Heintz, 1994), evidence suggests that satellite cells represent a multipotential SC population (Asakura *et al.*, 2001; Price *et al.*, 2007; Smith *et al.*, 1994; Wada *et al.*, 2002). Satellite cells activated in skeletal muscle could therefore give rise to myoblasts and continue differentiation into mature myofibers, or, depending on a different micro-environment and the circulating growth factors, contribute to non-muscle lineages (Gussoni *et al.*, 1999; Seale *et al.*, 2001). Satellite cells therefore fulfil the basic SC definition in that they are maintained by self-renewal and can give rise to a differentiated cell type.

The difficult morphological identification of satellite cells has resulted in the use of molecular markers to characterise these cells at various stages of commitment (outlined in *Chapter 4, Table 4.2*). None of these markers are however *absolute* to *quiescent, activated* or *proliferating* satellite cell stages and their identification is further complicated by the expression of these molecular markers also in other cell types that may be present in the muscle tissue. The majority of *quiescent* satellite cells express *M-cadherin*, the tyrosine-kinase receptor for hepatocyte growth factor (HGF), *c-met*, *Pax-3*, *Pax-7* and/or *CD34*, which is also an established marker of HSCs (Beauchamp *et al.*, 2000; Charge and Rudnicki, 2004; Grounds *et al.*, 2002). Schultz (1996) demonstrated that M-cadherin[+]-cells comprised only a small fraction (~20%) of satellite cells at a time when all cells were c-met[+] and suggested that these M-cadherin[+]/c-met^{+} cells represent a sub-population of quiescent satellite cells, possibly able to differentiate quickly upon stimulation. Other established satellite cell markers include *syndecan-3*, *syndecan-4* and *myostatin*, while more recent markers reported to identify *quiescent* and *activated* satellite cells include *lysenin*, a sphingomyelin-specific binding protein (Nagata *et al.*, 2006) and *caveolin-1*, a structural protein-component of caveolar-membrane domains and a novel regulator of satellite cell functions (Volonte *et al.*, 2005).

2.2.1.1 (B) Mechanisms of satellite cell activation

All processes of muscle growth, regeneration or adaptation to training require satellite cells to become activated, proliferate, form myoblasts and differentiate, finally fusing with existing myofibers or with other myoblasts to form multinucleated myotubes which then undergo terminal differentiation to develop into mature, functional muscle fibers (Grounds *et al.*, 2002; Seale and Rudnicki, 2000). The exact molecular mechanisms that regulate these processes, specifically activation and entry into the cell cycle, remain to be clarified. Satellite cell activation may result from the ligation of integrin molecules, such as VLA-4 on infiltrating

leukocytes with VCAM-1 on the resident satellite cells (Jesse *et al.*, 1998; Rosen *et al.*, 1992), which would then initiate genetic responses within satellite and immune cells to promote regeneration processes. Furthermore, damage to the basal lamina and extracellular matrix of myofibers may result in the release of HGF, a potent mitogen and chemotactic agent. It is postulated that HGF activates satellite cells through its associated receptor c-met which is predominantly expressed in quiescent satellite cells (Allen *et al.*, 1995).

Furthermore, chemotaxins are released from the damaged cells resulting in an inflammatory response. Lymphocytes and macrophages migrate to the area of tissue damage where the macrophages, the dominant immune cell, function to remove cell debris and secrete mitogenic growth factors, including the cytokines interleukin (IL)-6 and leukaemia inhibitory factor (LIF) which subsequently results in the stimulation of mpc proliferation (Cantini *et al.*, 1994; Merly *et al.*, 1999). Other growth factors involved in the expansion of the mpc-compartment include HGF, fibroblast growth factor (FGF) and insulin-like growth factor-I (IGF-1). IGF-1 has also shown to stimulate muscle hypertrophy simultaneously with satellite cell activation (Adams and McCue, 1998).

The activation of satellite cells from their state of quiescence and subsequent progression along the myogenic lineage are controlled by various transcription factors, the most important being the myogenic regulatory factors (MRFs) MyoD, Myf-5, myogenin and MRF4.

2.2.1.1 (C) Myogenic regulatory factors in satellite cell activation and differentiation
Myogenic differentiation involves the withdrawal of myoblasts from the cell cycle, induction of muscle-specific gene expression and formation of myotubes following fusion of myoblasts. These processes are controlled by a family of *basic* helix-loop-helix transcription factors which have proven to be essential in the determination and differentiation of mpcs into mature skeletal muscle (Rudnicki and Jaenisch, 1995; Weintraub *et al.*, 1991). These MRFs, which include MyoD, Myf-5, myogenin and MRF4 (also known as Myf-6 or herculin) (Dias *et al.*, 1994) are expressed exclusively in skeletal muscle and function by activating muscle-specific genes in response to extracellular growth factors to initiate myogenic differentiation. Whereas Myf-5 and MyoD are known as the primary MRFs, required for determination of myoblasts into the myogenic lineage, myogenin and MRF4 are the two secondary MRFs and function to regulate terminal differentiation (Arnold and Winter, 1998; Cooper *et al.*, 1999).

In established muscle cell-lines and during development, only subsets of the MRFs are active rather than all being expressed simultaneously. Once activated, satellite cells will first express either Myf-5, one of the earliest markers of myogenic commitment in dormant satellite cells (Beauchamp *et al.*, 2000; Grounds *et al.*, 1992; Megeney *et al.*, 1996) and/or MyoD, at which time daughter mpcs are generated, with subsequent transcription of *both* these genes. Nuclei *only* expressing Myf-5 are limited, although despite this early down-regulation of Myf-5, both MyoD and Myf-5 can still be detected during the early stages of myotube differentiation (Cornelison and Wold, 1997; Thayer *et al.*, 1989).

Following this proliferative burst, MRF4 and myogenin are exclusively expressed in cells entering their differentiation programme (Hollenberg *et al.*, 1993; Megeney and Rudnicki, 1995). These MRFs are required for mpcs to progress through stages of myogenic commitment, the formation of myotubes and subsequent terminal differentiation and fusion to form mature post-mitotic, multinucleated myofibers. Only myogenin is expressed during phases of early differentiation (Cornelison and Wold, 1997; Vivian *et al.*, 2000). To complete the terminal differentiation programme, muscle-specific proteins, including myosin heavy chain, are activated.

Studies analysing muscle regeneration in mice deficient of specific MRFs have revealed the essential role which these factors have on successful satellite cell progression through the process of myogenic differentiation. Myf-5 and MyoD deficient mice have neither differentiated skeletal muscle, nor a mpc population (Rudnicki *et al.*, 1993). Although the myoblasts of mice lacking MyoD grow more quickly, they show reduced numbers of proliferating mpcs, reduced fusion of myoblasts, a reduction in the number of generated myotubes, and therefore inefficient differentiation (Megeney *et al.*, 1996; Miller, 1990; Sabourin *et al.*, 1999). They do however show an increased number of satellite cells because of the greater tendency of these cells to self-renew rather than progressing further through the developmental programme (Megeney *et al.*, 1996). Conversely, Myf-5-null myoblasts proliferate poorly and differentiate inadequately (Montarras *et al.*, 2000). Results therefore suggest that Myf-5 functions toward myoblast self-renewal and proliferation, whereas MyoD promotes satellite cell progression to terminal differentiation (Charge and Rudnicki, 2004; Ishibashi *et al.*, 2005; Sabourin *et al.*, 1999; Seale *et al.*, 2001). The importance of MyoD as an essential regulator primarily responsible for inducing myogenic differentiation has been demonstrated in various studies: MyoD has the ability to activate muscle-specific genes in a variety of differentiated cell-lines, suggesting that no additional muscle-specific factors are needed to activate terminal muscle differentiation; MyoD-deficient myoblasts are incapable of

successfully differentiating into myotubes and have reduced regenerative capacity both *in vitro* and *in vivo*; *and* cells positive for differentiation markers express MyoD, but not Myf-5 (Cornelison *et al.*, 2000; Kitzmann *et al.*, 1998; Megeney *et al.*, 1996; Weintraub *et al.*, 1989).

Myogenin-deficient mice are capable of generating normal numbers of myoblasts, but these populations are arrested in their terminal differentiation programme, resulting in highly reduced myofiber formation (Arnold and Braun, 1996). Severe defects in embryonic muscle development of myogenin-knock-out mice have however prevented further study of the exact role of this MRF, as well as MRF4, in muscle regeneration since these mice usually die shortly after birth. Mice generated with a mutated myogenin gene are born immobile with severely reduced skeletal muscle mass (Hasty *et al.*, 1993; Nabeshima *et al.*, 1993). Since normal numbers of myoblasts are still produced in these mice, results from these studies indicate the importance of myogenin for initiating terminal differentiation, rather than for the commitment of cells to the myogenic lineage.

Additionally, Pax3 and Pax7, members of the paired-box transcription factor family, have also proven to play an essential role in muscle regeneration. Whereas Pax3 is required for the migration of muscle precursors from the somite during development (Tajbakhsh *et al.*, 1997), Pax7, which functions upstream of MyoD, is required for satellite cell specification (Seale *et al.*, 2000). Although its exact role in activation and regeneration has not yet been fully characterised, it has been suggested that Pax7 may be involved in maintaining proliferation and prevent selected differentiation, although it does not promote quiescence (Zammit *et al.*, 2006b). The unique requirement for this transcription factor is indicated in embryonic and foetal myogenesis which is largely not affected in Pax- mice, in contrast to postnatal muscle growth which is severely impaired (Seale *et al.*, 2000).

2.2.1.1 (D) Responses of satellite cells to physiological stimuli and disease

Understanding the functional responses of satellite cells to physiological stimuli and diseased conditions could contribute to the improved use of these cells in the development of therapeutic strategies.

Satellite cells are stimulated in response to *hypertrophic stimuli*, such as resistance training, to participate in proliferative and repair processes following exercise-induced myotrauma so that the nett result of the stimulus is greater force production (Nathan, 1987).

The initial response to *atrophic stimuli*, such as decreased muscle activity (e.g. caused by denervation of the muscle, malnutrition or muscle disuse), is an increase in satellite cell number. However, a prolonged period of inactivity will ultimately result in a significant decrease in satellite cell number, possibly due to satellite cell apoptosis or the lack of neurotrophic and growth factor input which negatively influences satellite cell function and content (McGeachie, 1989; Viguie *et al.*, 1997).

In *pathological conditions*, such as congenital myopathies and diseases causing muscle atrophy, satellite cell numbers and proliferation may decrease (Jejurikar and Kuzon, 2003) in an effort to restore muscle function: repeated cycles of muscle regeneration are brought on by repeated loss of differentiated tissue, and as such, failure to maintain muscle homeostasis (Luz *et al.*, 2002).

Aging results in a reduced capacity of skeletal muscle to regenerate following injury or disease (Grounds, 1998) which can be explained by a decline in satellite cell numbers, impairment of their intrinsic regenerative potential (Conboy and Rando, 2005; Mouly *et al.*, 2005), as well as effects of the aged immediate environment on satellite cell function. Such influences include reduced neural activation (Carlson and Faulkner, 1996), a declining systemic environment, increased fibrosis within the skeletal muscle (Marshall *et al.*, 1989), reduced vascularisation (Coggan *et al.*, 1992), and decreased levels of inflammatory and growth factors which are required for efficient immune responses (Danon *et al.*, 1989).

These physiological responses suggest that the self-renewal capacity of satellite cells is limited. Determining therapeutic applications which could negate these responses and enhance the muscle's regenerative potential are therefore required for the successful treatment of tissue injury and degenerative diseases.

2.2.1.2   Contribution of other stem cells to skeletal muscle repair and regeneration

Since its identification, the satellite cell has been presumed to be the only source of myonuclei in skeletal muscle repair. In regenerating muscle, however, the number of myogenic precursors exceeds that of resident satellite cells, implying migration and/or recruitment of undifferentiated progenitors from other sources (Ferrari *et al.*, 1998). As indicated in *sections 2.1.3.1* and *2.1.3.2*, multipotential SCs in various adult tissues have been characterised which have demonstrated potential to contribute to myogenic repair and regeneration (Figure 2.4).

2.2.1.2 (A) Muscle resident stem cells

Skeletal muscle contains a *side-population* (mSP) similar to that found in bone marrow, possibly derived from MSCs, migratory bone marrow cells or from the vasculature (Zammit and Beauchamp, 2001). *MDSCs* have shown multipotential capacity and myogenic conversion both *in vivo* and *in vitro* (Qu-Petersen *et al.*, 2002). Other muscle resident SCs include *post-mitotic nuclei*, *SSC* and *Pax3*^{+}*-cells* which have recently been identified in the interstitial compartment of the muscle (Kuang *et al.*, 2006). It has been suggested that these Pax3^{+} interstitial cells represent a novel myogenic population that is distinct from the satellite cell-lineage.

2.2.1.2 (B) Non-muscle resident stem cells

Transplanted progenitor cells isolated from the *bone marrow* have proven to contribute to muscle regeneration, although only to a limited extent (Bittner *et al.*, 1999; Ferrari *et al.*, 1998; Gussoni *et al.*, 2002; LaBarge and Blau, 2002). The possibility remains that under certain micro-environmental conditions or treatment with appropriate growth factors, the frequency of conversion can be increased. In addition, *bmSP* cells have also shown potential to give rise to skeletal muscle (Hawke and Garry, 2001; Zammit and Beauchamp, 2001), as well as contribute to the satellite cell pool (LaBarge and Blau, 2002). Whereas *neural* (Clarke *et al.*, 2000; Galli *et al.*, 2000) and *mesenchymal* (Young *et al.*, 2001) *progenitor cells* have shown differentiation potential into muscle cells *in vitro*, *adipose tissue-derived MSCs* can be directed towards a myogenic phenotype both *in vitro* and *in vivo* (Di Rocco *et al.*, 2006).

Research therefore confirm the capacity various ASC sources to contribute to myogenic differentiation. However, it remains to be determined to what extent these cells can contribute to *functional* regeneration and incorporation into regenerating musculature *in vivo*.

2.2.1.3 Stem cell applications to improve skeletal muscle repair and regeneration
2.2.1.3 (A) Transplantation of satellite cell-derived myoblasts

Primary myoblasts have been the principle source of muscle progenitors for cell-based therapies. The application of *myoblast transfer therapy* (MTT) has been used where skeletal myoblasts have been isolated, expanded *in vitro* and transplanted into the muscle, usually via intramuscular injection, to replace defective genes and cells of various myopathies. Successful MTT requires survival of the injected donor myoblasts in the host-environment and although this method is advantageous in that muscle biopsies are easily obtainable, a concern when applying MTT is overcoming the *in vitro-induced* immune problems (Morgan *et al.*, 1996; Watt *et al.*, 1982). Results from MTT have shown that there is a rapid rate of necrosis of the injected cultured myoblasts which can be as high as 90% (Beauchamp *et al.*, 1999; Smythe *et al.*, 2001). This massive myoblast death could be the result of tissue culture conditions affecting the cells to such an extent that when transferred to *in vivo* conditions, it elicits an acute adverse host-immune response. For consideration as a viable treatment option, enhanced myoblast-contribution, capable of multiple rounds of regeneration, and functional incorporation throughout the musculature therefore needs to be improved.

2.2.1.3 (B) Satellite cell transplantation

The ability to directly isolate a pure satellite cell population was only recently achieved (Montarras *et al.*, 2005). When injected into dystrophic muscle of mice, these cells have shown to restore dystrophin expression and contribute to the satellite cell-compartment. The use of a pure satellite cell population requires a smaller number of cells to obtain similar levels of regeneration when compared to the use of donor cells isolated from whole muscle. Limiting to this approach however is that *in vitro* cultivation of isolated satellite cells significantly reduces their *in vivo* myogenic regeneration potential (Price *et al.*, 2007). Changing the host environment to be more conducive to donor satellite cell migration could therefore improve the effectiveness of this transplantation strategy (Smythe *et al.*, 2001).

2.2.1.3 (C) Single muscle fiber

Resident satellite cells in whole muscle have shown the ability to initiate regeneration and contribute to the satellite cell compartment following transplantation into a new host (Hansen-Smith and Carlson, 1979; Roberts *et al.*, 1989). These transplanted satellite cells appear to migrate throughout the muscle in which the myofibers were implanted. Transplantation of donor *whole muscle grafts* into host muscle has also proven to be successful without adverse immune responses as an alternative approach to cell transplantation, also without the need for prior exposure to tissue culture conditions (Fan *et al.*, 1996a; Smythe *et al.*, 2000).

2.2.1.3 (D) Application of adult stem cells

In addition to satellite cells, as indicated in *section 2.2.1.2* (Figure 2.4), other resident and non-resident muscle SC sources have been identified which have shown myogenic potential, although only to a variable degree. In contrast to satellite cells and primary myoblasts, mSP cells and MDSCs are able to migrate from the vasculature into the muscle, a desirable feature for a therapeutic cell type, as is their multipotential nature.

Taken together, identifying the mechanisms responsible for satellite and adult stem cell activation and differentiation, maintenance in their quiescent state and self-renewal, are required to establish optimal satellite and stem cell-based therapies. In addition, for transplantation therapies to be successful, it would be required to determine greater levels of transplanted cell integration into the host, together with methods for long-term regeneration.

2.2.2 Cardiac Muscle Regeneration and Repair in Disease and Injury

Unlike skeletal muscle where injury can be repaired by the proliferation, differentiation and fusion of satellite cells and other mpcs, the response of cardiac tissue to ischaemic injury is predominantly scar formation (Sun *et al.*, 2000). To restore the structural integrity of the myocardium post-injury, myofibroblasts appear in the wounded area which originate from either interstitial fibroblasts, or from mesenchymal progenitor cells such as fibrocytes (Bucala *et al.*, 1994). Collagen (specifically type I and type III) is laid down by these myofibroblasts, accumulating in the wound and remodels the tissue to form a mature infarct scar. The problem facing individuals following myocardial infarction is that the scar does not function as normal contractile tissue, and as a result remodelling of non-injured tissue occurs, leading to a further reduction in cardiac output and ultimately heart failure. As previously outlined (*section 2.1.3.3*), there is however evidence for *in vivo* proliferation of cardiomyocytes after damage, either from pre-existing mature cardiomyocytes, or from resident cardiac SCs. Together with the recruitment of endogenous, extracardiac progenitor (stem) cells and cellular transplantation mechanisms, these local cardiac SCs provide potential for cardiac regeneration and repair strategies (Figure 2.5).

Figure 2.5. Sources of cardiac muscle stem and progenitor cells.

VASCULAR SYSTEM

Vascular endothelial stem cells

Mesangioblasts

SKELETAL MUSCLE

Skeletal myoblasts

Muscle side-population cells

CENTRAL NERVOUS SYSTEM

Neural progenitors

BLASTOCYST

Embryonic stem cells

LIVER

Hepatic stem cells

CARDIAC MUSCLE

Early committed cells *or* Multipotent cardiac stem cells

Adult heart-derived cardiac progenitor cells

Cardioblasts

ADIPOSE TISSUE

Adipose stromal *or* progenitor cells

Adipose tissue-derived MSC *or* Fibroblastic MSC

*IN VITRO-*DIFFERENTIATION

Skeletal muscle, HSC, MSC, etc......

BONE MARROW

Tissue-committed stem cells

Haematopoietic stem cells

Multipotent adult progenitor cells

Bone marrow side-population cells

Bone marrow-derived stromal cells *or* Bone marrow-derived MSC

VERY SMALL EMBRYONIC-LIKE STEM CELLS

PRIMITIVE EMBRYONIC-LIKE ADULT STEM CELLS *or* BLASTOMERE-LIKE STEM CELLS

31.

2.2.2.1   Contribution of resident stem cells in cardiac repair and regeneration

Growth of the heart during initial stages of embryonic and foetal development is generally characterised by cell-division, after which it enters a post-mitotic state. Further growth during normal development or in diseased conditions is achieved by means of the enlargement of cardiomyocytes (hypertrophy) rather than proliferation (hyperplasia).

Evidence of resident cardiac SCs has however revealed the presence of *ECC* or *multipotent cardiac SCs* capable of differentiating into cardiomyocyte or vasculature lineages (Beltrami *et al.*, 2003; Messina *et al.*, 2004). The presence of *primitive cardiac SCs* has also been demonstrated from evidence of myocyte hyperplasia which has shown to contribute to cardiac growth (Kajstura *et al.*, 1998; Urbanek *et al.*, 2003). Additionally, the use of cell proliferation markers such as Ki67 and PCNA has made it possible to identify replicating myocytes within the myocardium which supports the notion that a population of adult cardiac SCs can re-enter the cell cycle as a source for tissue self-renewal (Anversa and Nadal-Ginard, 2002). Two small populations, *adult heart-derived cardiac progenitor cells* (Oh *et al.*, 2004) and *cardioblasts* (Laugwitz *et al.*, 2005), have also been identified in the myocardium. Recently, a pool of *embryonic late plate mesoderm progenitor cells* were identified which yield both myocardial and endocardial cells during normal cardiac development (Ott *et al.*, 2007). These cells have shown the potential to differentiate both *in vivo* and *in vitro* into smooth muscle, endothelial- and cardiomyocyte lineages. It needs to be clarified whether these progenitor cells are related or form distinct myocardial cell populations.

2.2.2.1 (A) Cardiac-specific transcription factors

Similar to skeletal muscle, the expression of cardiac gene products occurs in a controlled programme during myocardial development. During early embryogenesis, the expression of cardiac-specific transcription factors GATA-4, Nkx2.5 and members of the myocyte enhancer family (MEF-2C) precedes and mediates the expression of markers for early, intermediate and terminal cardiac cell differentiation, including atrial natriuretic factor (ANF), myosin light chain, α- and β-myosin heavy chain and cardiac troponin-C. GATA-4, one of the first cardiac transcription factors, plays a key role in cardiomyocyte differentiation. Blocking GATA-4 transcription eliminates the formation of beating cardiac muscle cells, prevents the transcription of cardiac-specific markers and reduces levels of MEF-2C and Nkx2.5 (Marin-Garcia *et al.*, 2003). Nkx2.5 is expressed in cardiac progenitor cells during early development, acts downstream of GATA-4 and possibly plays a role in late cardiac differentiation events. MEF-2C likely acts downstream of both GATA-4 and Nkx2.5.

Despite being expressed at different stages of myocardial development, the transcription factors works in combination, also with other transcription factors, to activate the promoters of several critical genes involved in cardiac differentiation and postnatal development. The mechanisms by which these transcription factors are regulated, however, remain to be determined.

2.2.2.1 (B) Signalling pathways regulating growth in adult cardiomyocytes
Cardiomyocytes of the adult myocardium increase their cellular volume in response to various growth stimuli, including growth hormones, neuro-endocrine factors and increases in mechanical load (Schluter and Piper, 1999). Various signalling pathways have been characterised as important transducers of the growth response, including activation of G-protein-mediated pathways with further downstream signalling by protein kinase-C (PKC), mitogen-activated protein kinase (MAPK) and PI3-kinase. Growth factor-receptor pathways, such as that of transforming growth factor-β (TGF-β), have also been implicated as a potential hypertrophic transducer of cardiac signalling (Molkentin and Dorn, 2001). Downstream of TGF-β-receptor activation, intracellular signalling involves TGF-β-activated kinase (TAK1) which also regulates MAPK-kinase, leading to JNK and/or p38 signalling. Importantly, these signalling pathways, in addition to others, operate together to co-ordinate the relevant response (Solloway and Harvey, 2003).

2.2.2.2   Contribution of extracardiac stem cells in cardiac repair and regeneration
The generation of cardiomyocytes from various extracardiac ASC sources has received much attention as an alternative option to increase cell delivery to the damaged myocardium (Figure 2.5) and has been discussed above (*section 2.1.3*). Such ASC sources include various *bone marrow-derived-, endothelial-* and *skeletal stem and progenitor cells*. Also, the possibility of *transdifferentiation* of other non-resident SCs into cardiomyocytes, including *neural-, adipose-* or *hepatic* stem and/or progenitor cells, can be debated (Table 2.2).

2.2.2.3   Stem cell applications to improve cardiac muscle repair and regeneration
Experimental and clinical studies have focussed on three main approaches to improve cardiac repair and regeneration using SCs. Essentially, these include: **(A)** identification and activation of *resident* cardiac (stem) cells; **(B)** improvement in the recruitment, mobilisation and migration of *endogenous* SCs residing either in the bone marrow, circulation, or other extracardiac SC niches; *and* **(C)** cellular transplantation.

2.2.2.3 (A) Activation of resident cardiomyocytes

Studies from Quaini *et al.* (2002) and Laflamme *et al.* (2002) were the first to describe the presence of progenitor cells in the myocardium, although with large discrepancies regarding the number of cardiomyocytes (0.04%-18%). It has now been established that the heart contains different resident (stem) cell populations which can reconstitute the myocardium (*section 2.2.2.1*). Although these cells show variability in their capacities to contribute to cardiac regeneration, they are intrinsically programmed to generate cardiac tissue and therefore SC therapy directed to activate these resident SCs could prove to be beneficial in the treatment of heart disease (Barile *et al.*, 2007; Laflamme *et al.*, 2002; Quaini *et al.*, 2002).

2.2.2.3 (B) Recruitment of extracardiac progenitor cells

An initial study by Bittner *et al.* (1999) demonstrated that healthy *bone marrow cells* transplanted into dystrophic muscle, were found in the heart, indicating that these cells could migrate to the injured myocardium and differentiate into cardiac tissue. Subsequently, various bone marrow cell populations have shown the ability to repair the infarct heart, improving both function and survival of adult mice (Goodell *et al.*, 2001; Jackson *et al.*, 2001; Orlic *et al.*, 2001c; Toma *et al.*, 2002). The use of bone marrow-derived cells has been preferred mainly due to their autologous origin and potential for cardiomyocyte/endothelial *transdifferentiation* in response to the necessary environmental factors (Menasche, 2003). In these studies, cardiomyocytes were formed *in vivo* from circulating bone marrow-derived MSCs, HSCs or endothelial cells following engraftment of the transplanted cells into the irradiated bone marrow of the animal. It needs to be clarified whether transplanted endothelial cells improve cardiac function by means of these cells' contribution to improved vasculature-, rather than cardiomyocyte regeneration (Kocher *et al.*, 2001). For SCs recruited from distant niches, however, it is possible that the presence of myofibroblasts and the deposition of fibrous tissue post-infarct could prevent **(A)** these SCs from moving into the injured myocardium, *and* **(B)** the formation of new blood vessels in the scar tissue.

As alternative sources to bone marrow-derived SCs, a *hepatocyte* stem cell-line (Malouf *et al.*, 2001) and SCs from *neural* tissue (Clarke *et al.*, 2000) have shown to differentiate into cardiomyocytes *in vivo*, whereas a SC population within lipoaspirates has demonstrated *in vitro* myogenic potential (Zuk *et al.*, 2001). In addition to ASC sources, embryonic, neonatal and endothelial cells isolated from human umbilical veins have also shown good conversion potential into cardiomyocytes both in tissue co-culture and *in vivo* (Condorelli *et al.*, 2001).

2.2.2.3 (C) Transplantation of skeletal myocytes or alternative progenitor cells

In a further effort to replace cardiomyocytes lost after ischaemia, cellular transplantation has been investigated as a potential therapy. This approach, termed cellular cardiomyoplasty (Suzuki *et al.*, 2002) or myogenic cell grafting, involves the extraction of donor stem or precursor cells from a selected *in vivo* source, after which the cells are expanded in culture and then either injected into the myocardium bordering the infarct, or delivered via the circulation (intracoronary or intravenous). During intra-coronary delivery, cells must be delivered slowly to prevent cell clumping or embolism (Forrester *et al.*, 2003). In addition to the method of delivery, is the importance of timing, site of delivery, number and population of cells, and optimisation of cell survival, essential to ensure maximal engraftment. When ASCs or progenitor cells are transplanted into the myocardium, it is also essential that they encounter a suitable environment which will promote their appropriate differentiation and discourage any uncontrolled proliferation.

The selection of appropriate candidates for cardiac repair by means of cellular transplantation is essential as distinct stem or progenitor cells may respond differently to the post-infarct environment that prevails. Preferably, autologous SCs are selected to prevent problems of immune rejection. For transplanted cells to improve cardiac function, they must also feature contractile properties, therefore, although some positive data has been reported for transplantation of *fibroblasts* (Hutcheson *et al.,* 2000), *smooth muscle cells* (Sakai *et al.,* 1999), *endothelial cells* (Kim *et al.*, 2001) and *mesenchymal stem and progenitor cells* (Fukuda, 2001; Toma *et al.*, 2002), the best results have been obtained from contractile cells such as *foetal cardiomyocytes* and *skeletal myoblasts*. Foetal or neonatal cardiomyocytes can be regarded as a primary source for cellular transplantation since, being of myocardial origin, these cells differentiate towards an adult cardiomyocyte phenotype in the appropriate environment, whereas less differentiated cells delivered to the injured myocardium still need to undergo differentiation (Muller-Ehmsen *et al.*, 2002; Ruhparwar *et al.*, 2002; Scorsin *et al.*, 2000). Since Chiu *et al.* (1995) demonstrated the ability of *skeletal myoblasts* to be successfully delivered to the injured myocardium, these cells have extensively been examined and applied in both animal models of cardiac injury, as well as in human clinical trials (Chiu *et al.*, 1995; Hagege *et al.*, 2006; Menasche *et al.*, 2001; Menasche *et al.*, 2003; Murry *et al.*, 1996). The use of skeletal myoblasts are favourable since they can also be autologous of origin, are ischaemia-resistant which is essential in a post-infarct hypoxic environment, are highly proliferative, and form larger grafts in the injured heart (Murry *et al.*, 2002).

Skeletal myoblast transplantation studies were first initiated in humans by Menasche *et al.* (2001). Subsequently, the use of these cells for transplantation has demonstrated to be beneficial in humans over the short-term (Gavira *et al.*, 2006; Herreros *et al.*, 2003; Menasche *et al.*, 2003; Siminiak *et al.*, 2004) and for an extended period after transplantation (Dib *et al.*, 2005; Hagege *et al.*, 2006). Although these studies demonstrate that autologous skeletal myoblast transplantation is a feasible, straightforward procedure, a concern regarding their long-term clinical application is the risk of developing cardiac arrhythmias. Solutions to treat arrhythmias would include the co-implantation of defibrillators, prophylactic drugs, or possibly engineering skeletal muscle to express gap-junction proteins *in vivo* which may induce coupling with the host myocardium (Menasche *et al.*, 2006).

The use of *bone marrow-derived stem cells*, whether HSCs, MSCs or total unfractionated bone marrow (Shintani *et al.*, 2001; Tomita *et al.*, 1999) also holds certain advantages for cellular transplantation. They can be transplanted autologously without the need of immuno-suppression, and since they are multipotent, these cells can contribute to angiogenesis in addition to cardiogenesis. Similar to skeletal myoblast transplantation, several short-term and extended follow-up human clinical trials have applied intracoronary bone marrow cell-transfer to determine the extent to which these cells enhance recovery following myocardial infarction (Meyer *et al.*, 2006; Schachinger *et al.*, 2004; Strauer *et al.*, 2002). Most short-term studies, covering 3-6 months, have demonstrated improved functional regeneration to various extents. Although the long-term safety and benefits of multiple dosages of intracoronary bone marrow SC transfer still need to be established, cell-therapy trials using bone marrow-transplantation strategies in patients with myocardial infarction are on-going. These trials include *BOOST I* and *II* [bone marrow-derived] (Meyer *et al.*, 2006; Wollert *et al.*, 2004), *REPAIR-AMI* [bone-marrow-derived] (Schachinger *et al.*, 2006c), *STEMI* [bone marrow-derived] (Engelmann *et al.*, 2006), *TOPCARE-AMI* [bone marrow- and circulating blood-derived progenitor cells] (Assmus *et al.*, 2002; Schachinger *et al.*, 2004), *REVIVAL-2* [bone marrow-derived] (Zohlnhofer *et al.*, 2007), and *MAGIC* [peripheral blood stem cells] (Kang *et al.*, 2007; Kang *et al.*, 2004). Some of these trials suggest that mobilisation of bone marrow SCs by granulocyte-colony-stimulating factor can be used to improve cardiac regeneration (Ince and Nienaber, 2007).

Despite evidence that these ASCs can generate new cardiomyocytes after implantation, controversy still exists regarding the ability of transplanted cells, especially bone marrow-derived cells, to *transdifferentiate* into functional muscle fibers after injection into the myocardium (Davani *et al.*, 2005). Orlic *et al.* (2001) reported extensive cardiac regeneration after direct injection of Lin⁻/c-kit⁺ cells (haematopoietic cells) into infarcts. Similarly, Kajstura *et al.* (2005) also demonstrated that bone marrow-derived cells efficiently differentiate into myocytes and coronary vessels with no detectable differentiation into haematopoietic lineages or indications of cell fusion. In contrast, other studies have failed to detect tissue regeneration *or* transdifferentiation of either HSCs (Kuethe *et al.*, 2004; Murry *et al.*, 2004) or engrafted myoblasts/skeletal myocytes (Murry *et al.*, 1996; Reinecke *et al.*, 2002) when injected into normal and injured mouse hearts. Also, Nygren *et al.* (2004) demonstrated high levels of HSC engraftment into the ischaemic myocardium, however, on this occasion, engraftment was transient and haematopoietic in nature and although bone marrow-derived cardiomyocytes were indeed observed, this was at a low frequency outside the infarcted myocardium. These cells were derived exclusively through cell fusion, therefore also challenging the concept of *transdifferentiation*.

Taken together, despite progress, neither the ideal source and progenitor cell type, nor the cellular effect of the cytokine milieu present post-injury, has been identified satisfactorily to enhance skeletal and cardiac muscle regeneration. Stimuli that drive SCs into myogenesis and produce a highly proliferative environment therefore need to be identified which would improve the potential of efficient and functional repair and regeneration. Also, cellular transplantation strategies to improve migration from the injection site, integration into the host and complete functional differentiation, need to be established.

2.3   GROWTH FACTORS INFLUENCING MYOGENIC DEVELOPMENT, REPAIR AND REGENERATION

The regeneration processes following muscle damage or injury involve the activation of various cellular responses which require controlled regulation of muscle transcription factors and muscle-specific genes. Mechanisms which initiate these responses involve cell-to-cell and cell-to-matrix interactions. *In vitro* studies have indicated various secreted factors to be involved in both regeneration processes and maintaining a balance between growth and differentiation of mpcs (Hawke and Garry, 2001; Husmann *et al.*, 1996). The most prominent of these comprise cytokines and growth factors, including the interleukin-family member group, superfamily of transforming growth factor-β (TGF-β), tumour necrosis factor-α (TNF-α), hepatocyte growth factor (HGF), fibroblast growth factor (FGF), leukaemia inhibitory factor (LIF), insulin-like growth factor-1 (IGF-1) and platelet-derived growth factor (PDGF).

The effect of growth factors on myogenesis has been examined using mostly *in vitro* myoblast cultures and either individual, or a combination of growth factors. Such studies provide valuable information regarding the regulation of these cells. However, *in vitro* studies are limited when taking into consideration that the *in vivo* environment may exert different regulatory influences on cellular activity (Tatsumi *et al.*, 1998).

2.3.1 Cytokines and Important Growth Factors

Cytokines are small secreted protein growth factors which are classically known to mediate and regulate immunity, inflammation and haematopoiesis. Due to the different cells that secrete these cytokines, other names include lymphokines (cytokines produced by lymphocytes), monokines (cytokines produced by macrophages or monocytes), chemokines (cytokines with chemotactic activity) and interleukins (cytokines produced by one leukocyte to act on other leukocytes). In general, they act over short distances and time spans, and at very low plasma concentrations. Cytokines induce their effects by binding to membrane receptors, subsequently activating a signalling cascade to initiate gene expression in the cell nucleus (Lutz and Knaus, 2002).

The largest group of cytokines, the *interleukins* (ILs), regulates immune cell proliferation and differentiation, thereby playing an essential function in cellular immunity and inflammation. An important IL includes LIF which is regulated by serum- and other growth factors, including IL-1α, IL-1β, TNF-α, FGF-2 and members of the TGF-β-superfamily. Studies have shown LIF to stimulate myoblast growth *in vitro*, without affecting differentiation or fusion (Bower *et al.*, 1995; Spangenburg and Booth, 2002). *In vivo* administration of LIF results in enhanced myoblast proliferation, as well as increased myofiber size and number (Austin *et al.*, 2000; Barnard *et al.*, 1994).

Another group of cytokines, the *TGF-β-superfamily*, consists of over 40 diverse, multifunctional cell-to-cell signalling proteins (Kingsley, 1994; Meno *et al.*, 1996). Members of this family regulate a wide range of cellular functions, including proliferation, differentiation, migration, apoptosis, extracellular matrix (ECM) deposition and development (Derynck and Feng, 1997; O'Kane and Ferguson, 1997; Whitman, 1998).

FGF has shown, both *in vitro* and *in vivo*, to stimulate mpc *proliferation*, and together with HGF, these factors expand the mpc compartment for regeneration to occur, rather than to target the *differentiation* of myoblasts, a process which these factors inhibit (Allen and Boxhorn, 1989; Olwin and Rapraeger, 1992; Sheehan *et al.*, 2000). Similar to TGF-β, FGF is stored in the ECM in an inactive form. The inflammatory response following damage or injury to skeletal muscle is one method of FGF activation (Husmann *et al.*, 1996), while increased levels can also be measured in necrotic or regenerating muscle cells (DiMario and Strohman, 1988). Because of this factor's angiogenic properties, it additionally contributes to regeneration by means of revascularisation (Lefaucheur *et al.*, 1996).

HGF is an important regulator of satellite cell activity during muscle regeneration. Both *in vitro* and *in vivo*, this growth factor has shown to stimulate quiescent satellite cell activation, enabling these cells to enter the cell cycle and increase mpc proliferation, while simultaneously inhibiting differentiation (Allen *et al.*, 1995; Gal-Levi *et al.*, 1998). More over, HGF also functions to promote satellite cell migration to the site of injury. It therefore appears that HGF increases the mpc population by means of mitogenic and chemotactic activities to produce an optimal myoblast density after which fusion can commence (Zarnegar and Michalopoulos, 1995). Similarly, in cardiac tissue, HGF has also shown to promote mobilisation and facilitate the migration of cardiac stem and progenitor cells to an injured area (Urbanek *et al.*, 2005).

In vitro, *IGF-1* and *-2* are already well known for their function in promoting *both* the proliferation and differentiation/fusion of myoblasts. This hypertrophic effect of IGF-1 can be attributed to the increase in myonuclei numbers following satellite cell activation and proliferation, thereby regulating the cytoplasmic:myonuclei ratio. Also, together with insulin, IGFs have a general effect on muscle metabolism, specifically by stimulating protein synthesis which results in increased muscle protein and therefore augmentation of muscle mass (Adams and McCue, 1998; Bark *et al.*, 1998; Barton-Davis *et al.*, 1999).

Together with its main growth promoting activity in human platelets *in vitro*, *PDGF* has also shown to significantly promote proliferation of mpcs, whereas it inhibits their differentiation. *In vivo*, PDGF is released from injured vessels, macrophages and platelets to promote angiogenesis (Husmann *et al.*, 1996).

TNF-α acts as a mediator of muscle wasting, specifically by inhibiting processes of myogenic differentiation at the cell cycle level, the expression of muscle-specific transcription factors such as MyoD, and myotube formation (Langen *et al.*, 2004).

Many growth factors interact simultaneously during the process of muscle regeneration to activate muscle-specific transcription factors, thereby allowing new myofibers to be rebuilt after injury or disease. To various extents, most of these growth factors stimulate the proliferation of satellite cells and other mpcs while inhibiting their differentiation and fusion, with the exception of IGF, which promotes differentiation. The TGF-β-superfamily will be discussed in more detail in the next section.

2.4   TGF-β SUPERFAMILY

As mentioned earlier, the TGF-β-superfamily comprises a large and diverse group of proteins, the most prominent being the *TGF-β isoforms*, the *bone morphogenetic proteins* (BMPs), *activins* (A, B, and AB) and their negative regulators, the *inhibins* (A and B), *myostatin*, *decorin*, *growth and differentiation factors* (GDFs), and *Mullerian-inhibiting substances* (MIS) (Massague, 1990). The importance of this group of polypeptide proteins is exposed by the range of activities which they control as illustrated by the ability of a single factor to be responsible for diverse processes, including immuno-suppression and regulation of proliferation and differentiation to various extents depending on the cell type and environmental conditions.

The *TGF-β isoforms* were the first to be isolated from human platelets (Assoian *et al.*, 1983) and were named *transforming growth factors* after inducing morphologic transformation of fibroblastic cells in culture (de Larco and Todaro, 1978a; de Larco and Todaro, 1978b; Goustin *et al.*, 1986). They are multifunctional, pleiotropic proteins which play critical roles in the regulation of cell growth and development, especially in proliferation, differentiation, migration and angiogenesis, where the isoforms can induce both stimulatory and inhibitory effects (Lawrence, 1996). Other important processes which TGF-β isoforms are directly or indirectly involved in, include tissue repair and skin formation, apoptosis and tumorigenesis, atherosclerosis, bone metabolism and osteoporosis, and importantly, ECM production (Wahl, 1994). Various pathologies, such as inflammatory and fibrotic diseases, cancer, and tumour development (Roberts *et al.*, 1990a; Taipale *et al.*, 1998) have been linked to increases or decreases in the production of TGF-β, *or* is the result of mutations in the genes for TGF-β, its receptors or the molecules involved in its intracellular signalling pathway (Blobe *et al.*, 2000). Importantly, the activities of TGF-β isoforms are influenced by the state of the target cell, the immediate circumstances of the cell's environment and the presence of other growth factors (Massague, 1990).

The *bone morphogenetic proteins* (isoforms 1-8) primarily act to induce adequate bone formation during embryogenesis, often in concert with other factors, but are also required for growth and repair of skeletal tissue after birth (Massague, 1990).

In contrast to the diverse effects of the TGF-β isoforms, *myostatin* functions mainly as an endogenous inhibitor of muscle growth, negatively regulating satellite cell activation and self-renewal (McCroskery *et al.*, 2003; Wagner *et al.*, 2005). This is an important feature, since myostatin mutations can lead to unnatural, excessive growth, demonstrated by a hypertrophic (increased fiber size) *and* hyperplasic (increased fiber number) muscle phenotype (McPherron *et al.*, 1997). This inhibitory effect of myostatin could however potentially be used as a therapy for human diseases of muscle weakness and wasting.

Activins and *inhibins* primarily modulate the production of follicle-stimulating hormone from pituitary cells, gonadal steroids and placental hormones, although their actions are not restricted to gonadal and pituitary cells (Massague, 1990).

2.4.1　　The TGF-β Isoforms

Five isoforms of TGF-β have been isolated. The main isoforms, TGF-β1, -β2 and -β3, exist in mammals, whereas TGF-β1.2, a heterodimer containing one TGF-β1 and one TGF-β2 chain, has also been isolated in porcine platelets (Cheifetz *et al.*, 1987).

TGF-β1, a non-glycosylated homodimer protein, was the first mammalian isoform to be purified and is the most prevalent form, found almost ubiquitously (Assoian *et al.*, 1983; Frolik *et al.*, 1983; Roberts *et al.*, 1983). TGF-β2 was isolated thereafter from bovine bone, human glioblastoma cells and porcine platelets (Seyedin *et al.*, 1985; Wrann *et al.*, 1987). The third isoform, TGF-β3, was cloned in 1988 (Derynck *et al.*, 1988; ten Dijke *et al.*, 1988), although a similar homologue has been found in the chicken (Jakowlew *et al.*, 1988). TGF-β2 and -β3 are expressed in a more limited spectrum of cells and tissues than TGF-β1 (Lawrence, 1996).

Each isoform is encoded by a unique gene on different chromosomes (Lawrence, 1996). However, at genomic level, the TGF-β sequences have been very well conserved between isoforms and species (Derynck *et al.,* 1988), suggesting essential and specific roles for each isoform. Despite this high sequence conservation, the variability in effects which the isoforms exert on target cells is made possible by differences in their expression pattern and ability to interact with diverse cell surface receptors (Massague, 1990).

2.4.2 TGF-β Sources, Biosynthesis and Activity

TGF-β is synthesised and secreted, with only a few exceptions, by most cell types in the body (Lawrence, 1996). Blood platelets are the richest source of TGF-β (Derynck and Feng, 1997) and yield milligram amounts TGF-β/kg, whereas other tissues yield microgram/kg. These include predominantly spleen and bone tissues, with human milk also containing this factor. TGF-β is also synthesised and released by inflammatory (e.g. macrophages and lymphocytes) (Wahl, 1994), smooth muscle, endothelial, granulosa and leukaemia cells, as well as keratinocytes and chondrocytes (Lehnert and Akhurst, 1988; Wilcox and Derynck, 1988). High concentrations of TGF-β have also been found in the mitochondria of several cell types, suggesting the possibility that TGF-β could act as a linker between the energetics of the cell and its other activities (Heine *et al.*, 1991). Importantly, auto-induction of TGF-β has been demonstrated, providing a possible mechanism by which the biological effects of this growth factor might be amplified.

The three TGF-β isoforms are all synthesised as homodimeric pro-proteins (proTGF-β) of 75 kDa. The dimeric TGF-β-propeptides [the latency-associated peptide (LAP)], are cleaved intracellularly from the mature ~24 kDa TGF-β homodimer by furin-type enzymes. However, the TGF-β-propeptide remains non-covalently bound to the growth factor after the bonds between the propeptide and mature TGF-β have been cleaved. This complex, consisting of mature TGF-β and TGF-β-propeptide/LAP, is known as the small latent complex (SLC). Once the SLC has been targeted to the ECM (Figure 2.6 **A**), LAP forms disulfide bonds with the latent TGF-β binding protein (LTBP) to form the large latent complex (LLC), which subsequently gets covalently linked to ECM proteins by transglutaminase (Figure 2.6 **B**). LTBP may facilitate secretion of the SLC to promote targeting of TGF-β to the ECM, aid its final activation and also possibly play an important function in controlling the action of TGF-β. The TGF-β isoforms are primarily stored in this latent form (L-TGF-β) in the ECM and require activation before being able to bind to their cell surface receptors and exert a target function (Annes *et al.*, 2003; Rifkin, 2005).

As part of the LLC, TGF-β cannot bind with the TGF-β surface receptors because of the inhibitory function of LAP and therefore requires biological activation to release TGF-β from *both* the LAP and LTBP, a process termed latent-TGF-β activation or TGF-β formation. *In vitro,* disruption of LAP can be achieved by heat, acidic environments, oxidation of free radicals or reactive oxygen species (ROS), or detergents (Barcellos-Hoff and Dix, 1996). The nature of the activation mechanisms of L-TGF-β *in vivo* is unclear, however, possible mechanisms could include regulation by proteases, specifically plasmin, calpain, matrix-metaloprotein (MMP-9),

thrombospondin-1, transglutaminase, ROS produced after irradiation (Annes *et al.*, 2003), glycosidases (Miyazono and Heldin, 1989), the mannose 6-phosphate receptor (M6PR), and integrins (Gleizes *et al.*, 1997; Lawrence, 1996). It has been proposed that these activation molecules function as signals in response to disturbances in the ECM (e.g. inflammation, wound repair, cell growth or angiogenesis) that changes the cell's environment, thereby activating the release of TGF-β from LAP and LTBP to allow receptor binding and initiation of the desired signalling pathway and subsequent response. These multiple, seemingly unrelated activators of TGF-β possibly explains why the three TGF-β isoforms, having similar effects *in vitro*, display distinct effects *in vivo* (Annes *et al.*, 2003).

Latency is an important mechanism to control this growth factor's activity: by allowing TGF-β to circulate in an inactive form, it will prevent the isoform from eliciting a response until it reaches its target cell where it can then be converted into the active form. Latency also regulates TGF-β-bioavailability and may limit diffusion from the secreting cell, thereby controlling the autocrine and paracrine actions of this growth factor. Once released from the latent complex, active TGF-β can be bound by various ECM components and serum proteins such as *decorin*, which again allows it to be stored in a biologically inactive form. This mechanism could either protect TGF-β from rapid degradation or function as a long-term reservoir for sustained release and clearance (Massague, 1990).

Enhanced TGF-β expression therefore does not always correlate with increased levels of TGF-β activity (Theodorescu *et al.*, 1991): tissues can contain significant quantities of L-TGF-β, however, activation of only a small fraction of this latent form is required to generate cellular responses. It has also been shown that mature, active TGF-β can reversibly be dissociated from, and re-associated with its latency protein, LAP, resulting in gain and then loss of biological activity (Grainger *et al.*, 1995; Wakefield *et al.*, 1990). In our studies, TGF-β was supplied in its active form, indicating that the protective function of the LAP was not available.

Grainger *et al.* (1995) have developed assays to measure the active-, as well as the active plus latent ([a+l]TGF-β) forms of TGF-β in human serum and plasma, although results showed significant variability. In addition, assays detected TGF-β1 and -β3 with similar sensitivity, but were more than 10-fold less sensitive to TGF-β2. Their results indicated that the mean [a+l]TGF-β present in human serum was 330 pmol/ℓ, however, the range was very large (4-1400 pmol/ℓ). Similarly, the mean active TGF-β present was 230 pmol/ℓ (range 20-1400 pmol/ℓ) and the proportion of the active:total TGF-β present varied from <10% to 100%. This variability in TGF-β concentrations are also illustrated in Table 2.3 which indicates TGF-β levels in various conditions of disease (Grainger *et al.*, 1995).

Table 2.3. Average serum TGF-β concentrations in healthy individuals and diseased conditions.

TGF-β ISOFORM	DISEASE	CONTROL VALUES	REFERENCE
TGF-β1	autoimmune hepatitis: 230 ± 95 ng/mℓ	137 ± 81 ng/mℓ	Sakaguchi *et al.,* 2004
-	psoriasis: 42.9 ± 9.9 ng/mℓ	37.7 ± 6.0 ng/mℓ	Nockowski *et al.,* 2004
TGF-β1	breast cancer: 48.8 (18-82.4) pg/mℓ	51.6 (30.9-65.1) pg/mℓ	Lebrecht *et al.,* 2004
TGF-β1	invasive breast cancer: 498.7 ± 249.7 pg/mℓ	-	Sheen-Chen *et al.,* 2001
TGF-β1	haemodialysis patients: 26.64 ± 7.0 ng/mℓ coronary heart disease: 26.2 ± 4.9 ng/mℓ	42.31 ± 6.0 ng/mℓ	Stefoni *et al.,* 2002
TGF-β bioactive factor	chronic fatigue syndrome: 290 ± 46 pg/mℓ	104 ± 18 pg/mℓ	Chao *et al.,* 1991
TGF-β1	nephrotic syndrome: 1 549 ± 580 pg/mℓ	406 ± 424 pg/mℓ	Buyan *et al.,* 2003

In vivo, the latent form of TGF-β has a half-life of ~90 minutes, whereas active TGF-β has a short half-life of only ~2 minutes. *In vitro,* the TGF-β receptors have a longer turnover, as shown by their half-life of ~2 hours in cultured skeletal muscle cells (Ugarte and Brandan, 2006), ~12 hours in lung epithelial cells (Koli and Arteaga, 1997) and 2-7 hours in osteoblasts (Centrella *et al.*, 1996).

2.4.3   TGF-β Receptors, Signalling Pathways and Regulation

Most cells contain three main types of TGF-β surface receptors (type I, II and III) (Derynck and Feng, 1997). The two smaller receptors, TGF-β RI (65-70 kDa) and TGF-β RII (85-110 kDa), are the signalling mediators and possess transmembrane serine/threonine kinase activity within their cytoplasmic domains (Padgett *et al.*, 1998). Bound to active TGF-β (free of LAP), TGF-β RII induces recruitment of TGF-β RI to initiate TGF-β signalling responses to the nucleus. The largest TGF-β binding protein, TGF-β RIII or betaglycan (280-330 kDa), which is often the most abundant, is a non-signalling receptor and required to specifically enable TGF-β2 to become associated with the TGF-β RI/TGF-β RII-complex to either promote or inhibit TGF-β2 signal transduction (Lopez-Casillas *et al.*, 1993). This receptor is expressed in most foetal and adult tissues, although not in endothelial, primary epithelial, haematopoietic and

lymphoid cells, as well as certain types of myoblasts (Cheifetz *et al.*, 1986; Massague, 1985; Wang *et al.*, 1991). A splice variant of TGF-β RII, TGF-β RII-B, has recently been characterised (Rotzer *et al.*, 2001). Unlike TGF-β RII, this receptor is able to bind TGF-β2 in the absence of betaglycan. Although TGF-β RII-B is able to bind with all three isoforms, it is expressed in tissues where the predominant isoform is TGF-β2. More evidence is however required to determine whether TGF-β RII-B is the principle receptor for the TGF-β2 isoform. An additional TGF-β receptor, endoglin, is similar to TGF-β RIII and highly expressed in vascular endothelial cells. This receptor also binds activin-A and BMP proteins and therefore possibly functions to recruit other proteins into the TGF-β signalling pathway.

Following activation of the latent TGF-β complex, TGF-β either binds to TGF-β RIII which presents the active isoform to TGF-β RII, *or* the isoform directly binds to TGF-β RII. TGF-β RII then recruits, binds and transphosphorylates TGF-β RI, thereby stimulating its protein kinase activity (Figure 2.6 **C**). The TGF-β signal is then further propagated across the plasma membrane to the nucleus by its downstream signal-transducers, the Smad-family of tumour suppressors, specifically the receptor-regulated Smads (R-Smads), R-Smad2 or R-Smad3. Whereas R-Smad2 and -3 are activated by TGF-β, R-Smad1, -5 and -8 are BMP-activated (Lutz and Knaus, 2002). Activated TGF-β RI phosphorylates R-Smad2 or R-Smad3 which is then released from TGF-β RI and binds to Co-Smad4 (Figure 2.6 **D**). The resulting Smad complex can then translocate into the nucleus where it interacts in a cell-specific manner with other transcriptional co-activators, co-repressors and transcription factors at DNA sequence-specific binding sites to activate the expression of the target genes (Blobe *et al.*, 2000; Shi and Massague, 2003). The subsequent activation of these genes will manifest the multifunctional physiological behaviour of TGF-β (Figure 2.6 **E**).

Additional control is made possible by the inhibitory Smads (I-Smads), I-Smad6 and I-Smad7, which prevent TGF-β signalling by associating with TGF-β RI. Here, I-Smad6 or I-Smad7 interferes with the phosphorylation of the R-Smads to down-regulate signal transduction to the nucleus (Heldin *et al.*, 1997; Kawabata and Miyazono, 1999; Whitman, 1998). Since the expression of I-Smads is also induced by other TGF-β-superfamily proteins, Smads constitute an auto-inhibitory signalling pathway (Miyazono *et al.*, 2000).

Besides the Smad-mediated signalling pathway, TGF-β activates and cross-talks with other signalling cascades, including Wnt, Hedgehog and other tyrosine kinase-linked growth receptor-signalling pathways. Furthermore, TGF-β isoforms also activate MAPK, p38, ERK1/2 and JNK/SPAK signalling pathways, some of which regulate important cellular processes, including cardiac cell hypertrophy (Zhang *et al.*, 2000).

Figure 2.6. Schematic presentation of TGF-β signal transduction at various stages of the signalling pathway. Regulation of TGF-β can occur at various levels, including bio-availability of the ligand in the extracellular compartment, activation of the latent complex, receptor binding, influences on the Smad signalling pathway and gene expression in the nucleus. The final effect of a TGF-β response is therefore dependent on the balance between positive and negative influences and other interacting proteins which might be involved in the specific reaction.

[A] Following expression, the small latent complex and LTBP are secreted from the cell and assemble to form the large latent complex which attaches to the extracellular matrix.

[B] Almost all total plasma TGF-β is present in the latent (L-TGF-β complex) form.

[C] In response to specific signals, TGF-β is activated, released from the latent complex in the extracellular space, and subsequently either binds to **(i)** type III receptor (TGF-β RIII *or* betaglycan) which presents **(ii)** TGF-β to the type II receptor (TGF-β RII), or it binds directly **(iii)** to TGF-β RII on the cell membrane. The binding of TGF-β to TGF-β RII leads to phosphorylation of the type I receptor (TGF-β RI) **(iv)** and subsequent activation of the TGF-β RI-protein kinase. TGF-β RIII is required to specifically enable TGF-β2 to become associated with the TGF-β RI/TGF-β RII-complex **(i)**.

[D] This active receptor complex then phosphorylates the transcription factor R-Smad2 or R-Smad3 which binds to Co-Smad4. The inhibitory Smads, I-Smad6 and I- Smad7 lack the region normally phosphorylated by TGF-β RI and therefore these I-Smads interfere with the activation of R-Smad2 and R-Smad3 by TGF-β RI, repressing further Smad signalling.

[E] The R-Smad2/3-Co-Smad4 complex translocates into the nucleus where it interacts in a cell-specific manner with various other transcription factors to regulate the transcription of TGF-β responsive genes and mediate the effects of TGF-β at cellular level. Possible clearance organs for TGF-β include the liver (bile fluid) and kidney (urine).

[Adapted with modifications from Blobe et al., 2000; Gleizes et al., 1997; O'Kane and Ferguson, 1997]

Figure 2.6. Schematic presentation of TGF-β signal transduction at various stages of the signalling pathway.
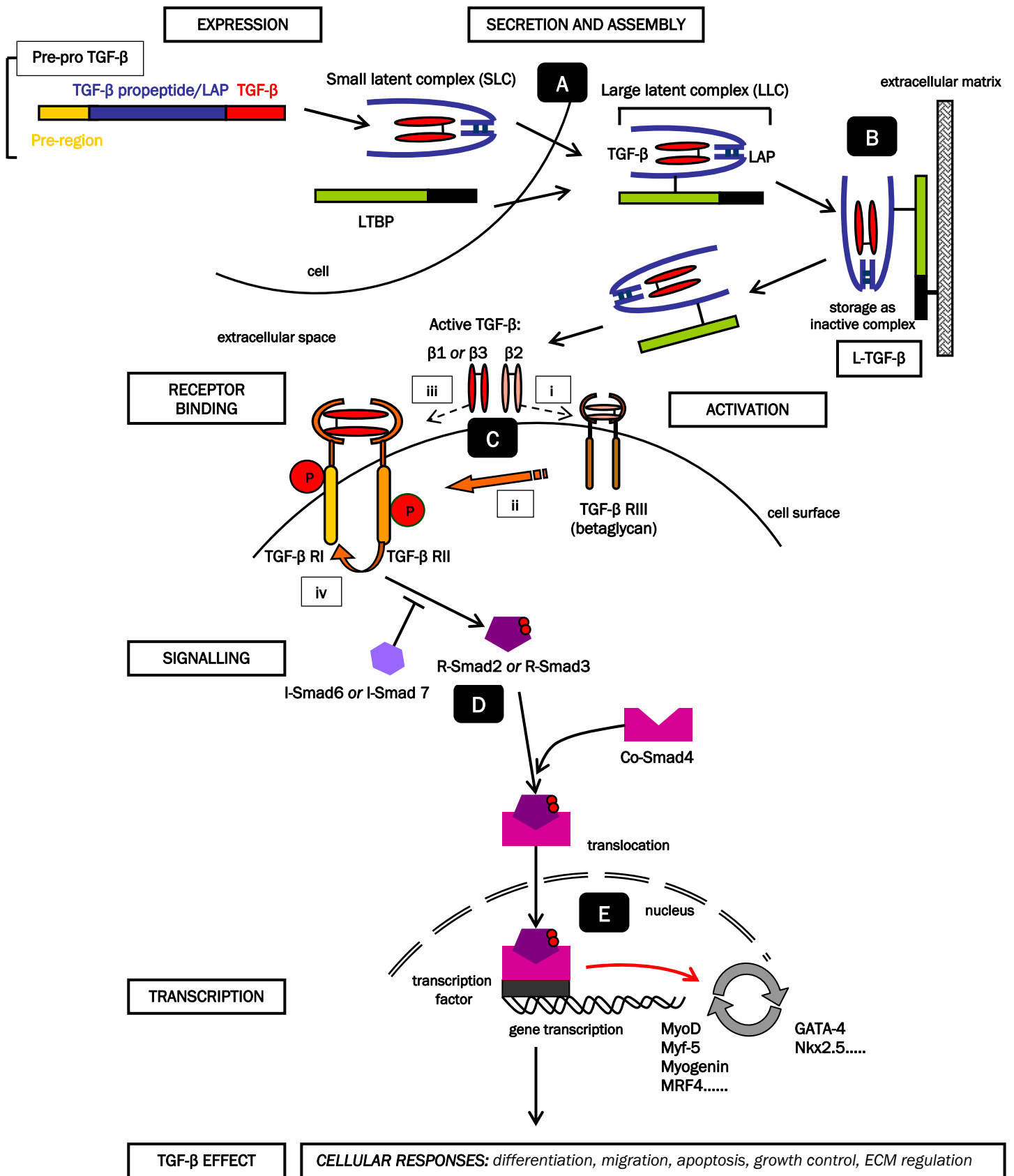
Because of the large diversity of processes in which the TGF-β isoforms are involved, their production, activation and signalling pathways are highly regulated and involve various levels of control mechanisms to enable the desired cellular response. Such mechanisms could include: **(A)** regulation of TGF-β gene transcription and gene dosage; **(B)** production of TGF-β as a latent form and activation in the extracellular space; **(C)** binding of active TGF-β to extracellular matrix and circulating proteins; **(D)** cell and tissue specific interaction with receptors; **(E)** signal transduction through Smads and cross-talk of TGF-β signalling with other signalling pathways; *and* **(F)** modulation of transcriptional activation in the nucleus (Derynck and Zhang, 2003; Droguett *et al.*, 2006; Massague and Wotton, 2000).

2.4.4 Role of TGF-β in Cell Growth, Proliferation and Differentiation

The TGF-β isoforms regulate three *main* activities: they control *growth* and *development*, exert *immunosuppressive* effects, and enhance the *formation* of *extracellular matrix* (Lawrence, 1996). Despite the similarity of their actions *in vitro*, each of the TGF-β isoforms appears to mediate distinct actions, and has a different distribution *in vivo*, with limited overlap (Schmid *et al.*, 1991).

The TGF-β isoforms have demonstrated multifunctional behaviour, even within the same cell-lineage, and depending on the differential stage and type of the target cell, the local environment, and the identity and dosage of the ligand, isoforms can either promote and/or inhibit cell proliferation, differentiation, migration and apoptosis (Barnard *et al.*, 1990; Lawrence, 1996; Roberts and Sporn, 1985). This variable behaviour is further influenced by the presence of other growth factors such as PDGF and EGF, e.g. in the presence of PDGF, TGF-β stimulates the growth of reader cells (fibroblasts), whereas in the presence of EGF, TGF-β functions as growth inhibitor (Roberts *et al.*, 1985). A potential mechanism by which TGF-β exerts this variable behaviour, is via the affinity by which the isoforms bind to their receptors. The order of relative receptor affinities between individual isoforms is TGF-β1~TGF-β3 > TGF-β1.2 > TGF-β2, with approximately a 10- to 20-fold difference in affinity between TGF-β1 and -β2 (Cheifetz *et al.*, 1990; Segarini *et al.*, 1987).

2.4.4.1   Effect of TGF-β on myogenesis

TGF-β isoforms play an important role in myogenic development. However, its role in *skeletal* muscle is unclear, since exogenous TGF-β has produced both positive and negative effects on muscle cell development. Also, both the *in vitro* and *in vivo* cellular responses to the three isoforms differ significantly (Letterio and Bottinger, 1998).

On the one hand, TGF-β isoforms have shown to depress proliferation and inhibit the progression of differentiation and fusion of skeletal muscle in neonatal myoblasts, primary satellite cells and myogenic cell-lines *in vitro* and *in vivo* (Allen and Boxhorn, 1987; Florini *et al.*, 1986; Greene and Allen, 1991; Lefaucheur and Sebille, 1995). Inhibition of growth by TGF-β is mainly the result of TGF-β lengthening or arresting the late G_1 phase of the cell cycle (Shipley *et al.*, 1985; Zhang *et al.*, 2002), possibly through its direct effect on MyoD (Liu *et al.*, 2001) or myogenin (Brennan *et al.*, 1991). TGF-β isoforms might therefore negatively regulate myogenic differentiation by inhibition of muscle-specific gene expression and protein synthesis (Massague *et al.*, 1986; Olson *et al.*, 1986). This inhibition of differentiation requires the continual presence of TGF-β and normal differentiation can therefore potentially resume once the TGF-β-stimulus have been eliminated.

The growth inhibitory effect of TGF-β, however, seems to be reversible in mitogen-rich environments (Zentella and Massague, 1992). TGF-β1 has shown to induce myogenic differentiation when added to L6E9 skeletal myoblasts in a mitogen-rich environment (20% foetal bovine serum, 10% bovine calf serum). In this study, the initial growth inhibitory response, due to delayed progression through the G_1-phase, was followed by elevated myogenin expression and cell commitment to terminal differentiation. It should be noted that other myoblast cell-lines (C2C12 and P2) have not shown this response when treated similarly. Furthermore, evidence have shown that TGF-β signalling, specifically through TGF-β RII, is required for distinct aspects of *myogenic* differentiation and that two effects of TGF-β, *stimulation* versus *inhibition* of myoblast differentiation, are mediated by different receptor systems and signalling pathways shown to be involved in muscle differentiation (Filvaroff *et al.*, 1994).

In *cardiac muscle*, TGF-β has shown to increase the expression of cardiac-specific genes and induce cardiac differentiation in mouse ESC *in vitro*, as well as direct differentiation *in vivo* (Behfar *et al.*, 2002; Boyer *et al.*, 1999; Pelton *et al.*, 1991). Studies have shown TGF-β1 and -β3 knock-out mice to have no major heart defects, whereas TGF-β2 knock-out mice suffer from cardiovascular abnormalities (Azhar *et al.*, 2003; Sanford *et al.*, 1997). In cell cultures, TGF-β1 has indeed shown to stimulate hypertrophic growth (Villarreal and Dillmann, 1992), but inhibit mitotic growth of cardiomyocytes (Kardami, 1990). Depending on the environment, all TGF-β isoforms are therefore involved and essential in several aspects of cardiovascular physiology. The contrasting results between TGF-β studies are mainly due to variable culture conditions *in vitro* and the influence of other regulatory factors *in vivo*, making the extrapolation from *in vitro* to *in vivo* myogenesis complex (McLennan and Koishi, 2002).

2.4.4.2   *Effect of TGF-β in other cell-lineages*

TGF-β induces strong growth *inhibitory* effects to variable degrees depending on the cell type, including most epithelial-, endothelial-, fibroblast-, neural-, lymphoid- and haematopoietic cell-lineages (Cheifetz *et al.*, 1987; Coffey *et al.*, 1988; Graycar *et al.*, 1989; Moses *et al.*, 1987; Tucker *et al.*, 1984). Differentiation of chondrocyte cultures (Ferguson *et al.*, 2004; Kato *et al.*, 1988), late-stage osteoblasts (Rosen *et al.*, 1988) and adipocytes (Torti *et al.*, 1989) are also *inhibited* by TGF-β. The effects of TGF-β on immune function are clearly demonstrated by its *negative* control of immune cell proliferation and differentiation *in vitro* and *in vivo*, as illustrated by its suppression of B- and T-lymphocytes (Kehrl *et al.*, 1986a; Kehrl *et al.*, 1986b) and the production of immunoglobulins. At the same time, TGF-β has both *activating* and *deactivating* effects on macrophages and is also cytotoxic to natural killer cells (de Martin *et al.*, 1987; Rook *et al.*, 1986; Tsunawaki *et al.*, 1988; Wrann *et al.*, 1987).

In contrast, TGF-β isoforms have shown *proliferative* effects on several other cell types, including various mesenchymal cells, connective tissue cells (Battegay *et al.*, 1990; Centrella *et al.*, 1987; Roelen and Dijke, 2003), and have also shown to promote *differentiation* in osteoblastic sarcoma cells (Pfeilschifter *et al.*, 1987) and early-stage pre-chondroblasts (Seyedin *et al.*, 1985). Such growth stimulation may indirectly result from the autocrine-induction as a secondary growth factor response.

Importantly, as illustrated by its effect on chondrogenesis and osteogenesis, TGF-β has shown to exert both positive and inhibitory responses within cell-lineages. The variable results could reflect real differences between cell types, or is a consequence of the timing of TGF-β addition and different culture conditions (cell-lines, species) used *in vitro*. Such variable effects which

TGF-β isoforms exert on cell types depending on their stage of differentiation can be demonstrated in ESC. As an example, during neural development of uncommitted ESC, once a precursor lineage is established, TGF-β signalling appears to accelerate the differentiation and lineage-commitment of the precursor cells. However, once selected neural cells are fully differentiated, TGF-β again inhibits growth to prevent tumorigenesis (Gangemi *et al.*, 2004).

2.4.5 Role of TGF-β in Human Disease

Normal homeostasis in human tissue is the result of continuous, highly controlled reactions between the cells, secreted proteins and the surrounding ECM. These co-operative interactions involve numerous signalling molecules and cytokines which act through specific cell surface receptors. The TGF-βs are of the most pleiotropic proteins involved in tissue homeostasis, mediating several physiological processes, including haematopoiesis, hormone secretion, immune function, angiogenesis, tissue morphogenesis and bone modulation. It also acts as a switch of many biological responses: TGF-β will facilitate the activation of *inactive* processes, conversely, within the same cell, once the process is activated, TGF-β can function to stop the signal (Sporn and Roberts, 1990). As such, the balance between the ECM, cells and increases or decreases in TGF-β production is highly regulated. Failure or disruption of TGF-β signalling produce changes in the activation of downstream signalling pathways, resulting in the onset of several disease states such as impaired wound healing, neurodegenerative disorders and tumorigenesis due to suppression of the immune system (Blobe *et al.*, 2000).

2.4.5.1 *Role of TGF-β in fibrosis, inflammation and wound healing*

All phases of wound healing are either directly or indirectly controlled by cytokines. Following injury, cytokines and other mediators are released, including TGF-β, PDGF and VEGF (vascular endothelial growth factor) to initiate the inflammatory response. Here, TGF-β is required for the chemotactic attraction of inflammatory cells and fibroblasts. The TGF-β isoforms are especially important in the regulation of this response: they promote tissue regeneration, however, since the isoforms induce differential effects on wound repair, fibrosis and scarring, their critical balance is required for *optimal* healing to take place. Failure to resolve the inflammation can lead to chronic non-healing wounds, whereas uncontrolled matrix accumulation can lead to excess scarring and fibrosis (Sporn and Roberts, 1993).

The initial release of latent TGF-β by degranulating platelets and activation by proteolytic and non-proteolytic mechanisms elicits the rapid chemotaxis of neutrophils and monocytes to the wound site in a dose-dependent manner (Wahl *et al.*, 1987). The neutrophils are the first to appear and reach peak levels ~24 hours after injury. Other sources releasing TGF-β include mast cells, monocytes, macrophages, fibroblasts, keratinocytes and endothelial cells. In addition to the auto-induction of TGF-β production, autocrine release of TGF-β by leukocytes and fibroblasts stimulate these cells to generate additional chemokines and cytokines, including TNF-α, IL-1β and PDGF (Amento and Beck, 1991; McCartney-Francis *et al.*, 1990).

After initiating the inflammatory response, the inflammatory cells become susceptible to TGF-β-mediated suppression to reverse the inflammatory process (McCartney-Francis and Wahl, 1994; Tsunawaki *et al.*, 1988). TGF-β subsequently contributes to the healing- and fibrotic processes by recruiting fibroblasts and stimulating their production of collagens I,III and V, proteoglycans, fibronectin and other ECM components (Branton and Kopp, 1999). It therefore modifies the ECM and blocks matrix degradation by decreasing the synthesis of proteases and simultaneously increasing the levels of protease-inhibitors, thereby preventing the proteolytic action of the proteases and subsequent breakdown of the ECM (Ignotz and Massague, 1987).

This apparent contradictory influence of TGF-β on cells of the immune system, both stimulatory and inhibitory, is party the result of the differential effects of TGF-β on resting and activated cells: in *general*, resting, immature cells are *stimulated* by TGF-β, whereas an activated population of the same cell group might be *inhibited* (Wahl, 1994).

2.4.5.2   *Lessons from wildtype and knock-out studies*

That TGF-β participates in wound healing was confirmed with *in vivo* studies which demonstrated that the administration of exogenous TGF-β could stimulate the formation of collagen and vascularised connective tissue found in healing wounds. In these studies, wound chambers made of stainless steel wire mesh were implanted into the backs of rats, into which preparations of TGF-β isoforms were injected and left for a required period. The contents of the chambers were then evaluated for collagen and histochemical expression (Roberts *et al.*, 1986; Sporn *et al.*, 1983).

Many *in vivo* studies followed using knock-out mice to further investigate the importance of TGF-β in wound healing and scar formation, especially since these processes occur in an isoform-specific manner. During the first 10 days, wound healing proceeds relatively normal in TGF-β1 null mice, after which they display an increased inflammatory cell response, together with a decrease in percentage wound closure and re-epithelialisation, granulation tissue formation, collagen deposition and vasculogenesis (Brown *et al.*, 1995). On the other hand, TGF-β2 knock-out mice die during, shortly before or shortly after birth, mainly due to developmental defects affecting angiogenesis, cell growth and ECM production. Wound healing studies are therefore difficult to perform on these animals (Sanford *et al.*, 1997). Wound healing studies on TGF-β3 knock-out mice have revealed an important role for this isoform in scarless would repair: whereas TGF-β3 null embryos display a scar during healing, wildtype embryos, in contrast, show scarless foetal wound healing (Ferguson and O'Kane, 2004).

The diverse effects of the TGF-β isoforms on wound healing have also been illustrated in various rodent incisional wound studies. *Neutralisation* of TGF-β1 and -β2 have shown to reduce the influx of inflammatory cells, deposition of fibronectin and collagen formation, while simultaneously increasing wound tensile strength and scar quality. In contrast, the *addition* of TGF-β3 has a similar effect (Shah *et al.*, 1995). The progressive increase of TGF-β3 over time and its association with scarless foetal healing suggests this isoform's involvement in the cessation of matrix deposition. The *addition* of *either* TGF-β1 or -β2 results in excess scarring (Ernst *et al.*, 1996), but the effect is less when compared to the *combined addition* of TGF-β1 and -β2, indicating that these isoforms enhance each others' action. In rabbits, the *addition* of recombinant human anti-TGF-β2 monoclonal antibodies has shown to significantly improve conjunctival scarring (Cordeiro *et al.*, 1999).

Taken together, these animal studies indicate that TGF-β1 and -β2 are *profibrotic* and function to *accelerate* wound healing, whereas TGF-β3 improves the *quality* of the scar and is less important in regulating the speed of repair. In fibrotic diseases, overproduction of especially TGF-β1 has shown to result in excessive deposition of scar tissue and subsequent fibrosis. Results therefore suggest that TGF-β3 can be used as a potential therapy agent in the prevention of scarring in humans (Ferguson and O'Kane, 2004; Sporn and Roberts, 1993).

2.4.5.3   Role of TGF-β in skeletal muscle repair and regeneration

In skeletal muscle, TGF-β is known as an inhibitor of differentiation of cultured myoblasts, however, the physiological significance of TGF-β signalling *in vivo* and in disease pathogenesis is largely unknown. This growth factor has been suggested to play a role in muscle regeneration, *preventing* advanced fusion of embryonic myoblasts, as well as satellite cell fusion into the main body of muscle tissue.

In muscle strain injury and diseases such as muscular dystrophy and inflammatory myopathy, the inflammatory response results in overproduction of TGF-β. In these conditions, TGF-β has been localised to the ECM and areas of inflammatory cell infiltration where increases in TGF-β production appears to be a major determinant of collagen synthesis, connective tissue proliferation and subsequent muscle fibrosis (Chan *et al.*, 2005a). This negatively affects the healing response by inducing scar tissue formation which also prevents possible incorporation of stem and progenitor cells for regeneration to occur, and as such propagates further skeletal muscle weakness.

In addition to the effect of TGF-β on skeletal muscle injury and fibrotic disorders, specific muscle diseases have also been associated with TGF-β signalling. In individuals with *Marfan's syndrome*, which is caused by a deficiency in the supporting connective tissue of the body, individuals are unable to increase muscle mass despite stimuli such as physical exercise. Evidence suggests that symptoms of the disease could be due to excessive signalling by TGF-β (Cohn *et al.*, 2007). Similarly, TGF-β-induced failure of muscle regeneration has been demonstrated in dystrophin-deficient mice and together with increased expression of TGF-β signalling in symptomatic patients, the involvement of TGF-β in *muscular dystrophy* has also been verified (Strober, 2006).

2.4.5.4   Role of TGF-β in cardiac muscle

TGF-β has proven to be involved in the maintenance and repair of cardiac muscle cells (Thompson *et al.*, 1988). Specifically, evidence suggests a function for TGF-β1: this isoform has been shown to be protective during the initial, acute phase of inflammation following myocardial infarction (Dean *et al.*, 2005; Ikeuchi *et al.*, 2004), it attenuates cardiac myocyte apoptosis (Chen *et al.*, 2003), and limits the infarct size (Baxter *et al.*, 2001). Further research has demonstrated that TGF-β1 inhibits migration and proliferation of macrophages, induces apoptosis in numerous cells involved in vascular lesions, and reduces adhesiveness

of the endothelium for inflammatory cells, illustrating further cardio-protective effects of this isoform (Stefoni *et al.*, 2002). In addition, despite the growth inhibitory effect of TGF-β1 and -β3 on endothelial cell cultures (Cheifetz *et al.*, 1990), TGF-β1 has shown to induce the formation of new blood vessels *in vivo*, either directly, or through other cells it attracts (Roberts *et al.*, 1986) which is essential during cardiac repair processes. Importantly, the beneficial effects of TGF-β1 might be lost when its expression is sustained and could potentially result in scar tissue formation and fibrosis (Ikeuchi *et al.*, 2004).

2.4.5.5   *Role of TGF-β in immune cell regulation*

As mentioned, TGF-β isoforms suppress growth and differentiation of most immune cell-lineages, including B- and T-cells and also inhibit immune cell activation by antigen presentation and/or interleukins. Immuno-suppressive activities may underlie a beneficial effect of systemic TGF-β-administration (Brandes *et al.*, 1991; Kuruvilla *et al.*, 1991): the essential function of TGF-β in the immune system is suppression of lymphocyte proliferation and differentiation to prevent inappropriate auto-immune responses and balance the requirements of appropriate immune cell levels during pathological conditions. This function has been demonstrated in TGF-β1 knock-out mice, where the lack of TGF-β resulted in a self-targeting inflammatory response, characterised by the overproduction of auto-immune antibodies which killed the animals in early life (Kulkarni *et al.*, 1993; Yaswen *et al.*, 1996).

2.4.5.6   *Bone and osteoporosis*

TGF-β plays a significant role in stimulating osteoblast proliferation and matrix synthesis both *in vitro* (Oreffo *et al.*, 1989; Pfeilschifter *et al.*, 1987) and *in vivo* (Marcelli *et al.*, 1990). Osteoclasts can activate latent TGF-β during bone resorption and subsequently release it in an active form which would stimulate osteoblastic function and bone formation.

2.4.6 Clinical Applications of TGF-β in Disease

Because TGF-β regulates such a diverse range of cellular processes, specifically with regards to injury and disease, this growth factor could potentially be used in multiple therapeutic interventions. Indirectly, TGF-β can be used as a therapeutic agent by adjusting local cellular concentrations of the isoforms by use of pharmacological agents that would regulate its synthesis, secretion, activation or inhibition. Clinical features in which modulation of TGF-β-activity may be useful, include scar-free wound healing, treatment of skeletal and cardiac muscle diseases, control of cancer- and tumour development, prevention of bone loss in osteoporosis, stimulation of bone formation in fracture healing, and inducing cartilage formation in patients with arthritis.

2.4.6.1 Inflammatory diseases

By manipulating the actions of the TGF-β isoforms, it may be possible to accelerate or modify *wound healing* in a variety of tissues (Ferguson and O'Kane, 2004). In cutaneous wound healing, possible mechanisms to reduce the effect of scarring could include combined neutralisation of TGF-β1 and -β2 by anti-fibrotic agents (Border and Noble, 1998; Wahl *et al.*, 1993), or alternatively, the local application and administration of exogenous TGF-β3 (Gorvy *et al.*, 2005). Similarly, antagonists of all TGF-β isoforms may be valuable in the treatment of *fibrotic disorders* which are associated with increased levels of TGF-β activity (Shah *et al.*, 1995; Wahl, 1992).

A role for TGF-β in atherosclerosis has also been investigated, where TGF-β1 has been found to be anti-atherogenic (Stefoni *et al.*, 2002). Atherosclerosis is an inflammatory, proliferative disease in which various cells are involved, including macrophages, and smooth muscle and endothelial cells which result in narrowing of the arteries. Studies by Grainger *et al.* have shown TGF-β1 serum levels to be depressed in patients with advanced atherosclerosis, suggesting this isoform as a possible inhibitor for this condition. Given TGF-β1's function as an inhibitor of smooth muscle and endothelial cell proliferation, it has been proposed that active TGF-β1 in the vascular wall is required to control the balance between inflammation and ECM deposition (Grainger, 2004; Grainger *et al.*, 1995).

2.4.6.2   Skeletal muscle diseases

Both *in vitro* and *in vivo* studies have demonstrated the beneficial use of anti-fibrotic agents to block the stimulatory effect of TGF-β on connective tissue proliferation and fibrosis. The administration of anti-fibrotic agents, such as *decorin* and *suramin*, has therefore been suggested to prevent scar formation and improve muscle regeneration due to the ability of these agents to antagonise the pro-fibrotic effects of TGF-β (Chan *et al.*, 2005b; Fukushima *et al.*, 2001). ECM proteoglycans also modulate TGF-β signalling by binding to this growth factor during skeletal muscle differentiation, thereby diminishing its bio-availability and could represent a further potential regulatory mechanism for use in therapeutic intervention (Droguett *et al.*, 2006).

Clinical applications for conditions such as muscular dystrophy and Marfan's syndrome could include the administration of a TGF-β-neutralising antibody which has shown to improve muscle repair and function *in vivo* (Cohn *et al.*, 2007).

2.4.6.3   Cardiovascular diseases

In cardiac muscle, the addition of TGF-β before or immediately after ischaemic injury can possibly prevent severe cardiac injury. Myocardial ischaemia is characterised by an increase in circulating TNF-α and production of superoxide anions, both of which TGF-β has shown to reduce after ischaemic injury. TGF-β might therefore prevent severe cardiac injury by alleviating damage mediated by increases in circulating TNF-α (Lefer *et al.*, 1990), as well as the generation of reactive oxygen species (Mehta *et al.*, 2002).

2.4.6.4   Immune response, cancer and tumour development

As an immuno-suppressive agent, the systemic delivery of TGF-β could potentially be used as possible treatment therapy in auto-immune and chronic inflammatory diseases. This inhibitory effect on immune cells also suggests an application for the use of TGF-β in the prevention of tissue rejection following organ-transplantation (Roberts and Sporn, 1993). Furthermore, the growth inhibitory response which exogenous TGF-β has on selected cell types may be a mechanism by which unregulated growth of transformed cells can be controlled. Several TGF-β signalling components have shown to be bona fide tumor suppressors with the ability to constrain cell growth and inhibit cancer development during its early stages (Mishra *et al.*, 2005).

Various studies therefore illustrate how increases or decreases in the production of TGF-β can be linked to numerous disease states. By manipulating the mechanisms and isoform-specific effects by which TGF-β mediates cellular functions, possible therapeutic interventions can be introduced. Furthermore, the timing, therapeutic dose and route of TGF-β administration in clinical settings are critical parameters that could play a role to either potentiate or suppress the response of TGF-β to enable a desired outcome.

Therefore, to address the question of how progenitor cells can be used to improve growth and regeneration in skeletal and cardiac muscle, and specifically, determine whether TGF-β isoforms differentially affect muscle development, the effect of TGF-β1, -β2 and -β3 on proliferation (*Chapter 5*), differentiation (*Chapter 6*), migration (*Chapter 7*) and fusion (*Chapter 8*) of muscle progenitor cells (*skeletal* and *cardiac*) were investigated using the C2C12 cell-line and P19 embryonal carcinoma cell-lineage as model systems. Markers and protocols were first established under control conditions (*Chapter 4*).

These studies could provide valuable information related to the regulation of TGF-β isoforms on skeletal and cardiac myogenesis. This knowledge could then be applied to improve treatments for diseases involving muscle degeneration and wasting such as muscular dystrophy, cancer, cardiovascular diseases and HIV.

CHAPTER 3

GENERAL METHODS AND MATERIALS

3.1 BUFFERS, STOCK REAGENTS AND GENERAL SOLUTIONS

3.1.1 LYSIS-Buffer

[RIPA^{++}-buffer: $^+$protein phosphatase inhibitors; $^+$protease inhibitors]	
1x Hanks Balanced Salt Solution (HBSS):	2.5 mM tris-HCl pH 7.4
	1 mM EDTA
	1 mM EGTA
	250 mM sucrose or mannitol
	50 mM NaF
	50 mM NaPPi
	1 mM DTT
add protease inhibitors:	0.1 mM PMSF
	4 µg/mℓ SBTI
	10 µg/mℓ leupeptin
	1 mM benzamidine
add detergents:	1% NP-40
	0.1% SDS
	0.5% Na deoxycholate
make up:	desired volume with dH$_2$O

3.1.2 10x SDS Running Buffer

mix:	60.6 g tris
	288 g glysine
	20 g SDS
make up:	2 ℓ with dH$_2$O

3.1.3 Transfer Buffer

mix:	10% 10x SDS running buffer
	20% methanol
make up:	desired volume with dH$_2$O

3.1.4　2x Sample Buffer

mix:	10% glycerol (v/v)
	5% β-mercaptoethanol (v/v)
	2.3% SDS (w/v)
dissolve in:	62.5 mM tris-HCL solution (pH 6.8)
add:	dH_2O to desired volume
add:	0.05% bromophenol blue

3.1.5　SDS Polyacrylamide Gels

	5% separating gel	*4% stacking gel*
dH_2O	5.7 mℓ	6.1 mℓ
40% degassed acrylamide-bis solution (Promega, H-5171)	1.7 mℓ	1.3 mℓ
1.5 M tris-HCl buffer (pH 8.8)	2.5 mℓ	2.5 mℓ
10% SDS	100 µℓ	100 µℓ
Temed	10 µℓ	10 µℓ
10% APS	100 µℓ	100 µℓ

3.1.6　Phosphate Buffered Saline (PBS) - pH 7.4

mix:	0.01 M phosphate buffer
	0.0027 M potassium chloride
	0.137 M sodium chloride
make up:	desired volume with dH_2O

3.1.7　10x Tris-Buffered Saline (TBS) - pH 7.6

mix:	48.4 g tris
	160 g NaCl
	500 mℓ dH_2O
set pH:	with required volume HCl
make up:	2 ℓ with dH_2O
0.05% TBS-T	0.5 mℓ Tween/ℓ TBS

3.1.8   Coomassie Blue

Coomassie Blue (Brilliant Blue R250, Bio-Rad) binds non-specifically to most proteins and was used to determine relative amounts of protein left on the gels following SDS-page electrophoresis, as well as illustrate equal loading of protein samples.

3.1.9   Ponceau-S

Ponceau-S (Sigma-Aldrich, P-3504) is a sodium-salt dye which was used to allow rapid, reversible staining of protein bands on the PVDF membranes after protein transfer to facilitate immunological detection and illustrate equal loading of samples.

mix:	1 g Ponceau-S
	50 mℓ 100% acetic acid
make up:	1 ℓ with dH$_2$O

3.1.10   α-Tubulin

α-Tubulin is a structural, cytoskeletal component of microtubules. It can be detected in all cells and was therefore also used to illustrate equal loading of protein samples following electrophoresis.

| Santa Cruz (B-7) | mouse monoclonal sc-5286; 55 kDa |
| make up: | $1/_{100}$ in 5% milk/TBS-T solution |

3.2 GROWTH FACTORS, ANTIBODIES AND MARKERS

3.2.1 Growth Factors and Antibiotics

3.2.1.1 *TGF-β*

Recombinant Human TGF-β1: 240-B; R&D Systems

Recombinant Human TGF-β2: 302-B2; R&D Systems

Recombinant Human TGF-β3: 243-B3; R&D Systems

Reconstitution of TGF-β (2 µg/mℓ stock solution)

prepare:	4 mM HCl	add 39.7 µℓ HCl to 99.96 mℓ dH$_2$O
	0.1% BSA in 4 mM HCl	add 50 µℓ 10% BSA to 5 mℓ 4 mM HCl - filter
make up:	2 µg/mℓ TGF-β	add 1 mℓ of 4 mM HCl containing 1 mg/mℓ BSA to 2 µg TGF-β stock

3.2.1.2 *IGF-1*

Recombinant Human IGF-1: 291-G1; R&D Systems

Reconstitution of IGF-1 (50 µg/mℓ stock solution)

prepare:	10 mℓ 10 mM ascetic acid	add 5.7 µℓ acetic acid to 10 000 µℓ ddH$_2$O
add:		100 µℓ BSA (for long-term storage)
make up:	50 µg/mℓ IGF	use 1 mℓ per 50 µg IGF stock

3.2.1.3 *Cycloheximide*

Glutarimide antibiotic from microbial source: C-7698; Sigma

Reconstitution of cycloheximide (5 mM stock solution)

molecular weight:	281.4
weigh off:	1.407 g
add:	1 mℓ sterile PBS
for 50 µM treatment concentration:	use 20 µℓ per 5 mM stock solution

3.2.2   Antibodies and Markers

Table 3.1. Primary antibodies for western blot protein analysis.

Antibody	Company	Clone	Size (kDa)	Dilution
α-Actinin	SIGMA	EA-53 *mouse* ascites fluid product A-7811	100	$^1/_{200}$
γ3-AMPK	Dr David Carling, MRC Cell Stress Unit, Hammersmith Hospital, Imperial College, London - developed in *rabbit*		63	$^1/_{1000}$
Connexin-43	SIGMA	developed in *rabbit* product C-6219	43	$^1/_{8000}$
MHC	*Developmental Studies Hybridoma Bank	myosin A4-1025 species: *mouse* origin: human - all fibers	200	$^1/_{100}$
MyoD	Santa Cruz C-20 Santa Cruz 1-318	*rabbit* polyclonal sc-304 sc-4080, positive control	38-45	$^1/_{100}$
Myogenin	Santa Cruz F-5D	*mouse* monoclonal IgG sc-12732	36-43	$^1/_{200}$
PCNA	Santa Cruz PC-10	*mouse* monoclonal IgG$_{2a}$ sc-56	36	$^1/_{200}$
p21	Santa Cruz F-5	*mouse* monoclonal sc-6246	21	$^1/_{100}$
ROCK-I	Santa Cruz H-85	*rabbit* polyclonal IgG sc-5560	160	$^1/_{1000}$

"The myosin heavy chain antibody (A4-1025) was obtained from the Developmental Studies Hybridoma Bank and developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242."

Table 3.2. Secondary antibodies for western blot protein analysis.

Antibody	Company	Dilution
Polyclonal *Rabbit* Anti-Mouse Immunoglobulins/HRP	P-0260, DAKO	$^1/_{1000}$
Polyclonal *Goat* Anti-Rabbit Immunoglobulins/HRP	P-0448, DAKO	$^1/_{1000}$ $^1/_{5000}$

Pre-stained Markers		
Kaleidoscope Prestained Standards	Cat no. 161-0324; Bio-Rad	
peqGOLD Prestained protein marker IV	Cat no. 27-2110; PeqLAB Biotechnologie	

Table 3.3. Antibodies for immunohistochemical analysis.

Antibody	Company	Specificity	Dilution
M-cadherin	H-71, Santa Cruz	*rabbit* polyclonal IgG sc-10734	$^1/_{50}$
MyoD	C-20, Santa Cruz	*rabbit* polyclonal sc-304	$^1/_{50}$
PCNA	PC-10, Santa Cruz	*mouse* monoclonal IgG$_{2a}$ sc-56	$^1/_{50}$
Biotinylated donkey anti-rabbit IgG	711-066-152, Jackson ImmunoResearch Laboratories, Inc.	*rabbit* IgG and immunoglobulins	$^1/_{200}$
Fluorescein Streptavidin (FITC)	711-096-152, Jackson ImmunoResearch Laboratories, Inc.	*rabbit* IgG and immunoglobulins	$^1/_{200}$ $^1/_{500}$
Texas Red Streptavidin	SA-5006 VECTOR Laboratories, Inc.	*mouse* IgG and immunoglobulins	$^1/_{200}$ $^1/_{500}$
Hoechst 33342	B2261, Sigma-Aldrich		$^1/_{200}$

All antibody-dilutions for western blotting purposes were made up in TBS-T. PBS was used to dilute antibodies for immunofluorescent staining to the required concentrations.

3.3 GENERAL METHODS

3.3.1 Tissue Culture

3.3.1.1 Cells

C2C12 cells are a satellite cell-line of murine origin, capable of proliferation, differentiation and fusion into myotubes. These cells, donated by the Cape Heart Centre, University of Cape Town, were used to investigate the effect of TGF-β isoforms on the proliferation, differentiation, migration and fusion of skeletal muscle.

Cells from the P19 cell-line are embryonal carcinoma cells which can change phenotype from malignant to non-malignant via cellular differentiation. These cells, originally isolated from an experimental embryo-derived teratocarcinoma in mice, are multipotent and can differentiate into cell types from all three germ layers. Retinoic acid (Edwards and McBurney, 1983), oxytocin (Paquin *et al.*, 2002) and dimethyl sulfoxide (DMSO) (Smith *et al.*, 1987) have been found to be inducers of P19 cardiomyocyte differentiation, possibly through the activation of essential cardiogenic transcription factors, such as GATA-4 and Nkx2.5 (Skerjanc, 1999; Srivastava and Olson, 2000).

Efficient differentiation of P19 cells depend of the prior formation of non-adhering embryoid bodies which resemble the inner cell mass of the embryo. In these embryoid bodies, initial differentiation occurs when the outer cells of the aggregates differentiate into endoderm-like cells that surround an undifferentiated core. With this capacity to form cardiomyocytes, P19 cells, obtained from M.W. McBurney, University of Ottawa, Canada, were used to study the effect of TGF-β isoforms on cardiac cell differentiation. DMSO was used to induce cardiac differentiation.

3.3.1.2   Medium

C2C12 culture medium:

- ‣ Dulbecco's Modified Eagle's Medium (DMEM; Highveld Biological (Pty) Ltd)
- ‣ 10% foetal bovine serum (FBS, CN-3107; Highveld Biological)
- ‣ 4% 2 mM L-glutamine (Sigma)
- ‣ 1% PenStrep (Highveld Biological)

C2C12 differentiation medium:

- ‣ DMEM
- ‣ 1% donor herd horse serum (CN-3089; Highveld Biological)
- ‣ 4% 2 mM L-glutamine
- ‣ 1% PenStrep

P19 culture medium:

- ‣ Alpha MEM with 1.5 g/ℓ NaHCO$_3$ (CN-3098; Highveld Biological)
- ‣ 7.5% newborn calf serum (N-4637; Sigma)
- ‣ 2.5% FBS
- ‣ 1% PenStrep

P19 differentiation medium:

- ‣ Alpha MEM with 1.5 g/ℓ NaHCO$_3$ (CN-3098; Highveld Biological)
- ‣ 7.5% newborn calf serum (N-4637; Sigma)
- ‣ 2.5% FBS
- ‣ 1% PenStrep
- ‣ 0.8% DMSO (D-5879, Sigma)

3.3.1.3   Passaging protocol

‣   Discard old medium from T75 flask (tissue culture flask 658175, Greiner Bio-One).

‣   Rinse cells with warm (~37°C), sterile PBS [to remove all traces of FBS] and decant. Use enough PBS to cover the monolayer of cells.

‣   Add 3 mℓ warm 0.25% trypsin-EDTA (T-4049, Sigma) and return flask to incubator (~37°C) [to increase enzyme activity] until cells have detached from the surface.

‣   Add 6 mℓ warm culture medium (or double trypsin-volume) [the serum in the culture medium inactivates the trypsin] to the cell-suspension and transfer the total volume to a 15 mℓ falcon tube - centrifuge 3 minutes at 1500 rpm.

‣   Decant medium and re-suspend cell-pellet in fresh culture medium.

‣   Return cell aliquots to new T75 flasks or plate desired amount as required.

‣   Cells were maintained in a humidified incubator at 37°C, 5% CO_2.

3.3.2   Protein Analysis Methods

3.3.2.1   Determination of protein concentrations

To estimate protein concentrations, a BSA-standard curve (0-20 μg protein) was prepared from a stock solution of 1 μg/μℓ. 1-5 μℓ of each sample to be analysed was diluted with dH$_2$O and 800 μℓ Bradford protein assay reagent (B6916, Sigma-Aldrich) added for a final volume of 1 mℓ. The absorbance was measured at 595 nm (UV-Visible Spectrophotometer, Cary 50) using the computer software *Simple Read* (version 2, WinUV, Cary 50).

3.3.2.2   Western blot analysis

Protein expression was determined by standard Western blotting techniques. Briefly, 50 μg whole cell homogenate of each sample was prepared with equal volume 2x sample buffer. Samples were boiled for 4-5 minutes, centrifuged and loaded onto 5% or 10% polyacrylamide gels for electrophoretic separation. Molecular weights were estimated by comparison with pre-stained molecular weight markers and confirmed with positive control samples (skeletal muscle or cardiac tissue). Mini-gels were run at 100 V for 90-120 minutes using the Mini-Protean 3 Gel System (Bio-Rad).

Following electrophoresis, proteins were transferred from SDS-page gels onto PVDF membranes (Immun-Blot 0.2 μm pore size, Bio-Rad; Immobilon-P, IPVH00010 Millipore) using the Mini Trans-Blot Cell (Bio-Rad) blotting apparatus. The PVDF membranes, which had been rinsed in methanol, and 3 mm chromatography/filter paper (Chr 303 0917, Whatman) were cut to the size of the gel (10 x 7.5 cm) and soaked in transfer buffer.

One fiber pad and two sheets chromatography/filter papers were carefully placed on the anode tray of the gel holder cassette, followed by the gel and PVDF membrane. Two additional sheets chromatography/filter paper and a fiber pad were placed on top and the cathode tray of the gel holder cassette placed over the stack and sealed, all the time taking care that no bubbles were introduced between the gel and PVDF membrane. Transfer of proteins was carried out by applying a current set at 100 V for 1 hour.

Following electroblotting, the gels were put into Coomassie Blue stain to determine the effectiveness of the transfer process. The PVDF membranes were incubated in TBS-T containing 5% skimmed milk powder for 60 minutes at room temperature to block non-specific binding sites. Thereafter, membranes were incubated in primary antibody for 2-3 days at $4^\circ C$. The PVDF membranes were routinely incubated in Ponceau-S to confirm equal loading, after which they were washed in TBS-T and re-probed in primary antibody.

For protein detection, membranes were washed in TBS-T for a total of three times, 5 minutes each, after which they were incubated in compatible horseradish-peroxidase conjugated secondary antibody at room temperature for 60 minutes. Secondary antibodies were diluted $1/_{1000}$ in TBS-T containing 5% skimmed milk powder. Membranes were again washed in TBS-T, twice for 8 minutes each, followed by TBS for 15-20 minutes.

Antigen-antibody complexes were visualised by enhanced chemiluminescence (ECL Plus) according to the manufacturer's instructions (Amersham Life Science Inc., Arlington Heights, IL, USA). Detection reagents were mixed 1:40 (vol/vol) in a sufficient volume to cover the complete surface of the membrane, which was exposed to this solution for 5 minutes. Thereafter, the membrane was placed in a cassette, covered with transparency paper and developed using hyperfilm (RPN-2103K, Amersham BioSciences; Fixing- and Developing solutions from Axim, 9X23013 and 9X23018, respectively).

3.3.2.3   Quantification of measurements

Protein expression levels were quantified using *Simple PCI*, version 4.0 (Compix Inc., Imaging Systems, USA) for densitometry. Each sample was evaluated in duplicate and all experiments were repeated a minimum of three times.

CHAPTER 4

DEVELOPMENT OF MODELS AND METHODS

4.1   INTRODUCTION

Analysis of the progress of proliferation and differentiation in stem cell-systems is often reliant on the expression of proteins as markers of the developmental and growth status of the system. Various *in vitro* molecular markers have been established to identify specific cell populations and/or the developmental stage of the cell which could provide information regarding the molecular regulation of the cell population during growth and regeneration (Table 4.1).

However, although several stem and progenitor cell markers have been identified, very few are restricted to an individual stage of development such as quiescence, activation or proliferation. Rather, they are expressed more broadly and not exclusive to one particular cell type or one developmental phase, which could lead to difficulties in the accurate characterisation of cell growth and differentiation stages. Specifically in skeletal muscle, the expression of selected molecular markers, used in *in vitro* and *in vivo* studies, has been demonstrated and suggested to be typical of particular stages of satellite cell myogenesis. The profile of gene expression of these markers (Table 4.2) often extends through phases of quiescence, activation and/or proliferation, clearly illustrating that markers typical of a developmental stage are largely inconclusive (Hawke and Garry, 2001).

Table 4.2 illustrates the expression-profiles of selected molecular markers typical of each stage during satellite cell myogenesis, as well as markers specific to cardiomyocyte differentiation (Charge and Rudnicki, 2004; Habets *et al.,* 2003; Hawke and Garry, 2001).
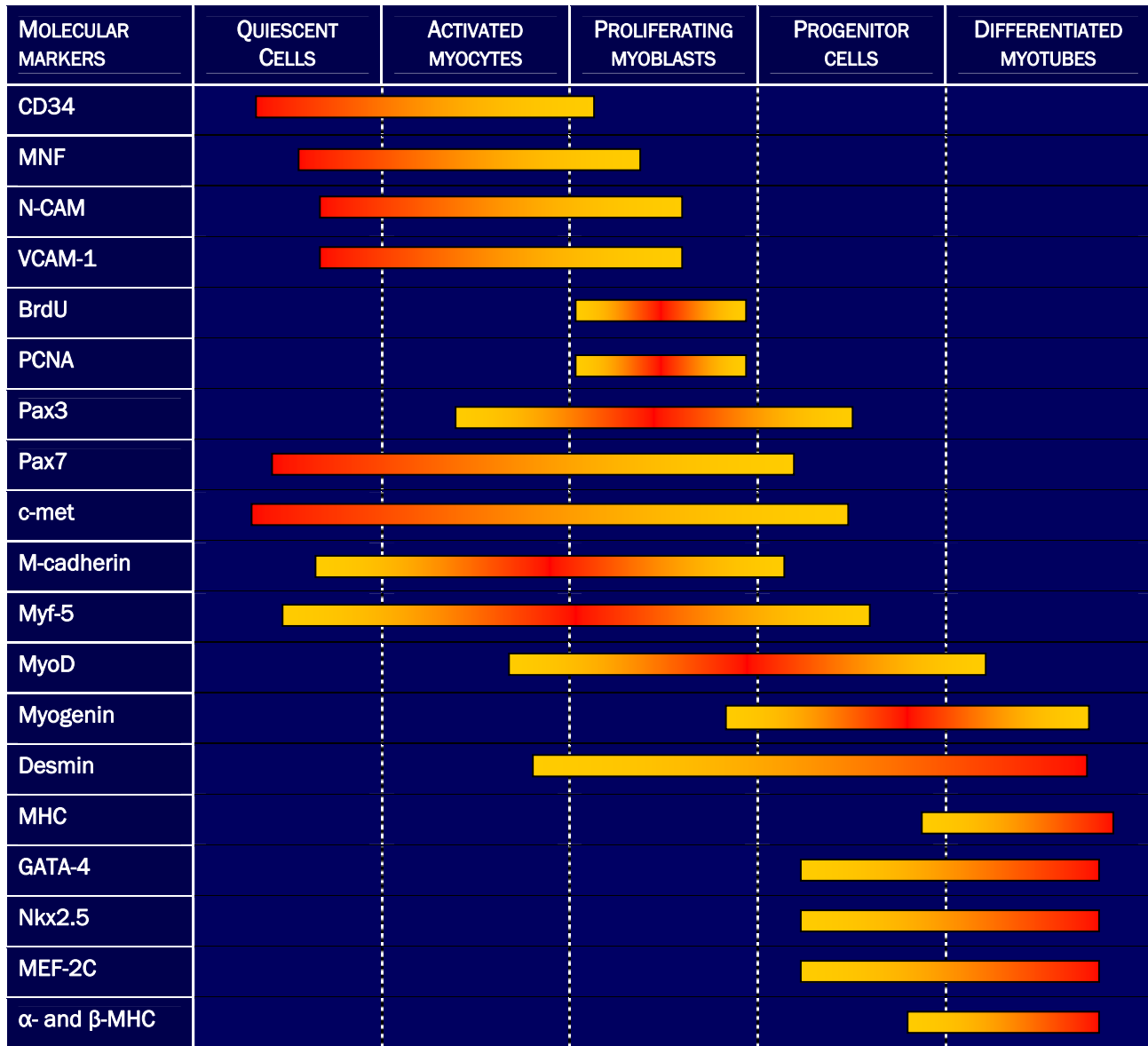
Table 4.1. Selected stem cell markers and expression for positive identification.

MARKER	FUNCTION	EXPRESSION IN:
CD34	gene, transmembrane glycoprotein	‣ quiescent satellite cells ‣ stem cells; endothelial/haematopoietic progenitor cells
CD45	gene, tyrosine phosphatase	‣ primitive haematopoietic stem cells
CD56/N-CAM	gene, cell adhesion glycoprotein	‣ quiescent and activated satellite cells ‣ neural tissue ‣ various leukocytes (e.g. natural killer cells)
CD133 (AC133)	gene, transmembrane glycoprotein (human cell surface marker)	‣ haematopoietic stem cells ‣ early endothelial progenitor cells ‣ neuronal stem cells
c-kit+	transmembrane glycoprotein, receptor for the haematopoietic growth factor, SCF	‣ cardiac stem cells ‣ haematopoietic stem cells
c-met	hepatocyte growth factor receptor	‣ stem cells; quiescent and proliferating satellite cells
CXCR4	cell surface chemokine receptor-4, specific for SDF-1	‣ stem cells - haematopoietic-, neural-, liver-, TCSC, and satellite cells
ETS1	transcription factor	‣ cardiac stem cells - endothelial-committed progeny
Lin-	refers to *"lineage negative"*	‣ bone marrow-derived cells that express no differentiation markers
GATA-4	transcription factor	‣ early cardiac stem cells - myocyte-committed progeny
GATA-6	transcription factor	‣ cardiac stem cells - smooth muscle-committed progeny
MDR1	transporter gene	‣ cardiac stem cells ‣ haematopoietic-, other bone marrow-derived stem cells
MEF-2C	transcription factor	‣ cardiac stem cells - myocyte-committed progeny
MyoD	skeletal muscle-specific transcription factor	‣ proliferating satellite cells and early stages of myogenic determination
Myogenin	skeletal muscle-specific transcription factor	‣ myogenic commitment of satellite cells
Myf-5	skeletal muscle-specific transcription factor	‣ quiescent, proliferating and early stages of satellite cell myogenic determination
M-cadherin	adhesion molecule	‣ quiescent and to a lesser extent activated satellite cells, all myogenic cells
MHC	skeletal muscle structural protein	‣ satellite cell terminal differentiation ‣ advanced cardiac differentiation
MNF	transcription factor	‣ satellite cells; activated myogenic progenitor cells
Nanog	transcription factor	‣ pluripotent ESC
Nkx2.5	transcription factor	‣ early cardiac marker expressed throughout myocardium
Oct-3/4	transcription factor/germline specific gene	‣ pluripotent (undifferentiated) ESC ‣ cardiac stem cells
Pax 3/7	transcription factors	‣ quiescent and activated satellite cells
Sca-1+	cell surface protein	‣ multipotent stem and progenitor cells ‣ cardiac and early haematopoietic stem cells
SDF-1	1-α, 1-β cytokines, CXCR4 ligands	‣ bone marrow stromal cells
Sox-2/8	transcription factor	‣ pluripotent ESC
Six-1/4	homeoproteins	‣ early skeletal myogenesis
Syndecan 3/4	cell surface proteoglycan	‣ satellite cells
UTF1	transcriptional co-activator	‣ pluripotent ESC

ETS1, external transcribed spacer 1; **Lin-**, lineage commitment; **MDR1**, multi-drug resistance protein 1; **MEF-2C**, myocyte enhancer factor-2C; **MHC**, myocin heavy chain; **MNF**, myocyte nuclear factor; **N-CAM**, neural cell adhesion molecule; **Sca-1**, stem cell antigen-1; **SCF**, stem cell factor; **SDF-1**, stromal cell-derived factor-1; **TCSCs**, tissue-committed stem cells; **UTF1**, undifferentiated embryonic cell transcription factor 1.

Table 4.2. Expression of molecular markers for various stages of differentiation in skeletal and cardiac muscle.

[Adapted with modifications from Chargé and Rudnicki, 2004; Hawke and Garry, 2001; Zammit et al., 2006]

MOLECULAR MARKERS	QUIESCENT CELLS	ACTIVATED MYOCYTES	PROLIFERATING MYOBLASTS	PROGENITOR CELLS	DIFFERENTIATED MYOTUBES
CD34					
MNF					
N-CAM					
VCAM-1					
BrdU					
PCNA					
Pax3					
Pax7					
c-met					
M-cadherin					
Myf-5					
MyoD					
Myogenin					
Desmin					
MHC					
GATA-4					
Nkx2.5					
MEF-2C					
α- and β-MHC					

BrdU, 5-bromo2'-deoxy-uridine; MEF-2C, myocyte enhancer factor-2C; MHC, myosin heavy chain; MNF, myocyte nuclear factor; N-CAM, neural cell adhesion molecule; PCNA, proliferating cell nuclear antigen; VCAM-1, vascular cell adhesion molecule-1 (predominant phase of expression in red).

C2C12 and P19 cell-lines express a variety of proteins which can be used to identify stages of their growth and development along the myogenic lineage. These cell-lines have been used as model systems of myogenic development in many laboratories. It needs to be kept in mind however that the expression of molecular markers by these cell-lines may differ from satellite cells and myoblasts *in vivo*.

Also, primary culture myoblasts isolated by tissue dissection may have a similar problem due to the rapidity with which molecular markers change during the process of isolation (Dhawan and Rando, 2005). Due to the added difficulty of primary culture isolation and maintenance, the C2C12 and P19 cell-lines were chosen as experimental models of myogenesis. In order to determine the effect which TGF-β isoforms have on the various phases of growth and development of these cell-lines, it was first required to identify and quantify the expression of markers under control conditions, as well as to establish reliable protocols for analysis. This is described below.

4.2 METHODS FOR ASSESSING PROLIFERATION AND DIFFERENTIATION

To establish specific proteins as markers which would reflect stages of *in vitro* skeletal and cardiac muscle proliferation and differentiation, protein expression was analysed in C2C12 and P19 cell-lines by means of standard western blotting techniques and immunohistochemistry. These cell-lines and analysis techniques were not established in our laboratory at the start of this research project.

4.2.1 Cell Culture

C2C12 and P19 cells were cultured and differentiated as described in Chapter 3. Profiles of protein expression were determined under control conditions, providing a baseline by which to measure the effect any treatment-condition would have on these culture systems.

4.2.1.1 *C2C12 differentiation*

C2C12 cells were maintained in culture medium and when ~40-50% confluent (in T75 flasks, day -1), all cells were pooled, counted and plated into six-well tissue culture-treated plates (3516, Corning Incorporated) in 2 mℓ culture medium at a density of 100 000 cells/well. When cells reached ~70% confluency (day 0), the medium was changed to mitogen-poor, differentiation-promoting medium. Thereafter, the cells were maintained in a humidified incubator at 37°C, 20% O_2, 5% CO_2 and the differentiation medium changed every 48 hours.

For western blotting analysis, the cells were harvested on days 0, 1, 3, 5, 7, 9 and 11. To prepare cells, the medium was removed and cells washed with PBS. While keeping the samples on ice, they were treated with lysis buffer, sonicated and stored at -20°C until later analysis. For immunofluorescent staining and determination of total nuclear count (TNC), C2C12 cells were differentiated, prepared and analysed as described in *section 4.4.1* and *4.4.2.*
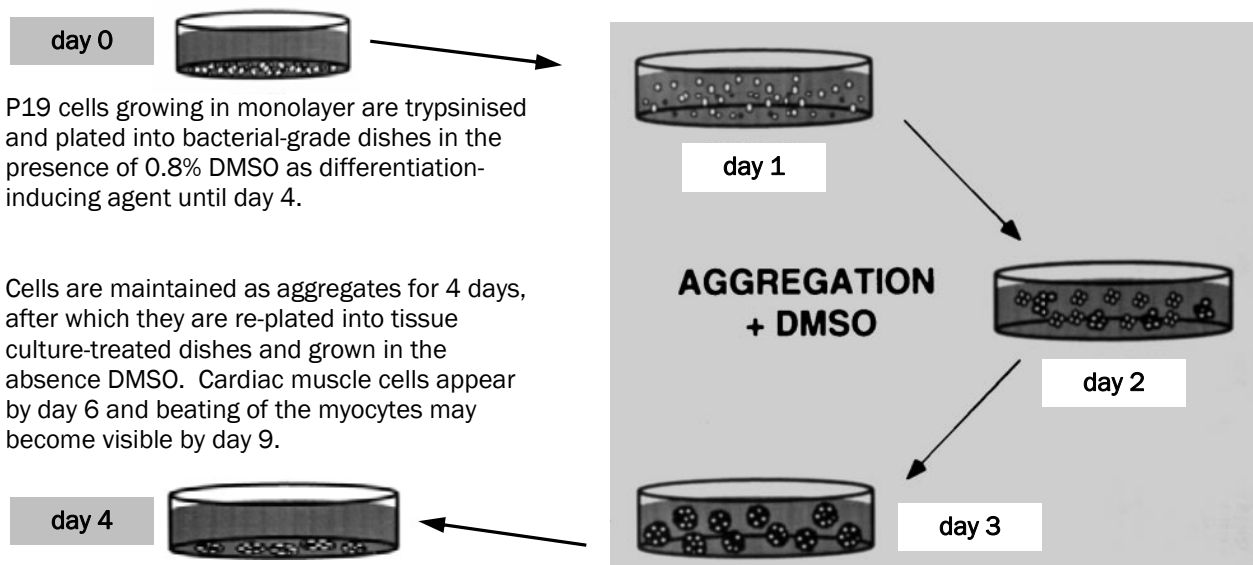
4.2.1.2   P19 differentiation

P19 cells were differentiated according to a modified version of the method of Skerjanc (McBurney, 1993; Skerjanc, 1999; Smith *et al.*, 1987). Briefly, differentiation was initiated by plating 500 000 P19 cells in 60 mm bacterial-grade dishes in the presence of 0.8% DMSO (D-5879, Sigma) and P19 culture medium (day 0). After 24 hours (day 1), the aggregates were transferred to 100 mm bacterial-grade dishes and new media containing 0.8% DMSO added. Aggregates were maintained in 100 mm bacterial-grade dishes, but the medium changed and fresh medium containing 0.8% DMSO again added on days 2 and 3. On day 4, the cells were re-plated into 100 mm tissue culture-treated dishes and differentiation of the cells continued in P19 culture medium which was changed every second day (Figure 4.1). The cells were maintained in a humidified incubator at 37°C , 20% O_2 and 5% CO_2.

Cells were harvested on days 6, 8, 10, 12 and 14 for western blotting purposes: after washing the cells with PBS, they were treated with lysis buffer, sonicated and whole cell-lysates stored at -20°C until later analysis.

Figure 4.1. Schematic representation of P19 cell differentiation into muscle. Differentiation is initiated by allowing the cells to aggregate in suspension in the presence of DMSO. Cells are plated into bacterial-grade dishes which prevent adherence of cells to the dish and promote aggregation. Aggregates are re-plated into tissue culture-treated dishes after 4 days and allowed to differentiate into cardiac muscle cells.
[Adapted with modifications from Skerjanc, 1999]

day 0

P19 cells growing in monolayer are trypsinised and plated into bacterial-grade dishes in the presence of 0.8% DMSO as differentiation-inducing agent until day 4.

Cells are maintained as aggregates for 4 days, after which they are re-plated into tissue culture-treated dishes and grown in the absence DMSO. Cardiac muscle cells appear by day 6 and beating of the myocytes may become visible by day 9.

day 4

day 1

AGGREGATION + DMSO

day 2

day 3

Following these cell culturing protocols, protein expression was determined by standard western blotting techniques as described in Chapter 3 (*section 3.3.2*). Myoblast development in the C2C12 cell-line was assessed using PCNA as a marker of proliferation, whereas MyoD, myogenin, the γ3-isoform of AMP-activated protein kinase, Rho-associated protein kinase and MHC were used as potential markers of differentiation. Cardiac development in differentiating P19 cells was assessed using connexin-43 and α-actinin. Following electrophoresis, either Coomassie Blue, Ponceau-S or α-tubulin was used to verify equal loading of protein samples.

In addition, the TNC was also used to assess proliferation in C2C12 cells by determining the number of nuclei in differentiating cells at various time-points.

All brightfield images were taken with an Olympus microscope and camera (Olympus CKX 31) at 10x or 20x magnification. Data are expressed as mean $\pm$ SEM.

4.2.2   Establishment of Markers for C2C12 Proliferation

The *in vitro* pattern of PCNA expression in differentiating cells is described below. Although MyoD is also expressed during proliferative stages of development, this protein plays an essential role committing cells to the myogenic lineage and is therefore discussed as marker of differentiation.

4.2.2.1   PCNA

The proliferating cell nuclear antigen (PCNA) is a 36 kDa nuclear protein also termed cyclin (Mathews *et al.*, 1984) and is expressed during the proliferative phase of the cell cycle (Hall *et al.*, 1990). PCNA interacts with various other proteins to be involved in important metabolic and cellular processes, including cell cycle control, apoptosis, and DNA replication and repair (Daimon *et al.*, 2002; Paunesku *et al.*, 2001; Scovassi and Prosperi, 2006). This protein acts as a co-factor for DNA polymerase-δ and functions by tethering the DNA polymerases onto the DNA template to accomplish progressive DNA synthesis during replication.

PCNA expression correlates directly with rates of cellular proliferation and DNA synthesis during the cell cycle: it appears in the nucleus during the late G_1-phase, becomes maximal during the S-phase and declines again during G_2- and M-phases (Celis *et al.*, 1986; Szuts *et al.*, 2005). Specifically, PCNA accumulates in the *nucleolus* during late G_1-/early S-phases (Louis *et al.*,

1991; Mathews *et al.*, 1984; Takasaki *et al.*, 1981) where it exhibits a granular distribution. Despite being mainly active during G_1- and S-phases, the relatively long half-life (in excess of 20 hours) leads to PCNA expression in cells which have left the cell cycle and are therefore not synthesising DNA (Bravo and Macdonald-Bravo, 1987).

PCNA is commonly used as a marker to follow proliferation and progression of satellite cells in the cell cycle (Johnson and Allen, 1993). The expression of this protein was determined in differentiating C2C12 cells, as illustrated in Figure 4.2.

Figure 4.2. PCNA expression in differentiating C2C12 cells.
Data are expressed as mean ± SEM; n = 2.
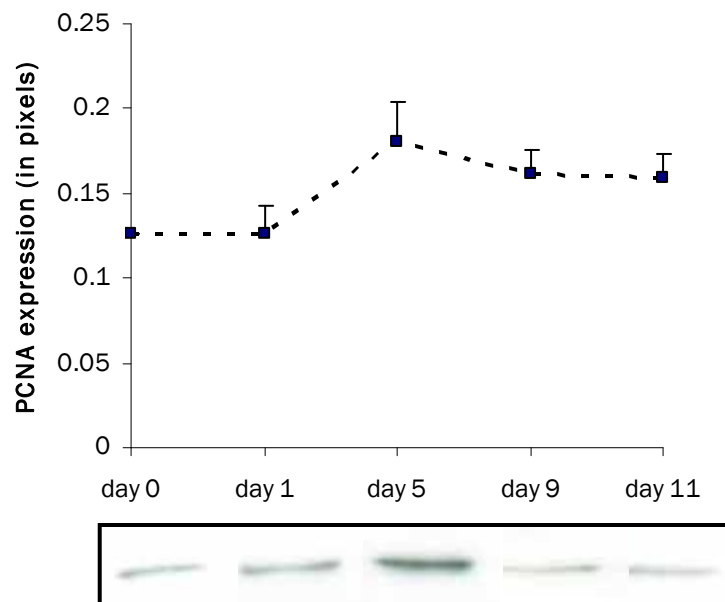
After cells were induced to differentiate, increased PCNA expression was evident only by day 5 and remained elevated until day 11. This result could possibly be explained by the lack of using synchronised cells: in response to the differentiation signal on day 0, the immediate reaction for those cells that are ready to differentiate is that they start doing so, whereas cells preparing to divide continue through the cell cycle which results in increased proliferation at a later stage. Alternatively, differentiating cells may signal to other still undifferentiated cells to continue proliferation and increase in number for optimal differentiation to take place at a later stage.

4.2.2.2   Total nuclear count

Terminal differentiation is characterised by the transition of hyperplastic growth (cell division and increased nuclear numbers) to hypertrophic growth (increase in cell size and fusion) (Poolman *et al.*, 1999). Nuclear staining provided a practical method for quantifying the total amount of nuclei (Figure 4.3) and possibly distinguishes between phases of proliferation (increased TNC and hyperplasia) and the onset of differentiation (cell growth through hypertrophy and limited changes in TNC) in a cell system.

Figure 4.3. Total nuclear count in differentiating C2C12 cells. Data are expressed as mean ± SEM; n = 1.
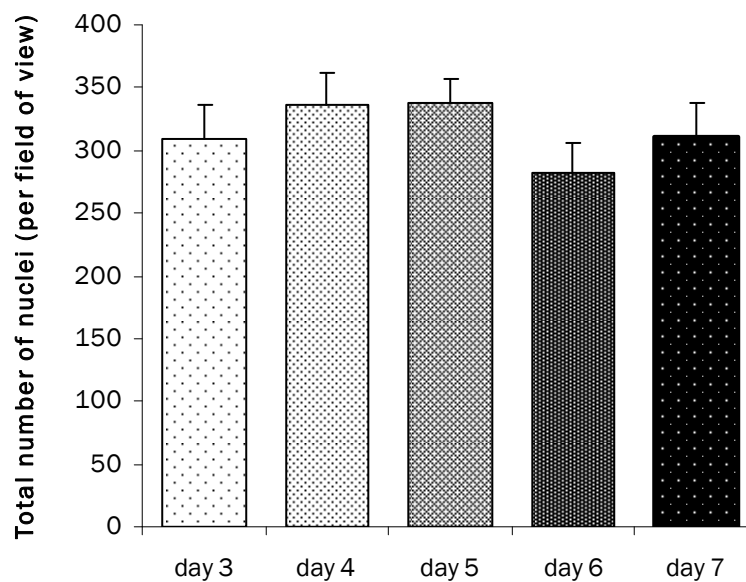
The TNC showed little change in the differentiating cells between day 3 and day 7. The results suggests that if measured, an increase in PCNA would have been evident at day 3 in Figure 4.2, or that, had TNC been measured at day 1, there would have been an increase in TNC to day 3. This observation could also suggest that an increase in apoptosis accompanied the increased proliferation (seen in Figure 4.2) and therefore the total nuclear number did not change. Unfortunately, apoptosis was not measured as this was not within the scope of the study.

4.2.3   Establishment of Markers for C2C12 Differentiation

C2C12 cells were differentiated *in vitro* to characterise the expression-profiles of selected cell cycle and myogenic regulatory factors. MyoD, myogenin and MHC were assessed as established markers of differentiation, whereas the effectiveness of the γ3-isoform of AMP-activated protein kinase (γ3-AMPK) and Rho-associated protein kinase (ROCK) were evaluated as novel markers.

The brightfield images in Figure 4.4 illustrate the progression of differentiation in C2C12 cells. Mononucleated myoblasts (A) rapidly proliferate, resulting in an increased number of myogenic precursor cells (B). These myocytes can then either fuse together or with existing myofibers to form multinucleated myotubes (C), *or* continue to proliferate and maintain the supply of myocytes, *or* alternatively, remain in an undifferentiated state and return to quiescence.

Figure 4.4. Morphological characteristics of differentiating C2C12 cells. Following initiation of differentiation, myoblasts (A) proliferate, allowing for expansion of the myocyte population (B). The proliferative phase is followed by terminal differentiation and fusion of myogenic precursor cells into elongated, multinucleated myotubes (C).

A   B   C
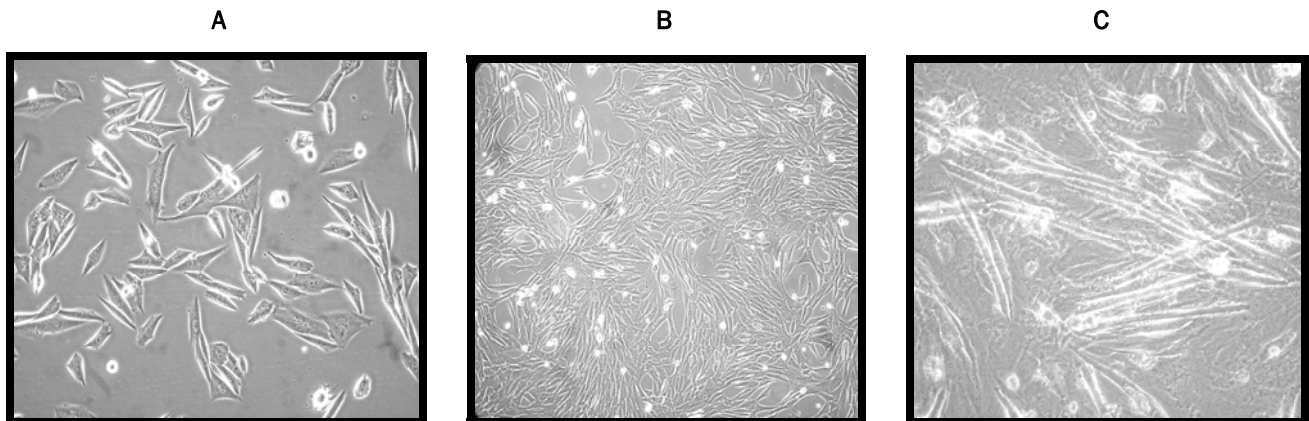
4.2.3.1   MyoD

As discussed in Chapter 2, MyoD is a member of the family of *basic* helix-loop-helix (bHLH) transcription factors and plays an essential role in the regulation of muscle cell development. This protein is expressed in the nucleus of proliferating myoblasts, as well as in differentiating myotubes (Tapscott *et al.*, 1988), though expression decreases once cells reach committed stages of differentiation (Cooper *et al.*, 1999; Cornelison and Wold, 1997).

In established muscle cell-lines and during development, only subsets of MRFs are active rather than all being expressed simultaneously. *In vitro*, either MyoD or Myf5 are initially up-regulated and expressed in proliferating myoblasts with subsequent transcription of both these genes (Cornelison and Wold, 1997; Weintraub, 1993). However, only MyoD expression continues during the committed stage of differentiation.

MyoD expression is controlled by cellular factors, extrinsic signals and has also shown to activate its own transcription (Salminen *et al.*, 1991; Thayer *et al.*, 1989). Together with myogenin, such auto-activation could either provide a positive feedback loop to keep cells committed to myogenesis, or function as a mechanism to increase its expression above a critical threshold which is required for activation of the myogenic program once the genes are activated by upstream factors. MyoD levels fluctuate within the cell cycle, the highest levels observed during the G_1-phase when differentiation can be initiated (Kitzmann *et al.*, 1998). If proliferative signals persist, MyoD expression eventually declines, whereas in response to cues for differentiation, the cells will exit the cell cycle, MyoD levels will increase and differentiation will be induced.

Therefore, as a reliable *in vivo* marker of stages of development, MyoD was assessed to identify its expression-pattern *in vitro*, and as such the use of this MRF as a marker of commitment to differentiation, as indicated in Figure 4.5.

Figure 4.5. MyoD expression in differentiating C2C12 cells.
Data are expressed as mean ± SEM; n = 2.
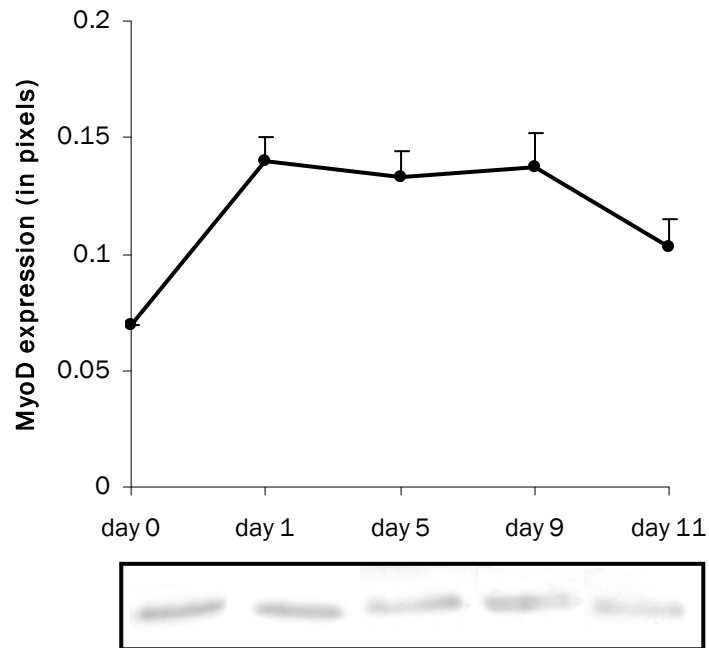
Following induction of differentiation, MyoD expression immediately increased and remained elevated during early stages of the differentiation programme. Expression decreased from day 9 after cells entered terminal differentiation phases.

4.2.3.2   Myogenin

Myogenin transcription in satellite cells or myoblasts is activated following expression of MyoD and/or Myf-5 in the committed stage once cells have been induced to differentiate (Olson, 1990; Weintraub *et al.*, 1991). *In vivo*, this would correspond to times of muscle repair and regeneration, whereas *in vitro* it occurs when serum is depleted from the culture medium. Therefore, this regulatory protein accumulates in differentiating myoblasts and myotubes (Salminen *et al.,* 1991) and is largely responsible for subsequent transcription of most muscle-specific structural genes.

Myogenin can therefore be used to detect relatively early stages of skeletal muscle differentiation (Hollenberg *et al.*, 1993; Miner and Wold, 1990), as shown in Figure 4.6.

Figure 4.6. Myogenin expression in differentiating C2C12 cells.
Data are expressed as mean ± SEM; n = 2.
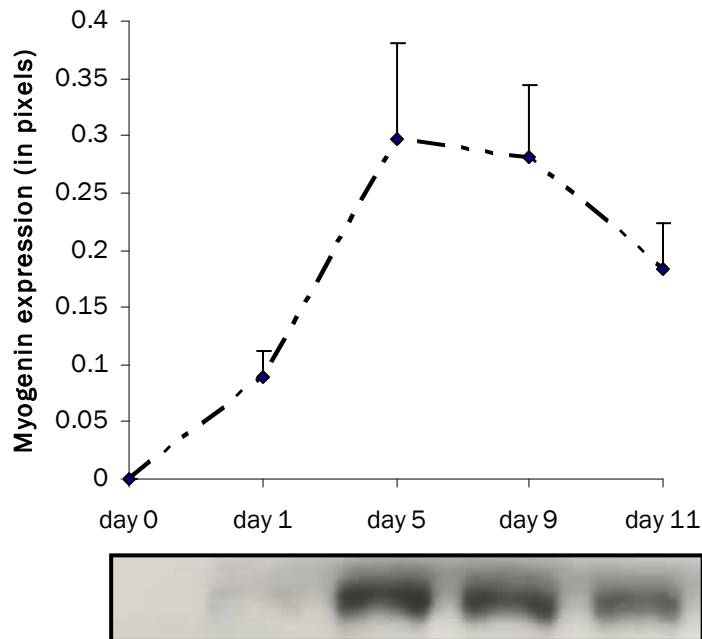
Low levels of myogenin were seen as early as day 1 post-differentiation, after which it increased to day 5, remained elevated until day 9 and slowly decreased thereafter, although it remained detectable until day 11.

4.2.3.3   AMP-activated protein kinase

The γ3-isoform of AMP-activated protein kinase (γ3-AMPK) is an important energy-sensing enzyme that monitors the cellular energy status. This protein plays an important role in stimulation of skeletal muscle fatty acid oxidation and muscle glucose uptake to regulate glucose and lipid metabolism in skeletal muscle (Barnes *et al.*, 2002). Specifically, activation of γ3-AMPK is required for insulin-independent stimulation of glucose uptake in resting skeletal muscle (Jessen *et al.*, 2003). Although γ3-AMPK is most abundant in skeletal muscle, it is expressed in most mammalian tissues (Ruderman *et al.*, 2003).

Initial results showed that although γ3-AMPK is expressed at a low level in C2C12 myoblasts (day 3), this expression increased with differentiation into myotubes (day 7), suggesting that γ3-AMPK could be used as a marker of terminal myogenic differentiation (Figure 4.7 A). However, subsequent repeat-analysis failed to show consistent measurable changes during differentiation of these cells (Figure 4.7 B) and therefore γ3-AMPK expression was not seen to be a reliable marker of differentiation in this cell-line.

Figure 4.7. γ3-AMPK expression in differentiating C2C12 cells.
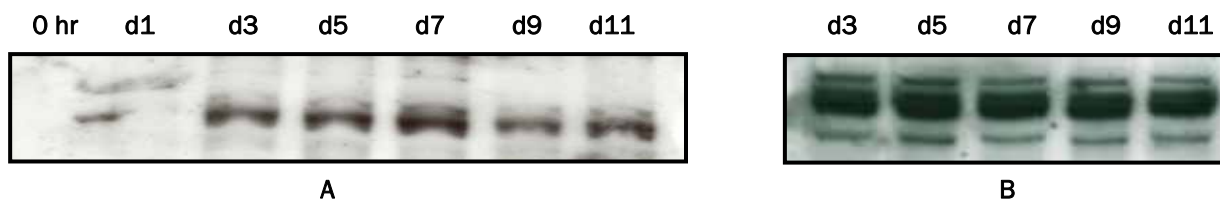
4.2.3.4   *Rho-associated protein kinase*

Rho-GTPases and one of its downstream effectors, Rho-associated protein kinase (ROCK), are involved in many aspects of cell motility, cell adhesion and are important regulators of cell growth, migration and apoptosis via control of the actin-cytoskeletal assembly (Ridley, 2001).

In the mammalian system, the serine-threonine kinase ROCK consists of two isoforms, ROCK1 and ROCK2 which are ubiquitously expressed in various tissues (Amano *et al.*, 2000). In addition to the above-mentioned functions, Rho and ROCK also play a critical role in skeletal muscle differentiation: Rho signalling prevents myoblast fusion by activating ROCK, thereby negatively regulating differentiation (Nishiyama *et al.*, 2004). Therefore, at the late stage of differentiation when myoblasts are ready for terminal differentiation, Rho/ROCK signalling must be *inactivated* to initiate myoblast fusion and allow progression of the differentiation programme (Nishiyama *et al.*, 2004).

As such, the expression of ROCK was analysed as a possible measure of C2C12 terminal differentiation (Figure 4.8), with a *decline* in expression anticipated with progression of differentiation.

Figure 4.8. ROCK expression in differentiating C2C12 cells.
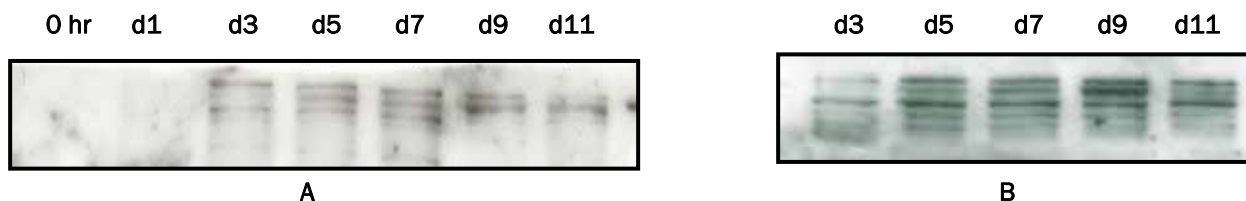
Initial blots failed to show consistent measurable results (Figure 4.8 A). As with γ3-AMPK, no differences in ROCK expression were observed in repeat trials (Figure 4.8 B) of differentiation and therefore this protein was also not included as a marker of differentiation in C2C12 cells.

4.2.3.5   *Myosin heavy chain*

Once mononucleated myoblasts fuse to form multinucleated myotubes, the muscle differentiation programme can be completed with the formation of muscle fibers. These highly organised cytoskeletal structures express various muscle-specific proteins, myosin heavy chain (MHC) being among the first of the sarcomeric proteins and thus has been used as a marker of muscle differentiation. MHC is the major component and most abundant protein of the sarcomere which is the basic contractile unit of myofibrils in skeletal and cardiac muscle fibers and responsible for converting chemical energy into mechanical force (Lu *et al.*, 1999).

Therefore, to confirm terminal muscle differentiation of the treated cell cultures in subsequent protocols, the expression of MHC was determined, as indicated in Figure 4.9.

Figure 4.9. MHC expression in differentiating C2C12 cells. Data are expressed as mean ± SEM; n = 2.
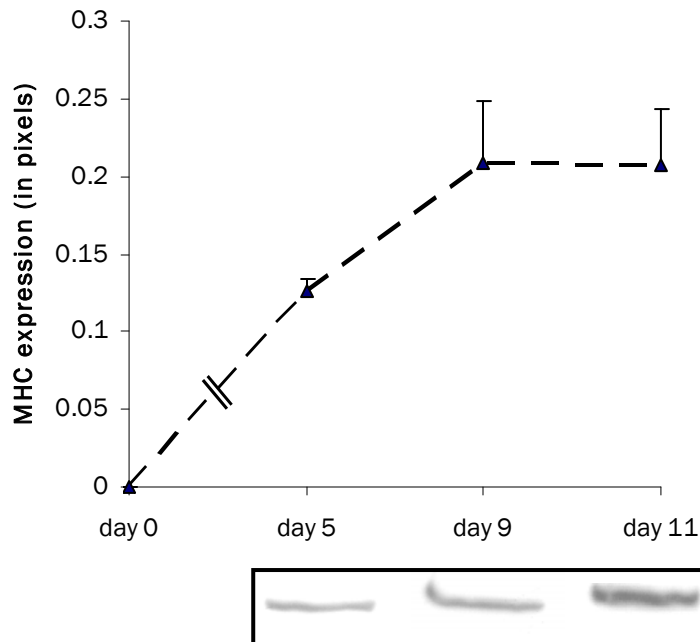
As expected, MHC was not expressed during early stages of development, but became detectable from day 5, after which the protein was strongly expressed by day 9 and day 11. MHC could therefore effectively be used as an indicator of terminal differentiation.

4.2.4　Establishment of Markers for P19 Differentiation

To identify reliable markers which would confirm successful differentiation in P19 cells, the expression of α-actinin and connexin-43 was assessed in this cell-line after induction of differentiation with DMSO. The brightfield images in Figure 4.10 illustrate the progression of differentiation in P19 cells.

Figure 4.10. Morphological changes in differentiating P19 cells. Differentiating P19 cells aggregate as early as day 1. After these aggregates are re-plated into tissue culture-treated dishes on day 4 of differentiation, they start forming embryoid bodies **(A, day 5)**. Once these embryoid bodies are grown to confluence **(B, day 9)**, beating of myocytes might become visible.

A   B

4.2.4.1　α-Actinin
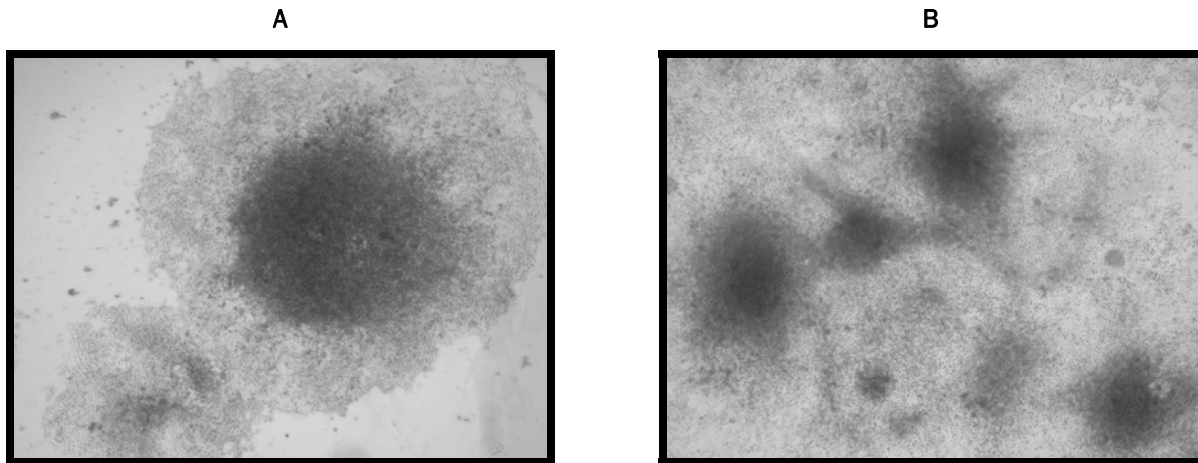
α-Actinin is a binding protein of ~100 kDa which is present in both muscle and non-muscle cells (Lazarides and Burridge, 1975). Functions of this protein include the binding of actin by cross linking two filaments together, associating with a number of cytoskeletal proteins, linking actin structures to membrane complexes, as well as binding cytoplasmic signalling proteins to the cytoplasmic domains of transmembrane receptors. Thus, α-actinin links signalling molecules to the cytoskeleton and participates in actin-organisation at the sites of signalling (Taylor *et al.*, 2000). In both cardiac and skeletal muscle, α-actinin is associated with the z-discs of the muscle sarcomeres (Goncharova *et al.*, 1992; Lazarides and Burridge, 1975). As such, α-actinin was assessed as possible marker of differentiated cardiac myocytes (Figure 4.11).

Figure 4.11. α-Actinin expression in differentiating P19 cells.

| d6 | d8 | d10 | d12 | d14 | cardiac muscle |
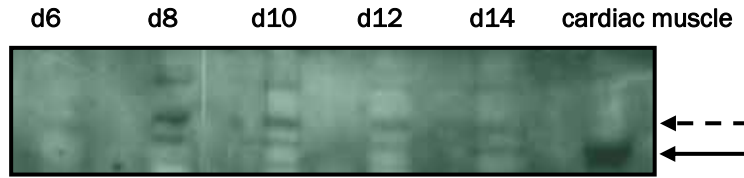
The α-actinin antibody was capable of detecting the protein in cardiac muscle (solid arrow), but not reliably in differentiating P19 cells (dashed arrow), with expression seemingly maximal at day 8, but diminishing thereafter. Multiple bands were also detected which were too vague to analyse and as such, this protein was not included as marker of differentiated P19 cells.

4.2.4.2   Connexin-43

A connexon, composed of six *connexin* proteins, forms intercellular channels known as gap junctions that connect the cytoplasm of adjacent cells. Connexins, also known as gap junction proteins and classified depending on their molecular weight, are translated by ribosomes and inserted into the membrane of the endoplasmic reticulum where the connexons are constructed. These connexons are then carried to the cell membrane where they connect with another cell's connexon to form the intercellular channel (Bennett and Zukin, 2004).

Connexin-43, a phosphoprotein, is one of the major connexins of the mammalian heart, although it is expressed by all myocytes, as well as non-muscle cells (Doble and Kardami, 1995). In the heart, these proteins are especially important for ensuring electrical and/or metabolic coupling between cells.

Figure 4.12. Connexin-43 expression in differentiating P19 cells.

| d6 | d8 | d10 | d12 | d14 | cardiac muscle |
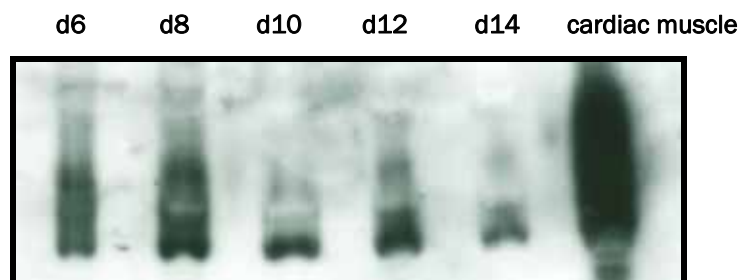
By immunoblotting, connexin-43 can be detected at 43 kDa in cardiac muscle and differentiating P19 cells (Figure 4.12). Connexin-43 was clearly expressed from day 6 and remained relatively unchanged for the duration of the differentiation protocol until day 14, at which point it decreased.

4.2.4.3   *Myosin heavy chain*

In addition to skeletal muscle MHC, two isoforms, consisting of either α- or β-homodimers of cardiac muscle-specific MHC, can also be distinguished in adult mammalian muscle (Hoh *et al.*, 1979). With its transcription activated by GATA-4, the α-isoform constitutes the major MHC-subunit of the adult heart (Molkentin *et al.*, 1994).

After probing the skeletal C2C12 cells with MHC which showed reliable expression (Figure 4.9), the antibody used which recognises all myofibers at all stages of development, was also applied to determine MHC expression in the P19 cell-line in the succeeding experimental protocols.

4.2.5   Summary and Conclusions

To determine the progress of proliferation and differentiation in cell culture systems, the expression patterns of cell cycle regulators, transcription factors and structural proteins were assessed in C2C12 and P19 cells to determine baseline profiles. Using these results, the effects which the three TGF-β isoforms exert on growth and development in skeletal and cardiac culture systems can subsequently be analysed under standardised conditions.

4.2.5.1   *C2C12 cells*

Skeletal myogenesis was induced in culture by depriving cells of serum, which resulted in the growth of multinucleated myotubes. During the differentiation programme, a co-ordinated induction of muscle-specific gene products occurs simultaneously with morphological changes of the cells (Olson, 1992). These changes in gene expression begin with the activation of primary MRFs, MyoD and Myf-5 during the committed stage, followed by myogenin and MRF4 as early markers for the entry of myoblasts into the terminal differentiation programme (Olson and Klein, 1994; Weintraub, 1993). Final stages of differentiation can then be indicated by the expression of contractile proteins such as MHC (Andres and Walsh, 1996). Figure 4.13 is a schematic representation of the cell cycle-related changes of the selected MRFs and proteins. By demonstrating their association with processes of quiescence, activation, proliferation and/or differentiation during the course of development and/or regeneration, the effect of TGF-β isoforms on the expression of these protein markers, as determined in the subsequent studies, could provide possible mechanisms by which TGF-β exerts its function during growth and development.

Under standard conditions of differentiation, C2C12 cells displayed characteristic expression of PCNA, MyoD, myogenin and MHC. C2C12 cells showed an increase in PCNA expression at day 5, after which levels were maintained until day 11, suggesting continued proliferation of undifferentiated C2C12 cells. Similarly, the TNC indicated constant proliferation between day 3 and day 7. In response to the differentiation stimulus, C2C12 cells showed immediate determination towards the myogenic lineage as demonstrated by increases in MyoD and myogenin expression from day 1. Myogenin increased to a maximum at day 5 and similarly to MyoD, remained elevated until decreasing at day 9. At this stage, evidence of structural differentiation was demonstrated with the increase in MHC expression at day 9 and day 11, indicating terminal differentiation stages.

In this preliminary analysis, γ3-AMPK and ROCK were also assessed as potential markers of differentiation. However, results revealed that their expression did not display consistent measurable changes which could be used to determine the progression of differentiation. As such, these proteins are not included in further experimental studies.

In the subsequent experimental procedures where the progress of differentiation in C2C12 satellite cells were analysed under different TGF-β isoform treatment conditions, days 1, 5, 9 and 12 were selected for determination of protein expression. These time-points were chosen to coincide with stages of undifferentiated myoblasts (day 1), myoblast shift prior to differentiation (day 5) and myotube stages (days 9 and 12) to represent terminal differentiation.

Figure 4.13. Schematic representation of the cell cycle, related changes in MRF expression and their association with processes of (A) quiescence, self-renewal and activation, (B) differentiation, (C) proliferation and (D) apoptosis.
For induction of differentiation, myogenic cells have to exit the cell cycle through the G_0-phase when cell cycle arrest of muscle division takes place. MyoD peaks mid-G_1, falls to a minimum at the G_1/S-phase transition and steadily increases again from the S- to M-phase to reach a lower peak on a second occasion, but is absent during G_0. As such, differentiation is prominent during the late G_1-phase. Signalling pathways driving proliferation, such as the expression of PCNA, must therefore be suppressed during the late G_1-phase to allow induction of differentiation. In addition, at the end of the G_1-phase, p21 up-regulation has been associated with permanent cell cycle arrest, allowing cells to exit the cell cycle. Since MyoD enhances p21 transcription, it is therefore possible that the decision for myoblasts to proliferate or differentiate relies on the effect of cell cycle signalling pathways influencing the level of MyoD expression. In contrast, Myf-5 expression is high during the G_0-phase decreases during the G_1-phase, re-appears at the end of G_1 and remains stable until mitosis. Therefore, cells in the G_1-phase express high levels of MyoD and enter differentiation, whereas in the G_0-phase, cells express high levels of Myf-5 and fail to differentiate. As an indicator of terminal differentiation and therefore the need of cell cycle withdrawal, myogenin is expressed during the G_0- and G_1-phases of the cell cycle.

Figure 4.13. Schematic representation of the cell cycle.
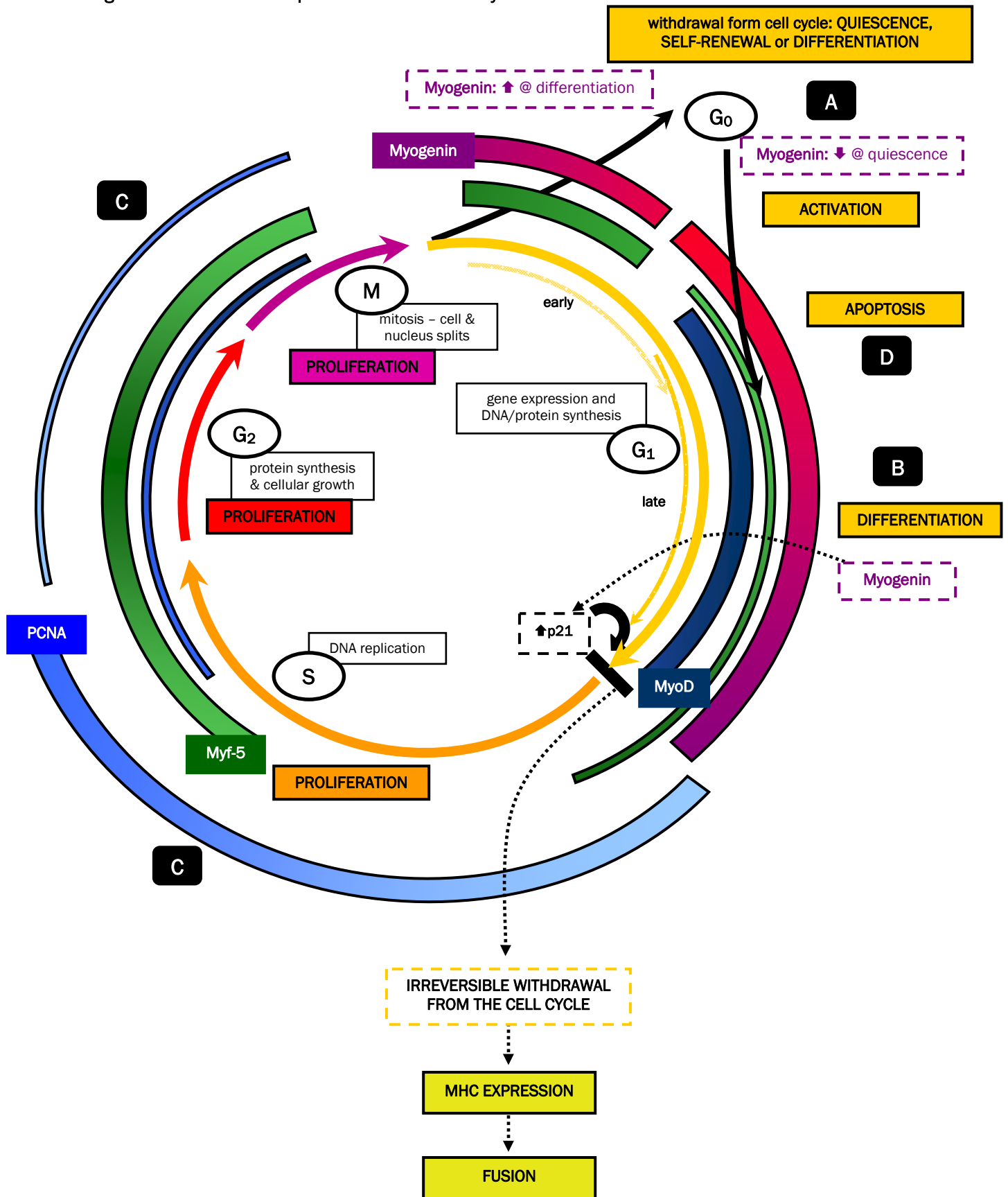
4.2.5.2 P19 cells

For the P19 cell-line, only terminal differentiated cells were analysed and as such, the structural proteins connexin-43 and α-actinin were assessed as markers for cardiomyocyte differentiation.

These proteins were established as positive markers, however, the α-actinin antibody did not express consistent results and as such, this protein was not included in further studies. Connexin-43, as well as MHC which demonstrated reliable expression in the C2C12 cell-line, were therefore used as positive markers of cardiomyocyte differentiation in the subsequent experimental procedures. Connexin-43 showed consistent expression from day 6 until day 14 and therefore day 12 was chosen to confirm successful differentiation of the P19 cells in these procedures.

4.3   METHODS FOR ASSESSING MIGRATION

Chemotaxis to the damaged area is promoted by factors released from the affected area. In order to establish the best method for cell migration analysis, various methods, treatment conditions and time-points were initially tested to set up a protocol for determining the effect of TGF-β on cell migration (*Chapter 7*). Cytokines such as IGF-1 and VEGF have shown to be involved in the regulation of tissue repair and to stimulate myocyte migration (Duan, 2003; Fiedler *et al.*, 2006; Germani *et al.*, 2003; Gockerman *et al.*, 1995; Grosskreutz *et al.*, 1999). In our initial experiments, VEGF was not consistently able to induce migration, whereas IGF-1 resulted in successful migration in all preliminary experiments and was therefore selected as growth factor to induce migration in all subsequent assays.

4.3.1   Cell Culture

Cells were allowed to migrate in a humidified incubator at 37°C, 20% O_2, 5% CO_2 and 80% humidity. Preliminary time-periods used to allow migration to occur, included 1-4 hours (Bischoff, 1997; Germani *et al.*, 2003; Grosskreutz *et al.*, 1999; Suzuki *et al.*, 2000) and 6-24 hours (Corti *et al.*, 2001; Kottler *et al.*, 2005).

For migration, cultured cells were trypsinised, washed with PBS, centrifuged and the pellet re-suspended in *standard medium*, consisting of DMEM with 0.1% BSA (Germani *et al.*, 2003). The volume required to plate out 50 000 cells was determined using a haemocytometer. Chemotaxis experiments were carried out using 8 μm pore size Falcon cell culture inserts (Becton Dickinson Labware; 35-3182) together with tissue culture-treated 12-well companion plates (Becton Dickinson Labware; 35-3503). After adding 2 mℓ of a treatment solution into each well of the plate, inserts were carefully placed inside the wells together with 50 000 cells suspended in 500 μℓ *standard medium*.
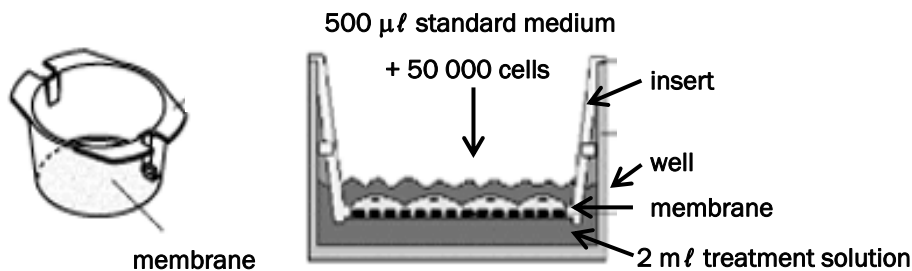
After incubation, inserts were taken out of the companion plate. The following protocols were evaluated to assess the number of migrated cells.

4.3.2   Evaluation of Migration Cell Counting Protocols

A.   **Haemocytometer counting:** After allowing the cells to migrate, 40 $\mu\ell$ samples containing migrated cells were taken from the well-solution and counted on a *haemocytometer.*

Problem: The majority of migrated cells which adhere to the insert-membrane were not taken into account and resulted in a low number of cells in the well- and therefore sample-solution. This high dilution resulted in limited cells being in the haemocytometer field of count and therefore an inaccurate estimation of cell migration number was achieved (cells outside the field were indeed clearly visible and had to be taken into consideration; drop-off of cells from the lower surface of the membrane appears to be negligible – Bischoff, 1997).

B.   **Grid counting** (modified - Bischoff, 1997): After allowing the cells to migrate, the *total* well-volume was pipetted onto a *grid* (drawn on 24-well plate) and the number of cells counted. This counting method was used to try eliminate the effect that, while you *see* cells on the haemocytometer, they must be discounted when outside the field of count. By drawing a bigger grid, all cells in the sample could be accounted for.

Problem: As above, the majority of migrated cells which adhere to the insert-membrane were not taken into account; not using a coverslip (as with haemocytometer) resulted in bubbles obstructing the view; the larger volume resulted in cells floating in different levels within the drop of media and all fields of focus could not be accounted for, resulting in unreliable counts.

C.   **Direct Hoechst staining:** After migration, 10 $\mu\ell$ *Hoechst* was added directly onto the membrane, incubated for 10 minutes and photos taken immediately thereafter.

Problem (Figure 4.14): Migrating cells formed aggregates which made it difficult to distinguish between individual cells; difficulty distinguishing between migrated versus non-migrated cells.

Figure 4.14. Typical image of nuclei stained with Hoechst, distinguishing migrated versus non-migrated cells.
The arrow indicates migrated cells, the arrowhead non-migrated cells, and the triangle indicates the pore size.
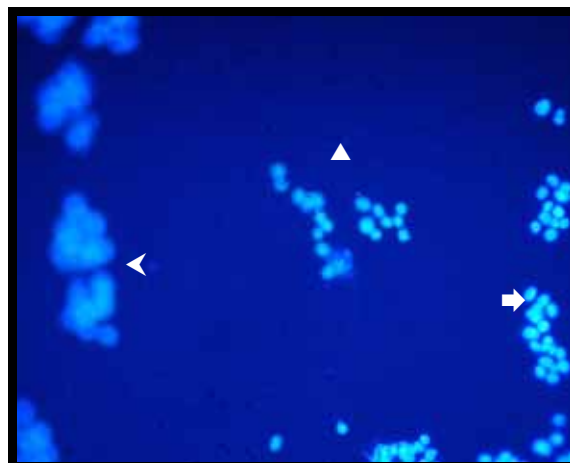
D. **Hoechst fixing and staining** (modified - Corti *et al.*, 2001; Germani *et al.*, 2003; Grosskreutz *et al.*, 1999; Suzuki *et al.*, 2000): After cell migration, all media was aspirated from the insert and the membrane rinsed 2-3 times with PBS. After gently scraping the top side to remove any non-migrated cells still attached to membrane and again washing it with PBS, the membrane was cut loose from the insert (Kottler *et al.*, 2005) and the top gently wiped to remove any additional non-migrated cells. Thereafter, the membrane was placed on a slide, with the side to which cells were added, facing down. Hoechst ($^1/_{200}$) was added and the membrane incubated for 10 minutes, after which it was again washed with PBS, fixed with a 1:1 acetone:methanol solution and mounted onto a coverslip with mounting media. Slides were stored at -20°C until later analysis. Photos were taken of six fields per membrane. *Problem* (Figure 4.15): As in protocol C, difficulty distinguishing between migrated versus non-migrated cells; migrated cells washed off when PBS and Hoechst were added (before fixing).

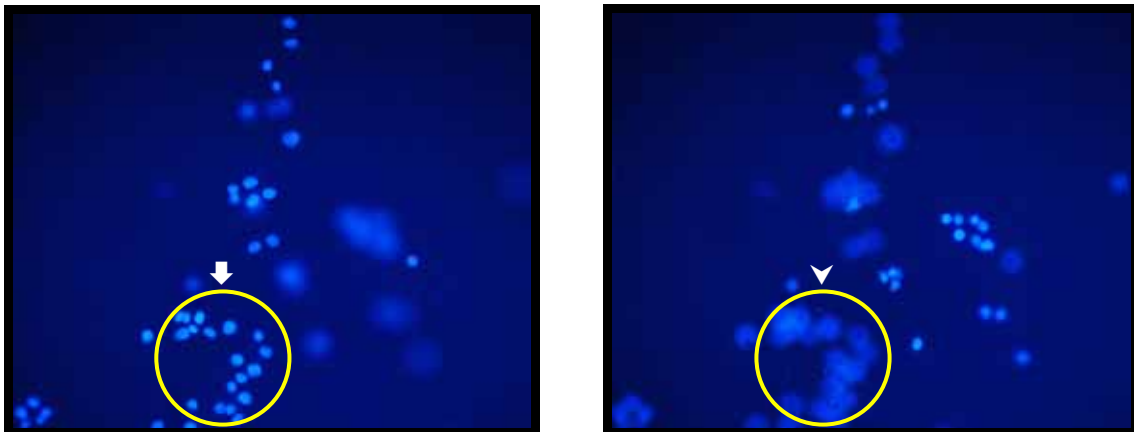**Figure 4.15. Typical images from the same field of view that illustrate the different levels at which the same group of cells can be viewed.** The arrow indicates migrated cells and the arrowhead non-migrated cells.

E. **Grid counting and trypsin:** 100 $\mu\ell$ drops trypsin was placed on each grid (as described in protocol B). After aspirating all media out of the insert, the insert was placed on top of the drop and incubated at 37°C for 10 minutes. The insert was then taken off and the membrane rinsed with 200 $\mu\ell$ media (containing FBS, to inactivate the trypsin), making sure the media and any additional cells mixed thoroughly with the cell-trypsin solution on the grid. Cells were counted using the grid to determine the migration number. *Problem:* Difficulty counting higher migration numbers; bubbles; inconsistent results, grid not calibrated (compare with haemocytometer).

F. **Haemocytometer and trypsin**

This methodology was the most successful and is fully described in Chapter 7 (*section 7.2.2*). This was the chosen method for analysis of migrated cells.

4.3.3   Summary and Conclusions

Table 4.3 summarises the described protocols which were evaluated and the results obtained from each. IGF-1 successfully increased the number of migrated cells (E) and was therefore selected as growth factor to induce positive migration in subsequent migration assays.

Short periods (less than 6 hours) did not allow sufficient time for migration, whereas longer periods (more than 12 hours) resulted in the proliferation of cells which made it difficult to accurately determine actual migration numbers. Therefore, 7 hours was chosen as time-period for cell migration in the final experimental protocol.

Table 4.3. Summary of initial methods used to assess migration in C2C12 cells.

CELLS	TREATMENT	TIME (hr)	NUMBER OF CELLS MIGRATED (cells/mℓ) AND METHOD OF COUNTING	
500 000	insert solution: SFM + cells well solution: SFM	0 1 4 8	0 0 0 35 000	A

500 000	insert solution: SFM + cells
	well solution: TGF-β1 at 100 pg/mℓ
	      300 pg/mℓ
	      1 ng/mℓ
	      3 ng/mℓ
	      10 ng/mℓ
		8	
			40 000
			30 000
			30 000
			30 000
			50 000
				A

50 000	insert solution: 1% HS well solution: 10% HS insert solution: 1% FBS well solution: 10% FBS	4 6	4 hr: 15 000 6 hr: 35 000 4 hr: 5 000 6 hr: 15 000	A
100 000	insert solution: 1% HS well solution: 2% HS insert solution: 1% FBS well solution: 2% FBS	1 2 4 22 1 2 4 22	0 0 0 90 000 (cells attach and start to proliferate) 0 0 0 60 000 (cells attach and start to proliferate)	A B A B

50 000	insert solution: DMEM/0.1% BSA
	well solution: DMEM/0.1% BSA
	
	insert solution: DMEM/0.1% BSA
	well solution: DMEM/0.1% BSA/10 ng/mℓ IGF-1
		7	control: 73 0
			
			IGF-1: 730 47
			        593 130
			        463 137
				E

SFM, serum free medium; **FBS**, foetal bovine serum.

4.4   METHODS FOR ASSESSING FUSION

Growth and regeneration of adult skeletal muscle requires activation, proliferation and differentiation of satellite cells. Following cell cycle withdrawal and phenotypic differentiation, myoblast fusion is required for further terminal differentiation, resulting in the formation of post-mitotic, multinucleated myotubes (Andres and Walsh, 1996). The increase in nuclei number within the myofiber is accompanied by a proportional increase in cytoplasm to allow myofiber size increases, indicating the importance of fusion for muscle growth (Horsley *et al.*, 2001; Mitchell and Pavlath, 2001). Determining indices to quantify fusion would therefore make it possible to assess successful progression through the entire differentiation programme.

4.4.1   Cell Culture

C2C12 cells were plated onto glass coverslips in each well of six-well tissue culture-treated plates (day -1) at a density of 50 000 cells/well and allowed to differentiate as described above (*section 4.2.1.1*). Cells were fixed and prepared for immunofluorescent staining on days 3, 4, 5, 6 and 7. At these time-points, while working on ice, the coverslips were rinsed with PBS and fixed in an acetone:methanol solution (1:1) for ~5 minutes. After removing the solution, the coverslips were left to dry and stored at -20°C until later analysis.

4.4.2   Immunohistochemistry

For immunofluorescent staining and analysis, cells grown on coverslips were allowed to defrost at room temperature, after which they were gently rinsed with 500 µℓ PBS (0.1 M, pH 7.4). To block non-specific binding sites, each coverslip was first incubated with 100 µℓ 5% donkey serum (Jackson ImmunoResearch Laboratories, Inc.) for 30 minutes at room temperature. Thereafter, the serum was drained off and the primary antibody, rabbit polyclonal M-cadherin, added and cells incubated overnight at 4°C.

Cells were then rinsed twice with PBS and further incubated with 100 µℓ biotinylated donkey anti-rabbit secondary antibody for 50 minutes at room temperature. Cells were again washed twice with PBS and further incubated with 100 µℓ Texas Red Streptavidin tertiary antibody for 40-60 minutes at room temperature. 100 µℓ Hoechst dye was added during the last 10 minutes of this step for nuclear staining. The sections were thoroughly rinsed with PBS, mounted in Fluorescent Mounting Medium (S3023, DAKO) and stored at -20°C until further analysis.

Sections were viewed under a fluorescence Nikon microscope (ECLIPSE E400) at 20x enlargement and photos taken with a digital camera (Nikon DXM1200). TNC and M-cadherin staining intensities were analysed using the computer software *Simple PCI*, version 4.0 (Compix Inc., Imaging Systems, USA).

4.4.3   Assessment of Fusion

Cadherin-proteins, which are transmembrane proteins mediating cell-to-cell interactions, are thought to play an important function in cell fusion and the regulation of intracellular cytoskeletal structures (Kaufmann *et al.*, 1999b). Specifically, M-cadherin, a cell adhesion protein, has been postulated as an important molecule involved in myoblast differentiation and fusion during myogenesis and muscle regeneration (Zeschnigk *et al.*, 1995). Since this protein is expressed in quiescent cells, myoblasts and myotubes (Irintchev *et al.*, 1994), cell fusion was studied by immunofluorescence using M-cadherin to identify all myocytes.

To quantify cell fusion, M-cadherin images of the cells were merged with the Hoechst-stained image of the nuclei from the same area to determine myoblast and myotube stages of differentiation. Total numbers of bi-nuclear myoblasts (two nuclei per cell) and myotubes (three or more nuclei per cell) were then counted (Figure 4.16). In addition, the total number of nuclei in these bi-nuclear myoblasts and myotubes were added and divided by the TNC (*section 4.2.2.2*) of that image to calculate the fusion index (%) (Nishiyama *et al.*, 2004; Park and Chen, 2005) (Figure 4.18). A minimum of six photos were taken from different regions of each slide. The experiment was performed in triplicate.

4.4.3.1   Total myoblast and myotube count

The total number of bi-nuclear myoblasts and myotubes is a direct reflection of the progress of differentiation (Figure 4.16).

Figure 4.16. Total number bi-nuclear myoblasts and myotubes in differentiating C2C12 cells. Data are expressed as mean ± SEM; n = 1.
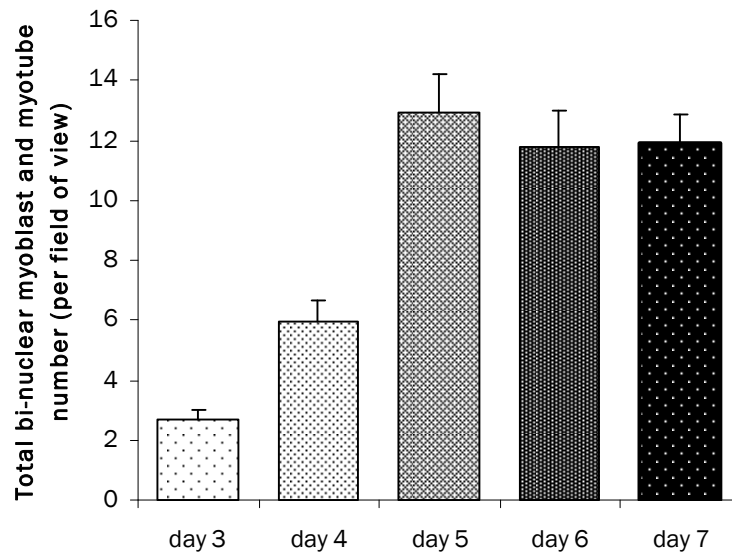
At the beginning of the differentiation programme, when proliferation is still predominant, the total number of bi-nuclear myoblasts and myotubes were minimal (day 3 and day 4), after which they showed a gradual increase. The maximum number of myoblasts and myotubes was observed at day 5 when myoblasts entered terminal differentiation phases and remained elevated until day 7. Figure 4.17 illustrates the increase in myoblast and myotube formation.

Figure 4.17. Typical images of myoblast and myotube formation in differentiating C2C12 cells.

day 3   day 4   day 5   day 6   day 7

A: myoblasts (2 nuclei/cell)
B: myotube (> 2 nuclei/cell)
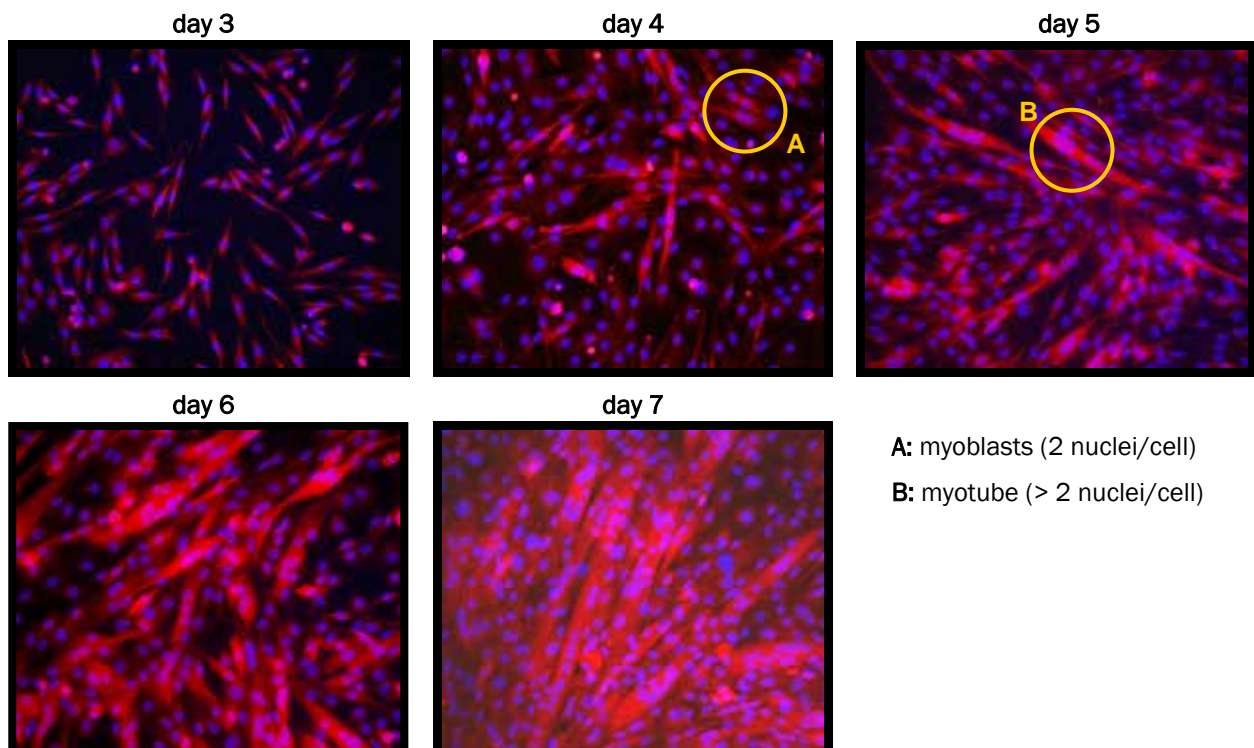
Mostly single nuclei myoblasts could be distinguished at day 3, after which the number of bi-nuclear myoblasts and myotubes gradually increased from day 4 to day 7. In addition, the number of nuclei per myotube also increased, and as such the amount of cytoplasm. At day 7, the number of nuclei per myotube had increased further as illustrated by the greater myofiber sizes.

4.4.3.2 *Fusion index*

The number of myonuclei *within* a myofiber determines its size by regulating the cytoplasmic volume which increases with myoblast fusion. The fusion index compares the proportion of nuclei within bi-nuclear myoblasts and myotubes to the TNC and enables a quantifiable and comparable measure of fusion. Greater fusion indexes would therefore be indicative of successful fusion and consequently also differentiation (Figure 4.18).

Figure 4.18. Fusion index in differentiating C2C12 cells. Data are expressed as mean ± SEM; n = 1.
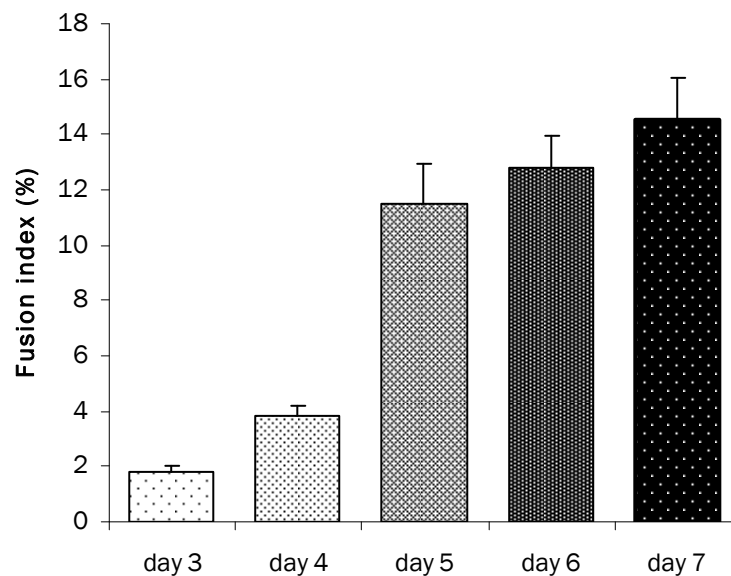
Differentiation and the formation of multinucleated myotubes increased significantly from day 3 to day 5 (Figure 4.17), after which gradual myotube increases were observed to day 7. Analysis of the fusion index (Figure 4.18) demonstrated a similar increase from day 3 to day 5. These results indicate an increase in the number of mononucleated myocytes entering phases of initial differentiation into bi-nuclear myoblasts and subsequently into myotubes. Consistent with this observation was the expression of the regulatory factor, myogenin (Figure 4.6) which was seen from day 1, but increased significantly thereafter to day 5 and remained elevated until day 9.

4.4.4   Summary and Conclusions

During the course of muscle development, myocytes are required to specifically fuse to each other to form bi-nuclear myoblasts and then multinucleated myotubes. Fusion is therefore critical to induce terminal differentiation to form syncytial muscle fibers (Andres and Walsh, 1996; Charge and Rudnicki, 2004; Nishiyama *et al.*, 2004) which cannot re-enter the cell cycle (Florini *et al.*, 1991; Olson, 1992).

In this preliminary model, limited C2C12 cell fusion was observed at day 3, after which significant increases occurred to day 5. Thereafter, the total number of bi-nuclear myoblasts and myotubes remained constant until day 7. The fusion index increased from day 3 to reach a maximal level at day 7. Days 3, 5 and 7 were therefore chosen for analysis of fusion in subsequent experimental protocols.

CHAPTER 5

PROLIFERATION

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5.1   INTRODUCTION

In adult skeletal muscle, satellite cells are the primary stem cell source which supply myonuclei to growing myofibers. Although quiescent under normal conditions, they are activated and begin proliferating in response to extrinsic signals to fulfil their function in growth, hypertrophy and repair of adult skeletal muscle. However, the extent of their contribution to processes of repair and regeneration is not always sufficient to restore functional integrity of the muscle.

Virtually any disturbance in the micro-environment could lead to satellite cell activation, proving the importance of extrinsic signalling. In this regard is the position of the satellite cell, where it is located beneath the basal lamina of the myofiber and in close proximity of interstitial cells, capillaries and the neuromuscular and myotendinous junctions, essential for communication between the cell and the adjacent myofibers. The influence of signals that are transmitted from these myofibers and the surrounding matrix, as well as from soluble endocrine, paracrine and autocrine factors, needs to be identified to distinguish possible mechanisms regulating the capacity of satellite cells to become activated from their natural quiescent state and proliferate. Such mechanisms would control the cell cycle of myogenic cells which is regulated by myogenic transcription factors (Weintraub, 1993), as discussed in Chapter 2 [*section 2.2.1.1 (C)*].

Once activated in response to stimuli such as muscle injury, satellite cells undergo rapid proliferation to progress along the myogenic lineage. The proliferative expansion of the progenitor cell population is required to provide sufficient myoblast numbers for subsequent cell cycle arrest, differentiation and fusion into myotubes, and importantly, also for satellite cells to self-renew and return to quiescence, assuring a sufficient reserve for future requirements. Taking into consideration the many phases of development, control of the cell cycle is essential, since deregulation would lead to uncontrolled proliferation and prevent further development and/or regeneration. Therefore, although myoblast proliferation is required to provide sufficient progenitor cells for terminal differentiation, proliferation and differentiation are mutual exclusive events and if the proliferative phase of development is not adequately controlled, effective myogenesis could be prevented.

Several growth factors have been identified which contribute to phases of myogenic development by inducing their specific signalling pathways at the appropriate stages. Hepatocyte growth factor (HGF) has shown both *in vitro* and *in vivo* to be essential for inducing the activation and early division of satellite cells (Allen *et al.*, 1995; Cornelison and Wold, 1997; Tatsumi *et al.*, 1998). Similarly, fibroblast growth factor (FGF) has also shown ability in recruiting satellite cells to break quiescence and enter the proliferative phase (Yablonka-Reuveni *et al.*, 1999), but in contrast, depress differentiation (Allen and Boxhorn, 1989; Spizz *et al.*, 1986). Insulin-like growth factor-1 (IGF-1) has demonstrated pronounced stimulation of both proliferation and differentiation (Allen and Boxhorn, 1989). The role of TGF-β isoforms in skeletal muscle is unclear since this growth factor has shown to have both positive and negative effects on muscle cell development. Depending on the individual responses of specific cells and the environmental conditions, the balance of such effects could lead to either the promotion or inhibition of proliferation. Despite the ability of TGF-β to affect different cell types in opposing ways, this may not reflect actual differences of initial cellular responses to TGF-β. Rather, variable cellular responses could be the end-result of TGF-β initiating a number of effects in all responsive cells, some of which may lead to proliferation and others to the inhibition thereof (Nilsen-Hamilton, 1990).

Transplantation models have shown that grafted myogenic cells contribute myonuclei, as well as produce myogenic precursors that can be activated following muscle damage to proliferate and enhance further regeneration (Gross and Morgan, 1999; Watt *et al.*, 1982). By altering the concentrations of selected growth factors such as these described, satellite cells and other mpcs can be treated to increase their potential for proliferation and differentiation and therefore be applied to enhance the capacity of stem and/or progenitor cells to contribute to functional regeneration processes.

The proliferating cell nuclear antigen (PCNA) is a protein essential for cellular DNA synthesis (Waseem and Lane, 1990). PCNA participates in different pathways of DNA metabolism and cell division, including co-ordinating DNA repair synthesis, DNA replication and cell cycle progression (Scovassi and Prosperi, 2006). It is suggested that PCNA influences proliferation by means of its association with the cell cycle regulator p21 (Maga and Hubscher, 2003; Paunesku *et al.*, 2001). Arrest of cell cycle progression by p21 and induction of terminal differentiation is dependent on the maintenance of high levels of p21, resulting in the inhibition or down-regulation of PCNA expression (Engel *et al.*, 2003). In contrast, proliferation requires increased expression of PCNA. Therefore, the decision of a cell to either proliferate or differentiate is dependent on the interaction between these two proteins.

The cellular distribution of PCNA is important and depends on its synthesis and activity, and on the cell cycle phase. This protein is produced in the cytoplasm and transported into the nucleus during the S-phase of the cell cycle. As mentioned in Chapter 4 (*section 4.2.2.1*), localisation of PCNA correlates directly with rates of cellular proliferation during the cell cycle, with highest levels observed during the late G_1- and S-phases in the nucleus and to a lesser extent during the G_2- and M-phases (Thomas *et al.*, 1993). It also forms complexes with various CDK-cyclins and checkpoint proteins within the cell cycle. This protein's expression is therefore not limited to proliferating cells and PCNA can be detected at various stages throughout the cell cycle (Thomas *et al.*, 1993).

During the S-phase, three different *nuclear* PCNA populations can be distinguished (Toschi and Bravo, 1988): an insoluble granular form involved in ongoing DNA synthesis which localises specifically to the nucleus in a chromatin-bound form associated with replication structures, a second, freely soluble (easily extractable) nucleoplasmic form seen in quiescent cells (Bravo and Macdonald-Bravo, 1987; Scovassi and Prosperi, 2006), and a third that is possibly only loosely associated with nuclear components as pre-synthesis complexes (Toschi and Bravo, 1988). The latter two are not involved in constant DNA synthesis, but might need to be additionally recruited for the initiation of DNA replication at a later stage (Szuts *et al.*, 2005). A granular form and a soluble, inactive form of PCNA can also be detected in the cytoplasm (Grzanka *et al.*, 2000; Scovassi and Prosperi, 2006).

In this chapter, C2C12 myoblasts were cultured in low serum conditions and treated with TGF-β isoforms, TGF-β1, -β2 and -β3. Thereafter, the expression and localisation of PCNA were assessed to determine the effect of TGF-β on myoblast proliferation and gain insight into the role of this growth factor during myogenic differentiation.

5.2 METHODS

5.2.1 Cell Culture

5.2.1.1 Assessment of total nuclear count

C2C12 cells were maintained in culture medium until they reached a confluency of ~40-50% in T75 flasks (described in *Chapter 3*). Thereafter, they were plated onto glass coverslips in each well of six-well tissue culture-treated plates (day -1) at a density of 50 000 cells/well in culture medium. On the following day (day 0), the cells were rinsed with PBS and the medium changed to mitogen-poor, differentiation-promoting medium (*Chapter 3, section 3.3.1.2*) supplemented with either TGF-β1 or -β2 or -β3 (5 ng/mℓ), and compared to control conditions (differentiation medium only) (short-term treatment, 24 hours). For long-term treatment (72 hours), the cells were *additionally* treated with TGF-β isoforms (5 ng/mℓ) on day 1 and day 2. After both short- and long-term treatment, cells were maintained in a humidified incubator at 37°C, 5% CO_2 in differentiation medium which was changed every second day.

In order to quantify the total nuclear number during stages of growth and differentiation, the cells were fixed and prepared for immunofluorescent staining on days 5 and 7, and additionally on day 3 following long-term treatment, as described in Chapter 4 (*section 4.4.1*). It should be noted that this method therefore does not take into account those cells which may have lifted off due to apoptosis. However, analysis of apoptotic nuclei on the coverslips prior to rinsing suggested minimal highly condensed apoptotic cells at the time-points analysed.

5.2.1.2 Protein analysis of proliferation

To determine the long-term effect of TGF-β isoforms on PCNA protein levels, C2C12 cells were prepared and plated as described in Chapter 4 (*section 4.2.1.1*) and treated with TGF-β isoforms for 72 hours as described above.

Cells were collected for immunoblotting purposes on days 0, 1, 5 and 9. Day 0 samples were collected before addition of differentiation medium to determine baseline values of antibody expression. To harvest the cells, the medium was removed and cells washed twice with PBS. While keeping the samples on ice, they were treated with 100-150 µℓ lysis buffer, sonicated, aliquoted and stored at -20°C until later analysis.

5.2.1.3   Immunofluorescent localisation of PCNA

To determine the cytoplasmic and nuclear localisation of PCNA in control- and TGF-β1-treated differentiating cells, C2C12 cells were plated into 8-chambered, cell culture-treated, coverglass units (155411, Lab-Tek, USA) at a density of 20 000 cells/chamber in 300 µℓ culture medium (day -1). After removing the medium and rinsing cells with PBS on the following day (day 0), differentiation medium was added and cells for TGF-β treatment were supplemented with 5 ng/mℓ TGF-β1 for comparison to control conditions. Further addition of TGF-β1 to the appropriate cells was repeated on days 1 and 2 and cells maintained as described above. For immunofluorescent staining, the medium was removed, cells rinsed with PBS and fixed in an acetone:methanol solution (1:1) on days 1 and 5. The chambers were covered and stored at -20°C until later analysis.

The concentration of the TGF-β used in these and subsequent protocols was taken from the literature. Although it would have been preferred to analyse more than one concentration of the TGF-β isoforms, it was felt that the number of parameters within the study (incubation time, different isoforms, analysis of 5 time-points, analysis of proliferation, differentiation, migration and fusion and multiple markers thereof, and two cell-lineages) were sufficient.

5.2.2   Proliferation Assays

5.2.2.1   Determination of total nuclear count

Quantification of the total nuclear count (TNC) in differentiating C2C12 cells was carried out by fluorescent nuclear staining. At the relevant time-points, Hoechst dye ($1/_{200}$) was added to the fixed cells for 10 minutes. The sections were then washed and mounted with Fluorescent Mounting Medium (DAKO). All incubation procedures were performed at room temperature. The complete assay has been described in Chapter 4 (*section 4.4.2*).

Sections were viewed under a fluorescence Nikon microscope (ECLIPSE E400) and photos taken with a digital camera at 20x enlargement (Nikon DXM1200). Photos were used to count the total number of nuclei per field of view using the computer programme *Simple PCI, version 4.0* (Compix Inc., Imaging Systems, USA). A minimum of six photos were taken from different regions of each slide. The experiment was performed in triplicate.

5.2.2.2   Western blot analysis of PCNA protein level

Protein analysis of C2C12 cells was carried out as described in Chapter 3. Total protein was assessed by the Bradford method to ensure equal loading concentrations of samples per lane (*section 3.3.2.1*). Western blot analysis (*section 3.3.2.2*) was used to evaluate PCNA levels following long-term incubation with TGF-β isoforms, whereas α-tubulin was used to assess consistency in loading of samples. Protein expression levels were determined by densitometry (*section 3.3.2.3*). Each sample was evaluated in duplicate and all experiments repeated a minimum of three times.

5.2.3   PCNA Localisation

Immunofluorescent localisation of PCNA in C2C12 cells was performed as follows. After defrosting the 8-chambered units, they were gently rinsed with 300 µℓ PBS and incubated with 50 µℓ 5% donkey serum for 20 minutes at room temperature. After the serum was drained off, the cells were stained with 50 µℓ anti-PCNA which was added as primary antibody (see *Table 3.3* for dilutions and supplier) and PBS as control, and the cells left to incubate for 90 minutes. The cells were then rinsed twice with PBS, followed by incubation for 30 minutes with 50 µℓ Texas Red Streptavidin secondary antibody. After thoroughly rinsing each chamber with PBS, a drop Fluorescent Mounting Medium (S3023, DAKO) was added, the units covered in a moist chamber and stored in a dark area.

Sections were visualised using the Motorised Inverted System Microscope (Olympus IX 81, Imaging Software Cell®) using a 60x oil immersion objective. Photos were taken with a monochromatic camera (F-View Soft Imaging Systems). No cross-reactivity of the secondary antibody was observed in control experiments in which the primary antibody was omitted.

5.2.4   Statistical Analysis

Statistical evaluations were made by one-way analysis of variance (ANOVA) and Bonferroni's multiple comparison test using STATISTICA. Significant differences were taken at $p < 0.05$. All data are expressed as mean $\pm$ SEM.

5.3   RESULTS

To determine the effect of TGF-β isoforms on C2C12 myoblast proliferation, the TNC was assessed in C2C12 cell cultures, as well as the expression of PCNA following western blot analysis. In addition, the localisation of PCNA in differentiating C2C12 cells is demonstrated following treatment with TGF-β1.

5.3.1   Assessment of Total Nuclear Count

Analysis of the total nuclei number indicated that, after both 24 hour and 72 hour treatment, TGF-β1, -β2 and -β3 significantly increased the proliferation of C2C12 myoblasts at all time-points analysed (Figure 5.1 and Figure 5.2) ($p < 0.01$).

Following the 24 hour treatment, the increase in TNC was similar for the three isoforms at day 5. At day 7, although no significant isoform-specific effects were demonstrated, TGF-β2 showed the greatest response of the three isoforms (Figure 5.1). Increases in TNC compared to control conditions ranged from 69% to 77% at day 5 and from 65% to 89% at day 7. Although a similar pattern was seen following 72 hour incubation with the isoforms, the effect of TGF-β was greater following 72 hour than 24 hour treatment for both day 5 and day 7 (Figure 5.2). Following 72 hour treatment, all three isoforms resulted in similar increases in TNC at day 3, ranging from 61% to 64%. At day 5, TGF-β2 again showed the greatest effect (113% TNC increase). Increases in TNC ranged from 88% to 104% at day 7.

Taken together, these results suggest that long-term (72 hour) incubation with TGF-β isoforms has a greater effect on C2C12 cell proliferation; the continued presence of the isoforms maintains the proliferative stimulus. Visual analysis of the cells confirmed that TGF-β1-treated cells were more proliferative than untreated cells (Figure 5.3). TGF-β2 and -β3 displayed similar images.

Figure 5.1. Incubation of C2C12 cells with TGF-β1, -β2 or -β3 for 24 hours results in an increase in proliferation. The total nuclear number was assessed by nuclear staining and image analysis in control- and TGF-β-treated differentiating C2C12 cells. #p < 0.01. Data are expressed as mean ± SEM; n = 3.
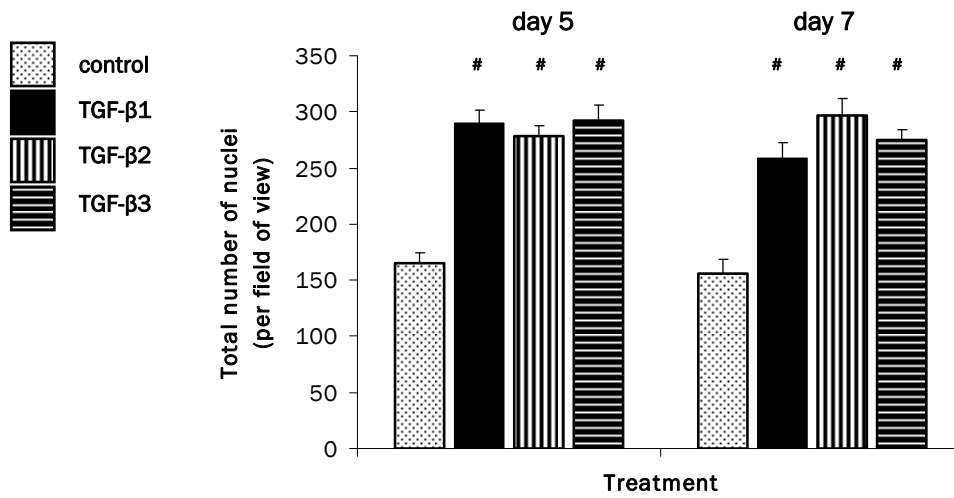
Figure 5.2. Incubation of C2C12 cells with TGF-β1, -β2 or -β3 for 72 hours results in an increase in proliferation. The total nuclear number was assessed by nuclear staining and image analysis in control- and TGF-β-treated differentiating C2C12 cells. #p < 0.01. Data are expressed as mean ± SEM; n = 3.
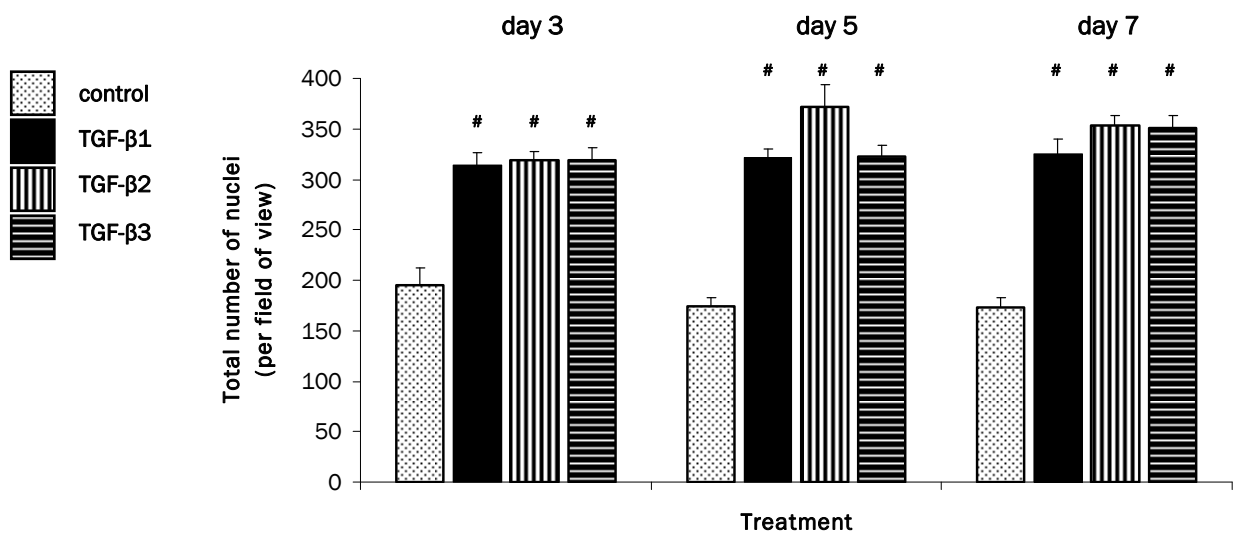
Figure 5.3. Typical images of nuclei (at 20x enlargement) in differentiating C2C12 cells at day 5 following 72 hour incubation with TGF-β1. **(A)** Control- and **(B)** TGF-β1-treated cells, stained with Hoechst to allow identification of the nuclei. TGF-β1-treated cells clearly illustrate an increase in total nuclei number at day 5.

105.
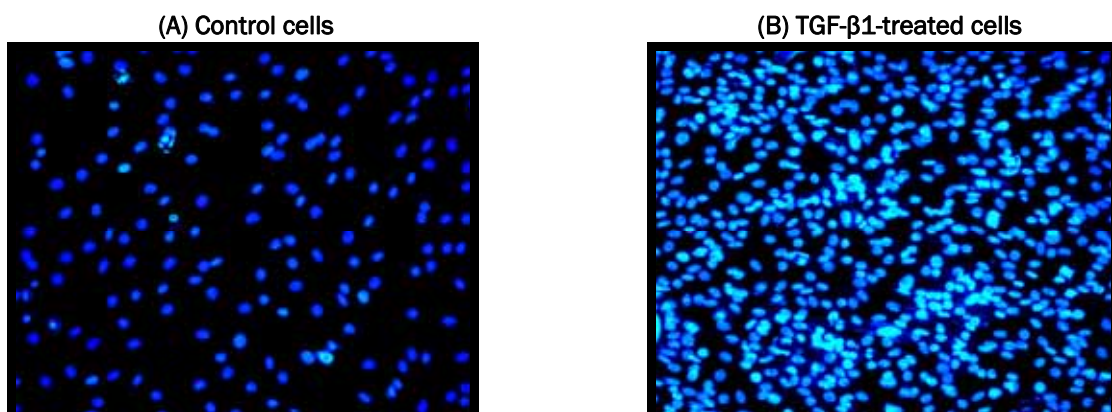
5.3.2   Effect of TGF-β Isoforms on PCNA Expression in C2C12 Cells

To determine a possible mechanism by which TGF-β isoforms increase proliferation, the expression of PCNA, a molecular marker of proliferation, was determined in C2C12 cells following 72 hour exposure to TGF-β isoforms. Data are expressed as a % of control.

Surprisingly, compared to control conditions, analysis of PCNA expression in TGF-β-treated cells displayed no significant differences on any day analysed (Figure 5.4). Despite not being significant, TGF-β1 displayed the greatest effect, increasing PCNA expression at day 1 compared to control conditions, as well as compared to TGF-β2 and -β3 treatment. At day 9, all three isoforms resulted in higher PCNA expression, however, this was also not significant.

Figure 5.4. Incubation of C2C12 cells with TGF-β1, -β2 or -β3 for 72 hours displays no significant differences in PCNA expression. PCNA protein levels were assessed by western blot analysis in control- and TGF-β-treated differentiating C2C12 cells. Data are expressed as mean ± SEM; n = 3.
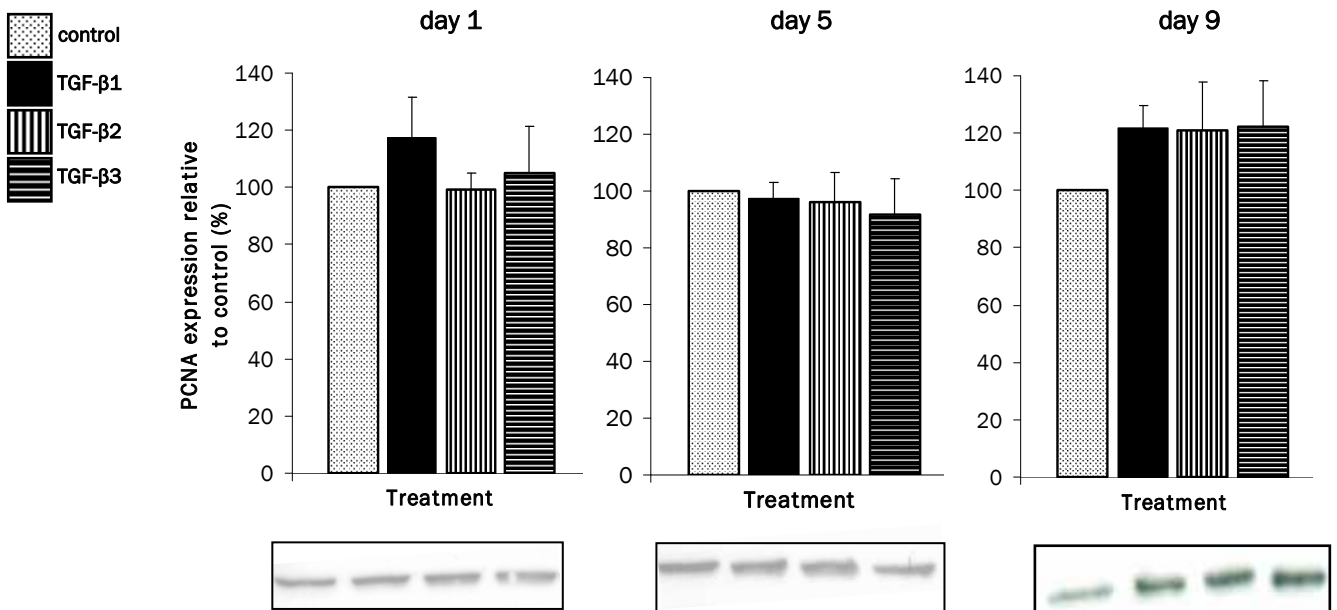
5.3.3   Immunofluorescent Localisation of PCNA in TGF-β1-treated C2C12 Myoblasts

As mentioned in *section 5.1*, PCNA is produced in the cytoplasm (an inactive soluble and granular form can be distinguished), but is transported to the nucleus to regulate proliferation during the S-phase of the cell cycle (both soluble and *active* granular form). This infers that, although total PCNA expression levels may not change, the *localisation* of PCNA may change in such a way so as to alter proliferation. As a result, the cellular distribution of PCNA was analysed between the cytoplasm and nucleoplasm by means of immunofluorescence in order to determine whether TGF-β isoforms could alter the distribution of PCNA within the cell without changing the total expression levels. Whereas the active, insoluble form of PCNA is strongly associated with the nuclear regions where DNA synthesis is occurring, the soluble, nucleoplasmic form degrades in the presence of organic solvents, making it undetectable when using organic fixatives (such as methanol) in cell cultures. This does however provide a method for only visualising the DNA synthesising form (Bravo and Macdonald-Bravo, 1987). Since an acetone:methanol fixing solution was used, only the synthesising form is visible in the images (Figure 5.5).

Figure 5.5 illustrates the immunofluorescent localisation of PCNA in control- and TGF-β1-treated C2C12 cells at day 1 and day 5 of differentiation. At day 1, control cells display both cytoplasmic and, to a lesser extent, nuclear staining (Figure 5.5 A - i), whereas TGF-β1-treated cells display primarily granular cytoplasmic staining (Figure 5.5 B - i). However, by day 5, TGF-β1-treated cells display increased PCNA expression throughout the cell, especially in the nucleus (Figure 5.5 B - ii), whereas control cells continue to express primarily cytoplasmic and limited nuclear PCNA (Figure 5.5 A - ii). This change in distribution and increased expression of nuclear PCNA in TGF-β1-treated cells could be explained by Szuts *et al.* (2005) who demonstrated that PCNA from a soluble nuclear pool is required for initiation of DNA replication. Although these images were not analysed, the increased nuclear localisation observed in TGF-β1-treated cells at day 5 (Figure 5.5 B - ii) illustrates a possible increase in proliferative capacity following TGF-β treatment, as demonstrated by the higher TNC in TGF-β-treated C2C12 myoblasts (*section 5.3.1*).

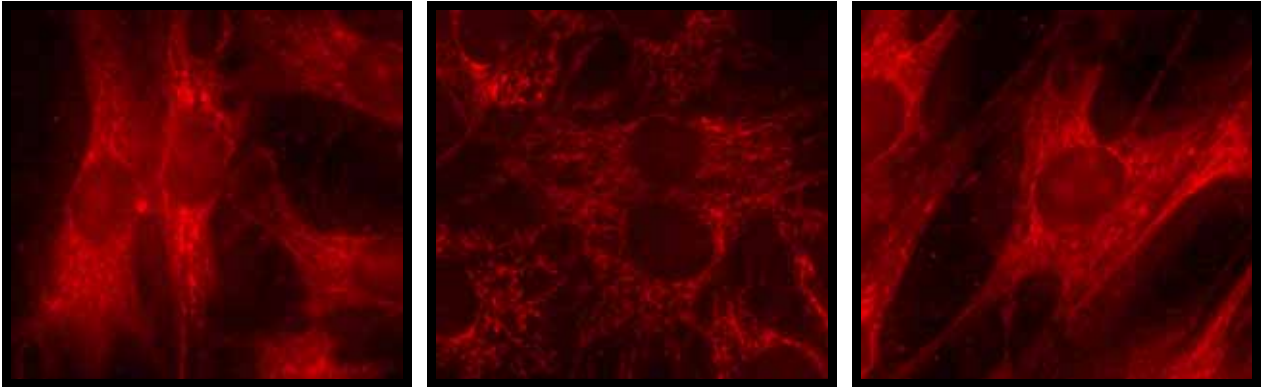Figure 5.5. Immunofluorescent localisation of PCNA (at 60x enlargement) in differentiating C2C12 cells following 72 hour incubation with TGF-β1. Typical images of (A) Control- and (B) TGF-β1-treated cells at (i) day 1 and (ii) day 5 of differentiation. n = 1.

(A - i) Control day 1

(B - i) TGF-β1 day 1
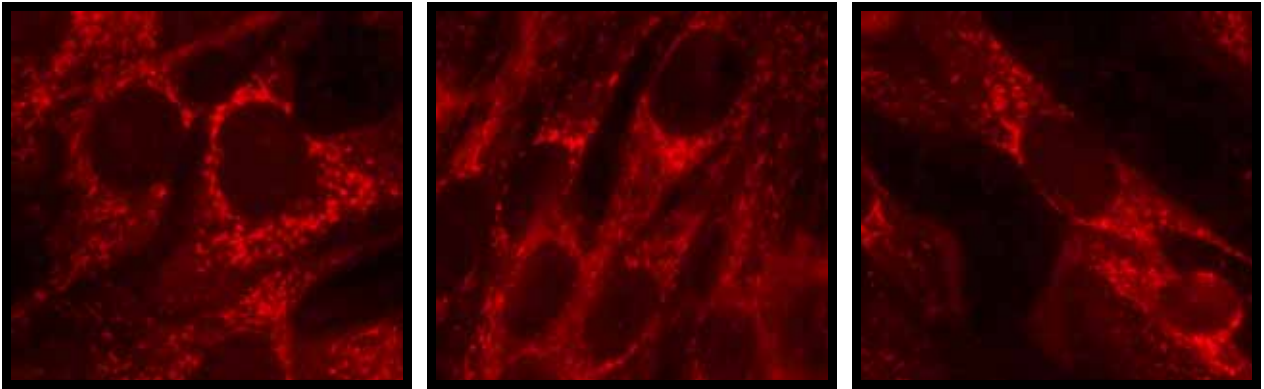
(A - ii) Control day 5
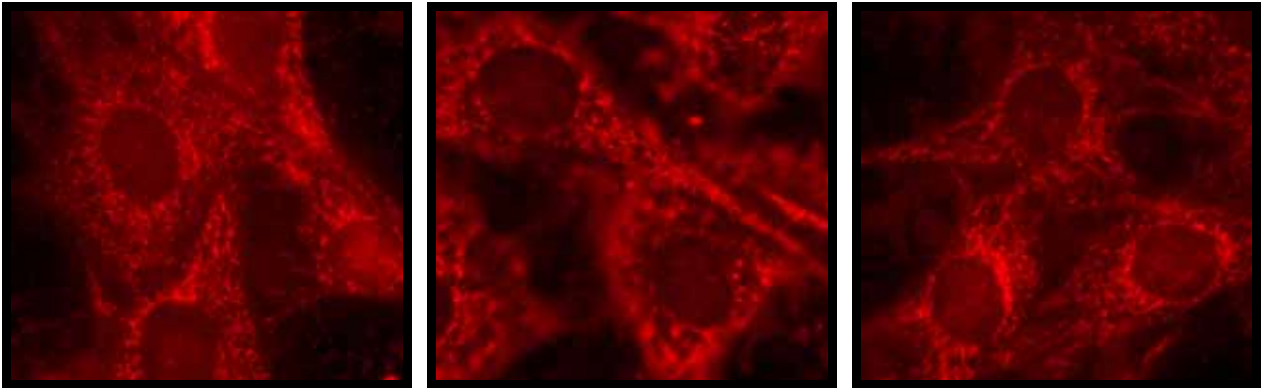
(B - ii) TGF-β1 day 5
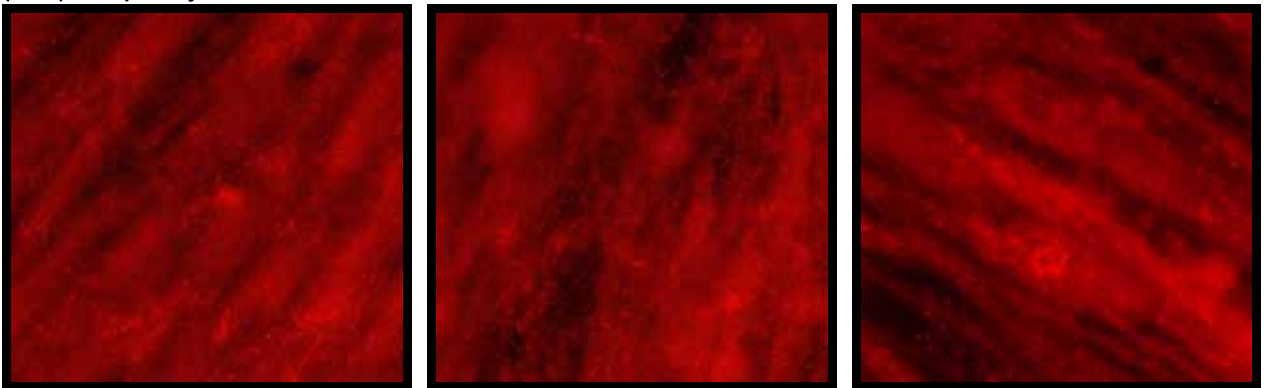
5.4   DISCUSSION

The decision of a cell to proliferate, differentiate or migrate during development is closely connected to its environment. Transplantation studies have shown the importance of cell-to-cell interactions and the extracellular milieu in determining cell fate (Greenwald and Rubin, 1992; Gurdon, 1992). Specifically, during phases of growth and development, proliferation of the relevant cell type is essential to provide sufficient cell numbers for subsequent differentiation. Similarly, during stages of regeneration following injury, proliferation of resident or transplanted stem or progenitor cells is necessary to aid in the repair process.

A current limitation in stem cell therapy is the need for greater cell numbers to be functionally incorporated into the damaged tissue (Zammit *et al.*, 2006a). Possible means to increase the number of donor cells could either be achieved by extended culturing and proliferation of cells *ex vivo* before transplantation, or by manipulating the cellular conditions *in vivo* following transplantation to generate a stem cell-prone micro-environment which would favour the proliferation of donor cells. Since it has been shown that extended *in vitro* culturing of stem or progenitor cells result in a loss of these cells' differentiation potential (Price *et al.*, 2007), an alternative would therefore be to control the immediate cellular environment to favour conditions for more efficient proliferation. Such a strategy would be of benefit to both the local (resident) stem or progenitor cells of the host, as well as a transplanted population.

Several growth factors have been shown to play a role in growth and regeneration processes (Allen and Boxhorn, 1989; Jessell and Melton, 1992). TGF-β is one such factor which affects proliferation and differentiation of many cell types to various extents both *in vitro* and *in vivo*. The effect which this multi-functional growth factor has on cellular growth and repair varies greatly, being dependent on the cell type and its stage of development, the environmental conditions, the concentration and isoform released, and the presence of other (growth) factors. Pleiotropic effects produced by TGF-β include induction of growth in mesenchymal cells (Roberts *et al.*, 1985), inhibition of adipocyte and osteoblast differentiation (Ignotz and Massague, 1985), induction of collagen and fibronectin synthesis (Ignotz and Massague, 1986), and inhibition of proliferation but stimulation of differentiation in epithelial cells (Masui *et al.*, 1986). In skeletal muscle, the role of TGF-β is unclear, having shown the ability to either inhibit (Massague *et al.*, 1986) or induce (Zentella and Massague, 1992) myoblast differentiation *in vitro*. Also, combined with either IGF-1 or FGF, TGF-β inhibits differentiation, whereas maximal stimulation is observed in the presence of both IGF-1 and FGF (Allen and Boxhorn, 1989).

The responsiveness of cell populations such as myoblasts to TGF-β, whether endogenously synthesised by the cells or added to the media, could have variable effects on their growth and differentiation. To determine the effect of TGF-β isoforms on skeletal muscle proliferation in an *in vitro* system, C2C12 cells were cultured in the presence of TGF-β. Despite being induced to differentiate, significant results from this chapter showed an increase in total nuclei number of the cell culture following both short- and long-term treatment with TGF-β isoforms which were interpreted as an increase in cell proliferation status. No isoform-specific effects were evident, although TGF-β2 showed a greater response at day 7 and day 5 following short- and long-term treatment, respectively. Similar conclusions were made by Filvaroff *et al.* (1994 and unpublished data) who suggested that TGF-β may be used to assist in myoblast proliferation; this group however analysed the general effect of TGF-β1 on C2C12 cells without looking closely at isoform-subtype.

Following tissue damage, such an increase in stem or satellite cell proliferation in response to TGF-β treatment could prove to be of benefit if regulated correctly to prevent uncontrolled proliferation and taking into consideration the possible detrimental effects which TGF-β exerts during wound healing processes. Although animal models have indicated that TGF-β1 and -β2 are profibrotic, often expressed at higher levels and function to accelerate wound healing, whereas TGF-β3 improves the quality of the scar and is less important in regulating the speed of repair (O'Kane and Ferguson, 1997), it is clear that excess TGF-β within a lesion will result in unresolved inflammation and fibrotic events (Border and Ruoslahti, 1992) and therefore any disturbance in TGF-β activity may result in pathological consequences (Wahl, 1994).

As such, although TGF-β could increase proliferation and consequently satellite cell or myoblast numbers to produce more progenitors for subsequent differentiation which will aid in the repair process, excess TGF-β would result in fibrosis and scar formation. Increases in scar tissue could further prevent these progenitor cells from being incorporated into the damaged area. For therapeutic purposes, circulating and/or treatment levels of TGF-β would therefore need to be carefully modulated in order to result in any beneficial effect on repair and regeneration processes. In the current study, all three isoforms promoted C2C12 proliferation, implying a possible use of the less pro-fibrotic TGF-β3 isoform to increase cell numbers prior to transplantation.

Taking into consideration the TNC results, greater changes in PCNA expression levels were expected following treatment with TGF-β isoforms compared to control conditions. However, despite the increase in total nuclear number, no significant changes were seen in total PCNA protein levels. Possible explanations for this lack of significant differences between control- and TGF-β-treated cells could include the prolonged half-life of PCNA which results in PCNA expression of cells which have left the cell cycle, the involvement of PCNA in cellular processes other than proliferation, the fact that an unsynchronised cell culture was used, and finally that analysis of whole cell-lysates by western blotting does not distinguish between the active, nuclear form and the inactive cytoplasmic population (Celis and Celis, 1985a; Celis and Celis, 1985b; Hall *et al.*, 1990; Toschi and Bravo, 1988). The current study suggests that, despite the absence of change in total PCNA levels, translocation of PCNA from the cytoplasm to the nucleus in response to TGF-β1-incubation would favour proliferation, whereas in control cells, the prominent granular cytoplasmic expression would result in a lower rate of proliferation. This agrees with Iyengar (Iyengar, 1994) who suggested the possibility that pools of PCNA in the cytoplasm, when transported into the nucleus, could result in rapid proliferation, similar to the effect observed in the TGF-β-treated cells.

Possible ways through which the isoforms regulated proliferation, could involve the TGF-β-receptor system. Although TGF-β receptor expression was not analysed, the type I and type II TGF-β receptors have shown to mediate many of its biological effects (Geiser *et al.*, 1992; Laiho *et al.*, 1991). Especially the type II receptor, which has been shown to be a functional kinase (Wrana *et al.*, 1992) and required for activation of the type I receptor (Bassing *et al.*, 1994), could prove to be essential in mediating the effects of TGF-β during myogenesis. Although most cells in culture have both receptors, the type II receptor is expressed *in vivo* at higher levels in differentiated muscle tissue (Lawler *et al.*, 1994) and as such, studies have suggested that signalling through the type II TGF-β receptor is essential for certain changes associated with myoblast growth and myotube formation (Filvaroff *et al.*, 1994).

As mentioned, the effects which TGF-β isoforms exert on muscle formation have shown conflicting inhibitory and stimulatory results (Brennan *et al.*, 1991; Massague *et al.*, 1986; Vaidya *et al.*, 1989; Zentella and Massague, 1992). Explanations can only be hypothesised. One possibility is that the effect of TGF-β could depend on the intrinsic developmental phase of the cell. In this regard, TGF-β would promote the proliferation of myoblasts while maintaining them in an immature and activated state, still capable of differentiation (by means of MyoD expression), but preventing further development by means of the inhibitory effect of TGF-β until differentiation can be induced by an appropriate signal, or until cells have

migrated away from areas of TGF-β production. This could explain the lack of significant MyoD results observed between control- and TGF-β-treated C2C12 cells (*Chapter 6, section 6.3.1.1*), as well as the inability of TGF-β to induce migration in both C2C12 and P19 cell-lines (*Chapter 7, section 7.3).* Thus, TGF-β could maintain proliferation of committed cells, ready for fusion into myotubes, while simultaneously preventing premature myoblast differentiation until sufficient myoblast numbers have been achieved (Massague *et al.*, 1986; Olson *et al.*, 1986).

Two effects of TGF-β, stimulation and inhibition of myoblast differentiation, could be mediated by the different TGF-β receptor systems (briefly discussed in *section 2.4.3*) (Figure 5.6). Whereas the type II receptor (TGF-β RII) is believed to be required for growth inhibition induced by TGF-β, the type I receptor (TGF-β RI) may be involved in TGF-β-induced ECM production (Chen *et al.*, 1993). Since collagens inhibit myogenesis (Heino and Massague, 1990) and TGF-β stimulates collagen production during stages of wound healing, signalling through TGF-β RI could indirectly prevent myoblast proliferation and differentiation by inducing expression of collagen and other inhibitory factors. Any positive effect which TGF-β may exert on skeletal muscle growth could therefore preferentially involve signalling through TGF-β RII (Gu *et al.*, 1993). As such, the multiple effects which TGF-β isoforms exert on myoblasts might be mediated by *at least* two signalling pathways involving the two primary TGF-β receptor systems, TGF-β RI and TGF-β RII. However, two additional receptors, TGF-β RIII or betaglycan, which is required for TGF-β2 signalling, and a splice-variant of TGF-β RII, TGF-β RII-B, could further add to the variable outcomes in TGF-β signalling and the isoform-specific effects demonstrated by this growth factor.

In this regard, whereas most cells *in vitro* respond equally to the three isoforms, some myogenic cell-lines respond to TGF-β1 and -β3 but not to TGF-β2. The molecular basis for this specificity could be explained by the binding affinity of TGF-β2 to the receptors described: TGF-β RI and TGF-β RII must co-operate to transduce the TGF-β signal, with betaglycan only being required when the ligand is TGF-β2 (Lopez-Casillas *et al.*, 1993). TGF-β RII-B is able to bind TGF-β2 in the absence of betaglycan and is associated with tissues where TGF-β2 is the predominant isoform, although this receptor does also bind with the TGF-β1 and -β3 isoforms (Rotzer *et al.*, 2001). Under normal conditions, a balance may therefore exist between the receptor systems, signalling pathways and TGF-β isoform involved (Filvaroff *et al.*, 1994).

Figure 5.6. Schematic illustration of the specificity of TGF-β receptor binding.
(A) The combination of TGF-β RI and TGF-β RII is responsive to TGF-β1 and TGF-β3 and must associate with each other to transduce the TGF-β signal to the cell nucleus. **(B)** The addition of TGF-β RIII is required for TGF-β2 to associate with the TGF-β RI/TGF-β RII receptor complex. **(C)** The combination of TGF-β RI and TGF-β RII-B, a splice-variant of TGF-β RII, binds all three TGF-β isoforms and therefore also TGF-β2 in the absence of betaglycan. This receptor is associated with tissues where the predominant isoform is TGF-β2, although it still needs to be determined whether TGF-β RII-B is principally a receptor for TGF-β2.
[Adapted with modifications from Derynck and Feng, 1997; McLennan and Koishi, 2002]
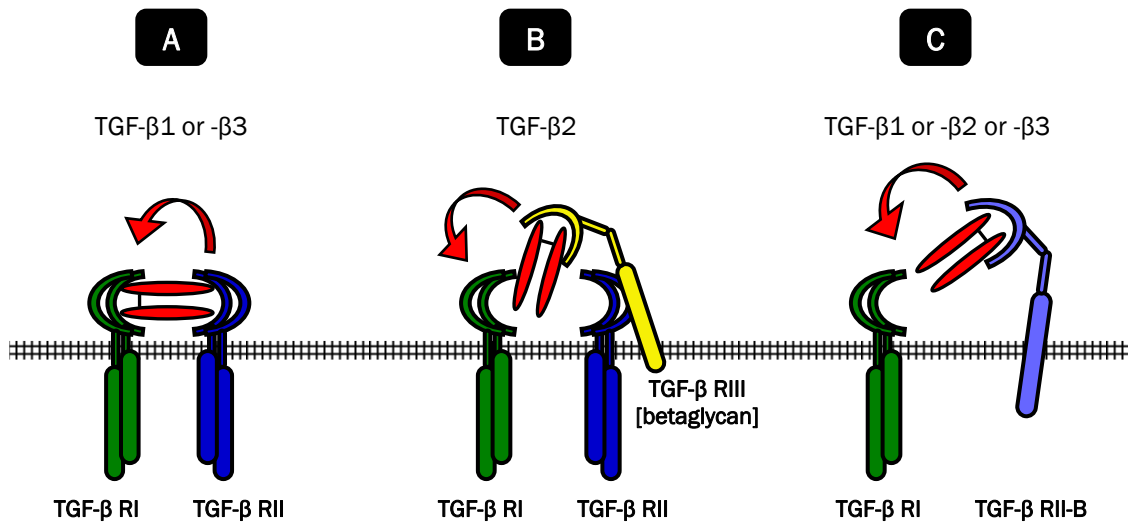
In vitro results could also be influenced by the cell population: with regards to the C2C12 cell-line, these cells in culture *produce* all three TGF-β isoforms (Lafyatis *et al.*, 1991) which could add to the effect of the TGF-β treatment protocol, influencing the balance between signalling pathways, receptor expression within the cell, extracellular concentrations of the ligand, and as such, the final effect produced by TGF-β isoforms.

5.5   SUMMARY

In summary, results have shown that all three isoforms of TGF-β increase proliferation of C2C12 cells when stimulated to differentiate in culture. Furthermore, the increase in proliferation shown in this chapter was not due to elevated total PCNA protein expression, but may rather be attributed to a change in cellular localisation of the proliferating cell nuclear antigen protein. In the following chapter the effect of TGF-β on differentiation itself is analysed.

CHAPTER 6

DIFFERENTIATION

6.1   INTRODUCTION

Stem cell therapy holds the potential to treat various conditions of muscular disease and injury. In theory, only a small number of cells and a stimulatory signal for expansion are required to elicit a therapeutic effect. To achieve clinical relevance, candidate stem or progenitor cell populations must be easily obtained, capable of efficient myogenic differentiation *in vitro* and *in vivo*, and, once transplanted, integrate into the musculature to improve function of the affected tissue. Stem cell populations with myogenic potential can be isolated from multiple regions in the body and at different stages of development (*Chapter 2*). Also, the (apparent) ability of cells to *transdifferentiate* has added additional possible sources of progenitor cells with myogenic potential to those populations resident within the muscle. However, despite such diverse stem cell populations which have shown the ability to contribute to muscle regeneration, the cell type best suited for therapeutic use remains to be established (Price *et al.*, 2007).

In *skeletal* muscle, the use of stem cells for therapeutic purposes, specifically satellite cells, has shown some degree of contributing to regeneration processes following myoblast transplantation into diseased muscle (Gussoni *et al.*, 1999; Yao and Kurachi, 1993). Greater efficiency of this treatment is however prevented by problems such as limited migration of donor cells into the damaged area, poor donor cell survival, immune-rejection and inefficient functional engraftment of transplanted cells.

Cellular cardiomyoplasty has also shown the potential to contribute to improved perfusion and contractile function of injured *cardiac* regions following myocardial infarction. Several stem and progenitor cell populations have been applied with varying degrees of success, including embryonic stem cells, and progenitor cells of skeletal muscle, bone marrow, cord blood and adipose tissue origin (Anversa *et al.*, 2002; Ghostine *et al.*, 2002; Min *et al.*, 2002; Orlic *et al.*, 2001a; Orlic *et al.*, 2001b; Toma *et al.*, 2002). Skeletal myoblasts and satellite cells have also proven to repair significant portions of the infarcted myocardium (Horackova *et al.*, 2004; Murry *et al.*, 2002; Taylor *et al.*, 1998) and possess many advantages as cardiac donor cells which include their resistance to ischaemic conditions and high proliferative capacity within

the injured muscle (Partridge, 2000). Furthermore, results from clinical trials using myoblast transplantation to treat ischaemic heart failure have thus far proven to be beneficial over the short-term (Hagege *et al.*, 2006; Menasche *et al.*, 2003). However, to verify results and safety measurements, randomised, placebo-controlled studies need to be conducted over an extended period to further characterise the risk/benefit ratio of this approach.

To improve clinical significance, further studies are required to characterise the pathways activated for homing and incorporation of stem and progenitor cells into the injured area. The application of growth factor *pre-treatment* to myogenic stem cell populations may improve their potential for functional incorporation. In addition, when these cells are transplanted into the damaged skeletal muscle or myocardium, it is essential that the micro-environmental conditions *post-injury* are suitable for appropriate differentiation of stem cells into the required cell type. Factors that can induce such selective differentiation and increase the number of functional skeletal- or cardiomyocytes therefore need to be determined (Behfar *et al.*, 2002; Torrente *et al.*, 2003; Vandervelde *et al.*, 2005).

TGF-β is a growth factor which has shown to be involved in various aspects of growth and development. *In vitro*, TGF-β *inhibits* differentiation of myoblasts (Lafyatis *et al.*, 1991; Olson *et al.*, 1986) depending on the environmental conditions and subsequently prevents activated satellite cells from leaving the proliferative stage of the cell cycle. In contrast, TGF-β *induces* both cardiac differentiation and angiogenesis of embryonic stem cells (Roberts *et al.*, 1986), and it is also known to regulate cell growth, differentiation and migration during embryonic development in an isoform-specific manner (Akhurst *et al.*, 1990; Behfar *et al.*, 2002; Pelton *et al.*, 1991). In addition, within a post-mitotic cardiomyocyte environment, stem cells have shown increased cardiac differentiation following treatment with TGF-β (Behfar *et al.*, 2002). TGF-β expression has also been shown to increase during cardiac hypertrophy (Kuwahara *et al.*, 2002), as well as in the post-infarct myocardium (Deten *et al.*, 2001).

Despite the regenerative potential shown by TGF-β in the myocardium, increased expression of the isoforms post-infarct could also adversely affect remodelling and result in progressive cardiac failure. Damage to the muscle stimulates an influx of inflammatory cells to remove necrotic tissue and facilitate the synthesis of fibronectin, collagens and other extracellular matrix proteins (Ignotz and Massague, 1986; Roberts *et al.*, 1990b). TGF-β is released by inflammatory cells, fibroblasts and to a large extent by de-granulating platelets at the wound site. Throughout the regeneration period, TGF-β is responsible for reconstruction of the basement membrane and extracellular matrix surrounding the damaged myofibers and

activated satellite cells (Streuli *et al.,* 1993). Excessive amounts of TGF-β produced at the site of injury would however result in progressive fibrosis and consequently, myocardial scarring. In addition, fibrosis could influence stem and progenitor cell mobility, whether recruited locally or transplanted, by preventing them from being incorporated into the damaged muscle (Frangogiannis *et al.*, 2002; Nian *et al.*, 2004; Vandervelde *et al.*, 2005). The inflammatory response could therefore negate the potential beneficial therapeutic effect demonstrated by TGF-β following myocardial infarction (Behfar *et al.*, 2002; Lefer *et al.*, 1990).

It is clear that TGF-β is important in the muscle repair process and could contribute to the regulation of stem and progenitor cell responses post-injury. However, most studies have analysed the role of TGF-β without distinguishing specifically between its three isoforms despite the fact that they may be able to elicit differential effects (see *Chapter 2, section 2.4.1; 2.4.5.1*). The aims of the experiments in this chapter were therefore to specifically determine the effects of the three isoforms, TGF-β1, -β2 and -β3, on the differentiation of muscle progenitor cells. The C2C12 and P19 cell-lines were used as *in vitro* model systems and cells incubated for either 24 hours or 72 hours to determine how an acute increase of TGF-β, or its presence over a longer period would affect differentiation and the recovery of the cells once the stimulus had been removed. The results presented from this chapter may give further insight into the selection of optimal candidates and micro-environmental conditions for repair by means of cell transplantation strategies.

6.2 METHODS

6.2.1 Cell Culture

6.2.1.1 C2C12 differentiation

C2C12 cells were cultured, treated and cell lysates prepared as described in Chapter 5 (*section 5.2.1.2*) to determine the short- (24 hour) and long-term (72 hour) effect of TGF-β isoforms on C2C12 differentiation. For short-term analysis, cells were treated once with TGF-β (5 ng/mℓ) on day 0, and additionally on day 1 and day 2 for long-term analysis. Cells were collected for immunoblotting purposes on days 0, 1, 5, 9 and 12 (day 12 long-term only).

6.2.1.2 P19 differentiation

      6.2.1.2 (A) Short- and long-term TGF-β treatment

To compare the short- and long-term effect of TGF-β treatment between the C2C12 skeletal cell-line and that of cardiomyocyte differentiation, P19 cells were induced to differentiate as described in Chapter 4 (*section 4.2.1.2*) and treated with TGF-β isoforms.

Briefly, differentiation was initiated by plating 500 000 cells in 60 mm bacterial-grade dishes in the presence of 0.8% DMSO (day 0). On day 1, the media with aggregates were transferred to 100 mm bacterial-grade dishes and additional differentiation medium added. Differentiation medium was also added to the aggregates on day 2 and day 3. In addition, 5 ng/mℓ TGF-β1, -β2 or -β3 was added to the differentiation medium on either day 0 only (short-term) or days 0, 1 and 2 (long-term) and compared to control conditions (P19 differentiation medium only). On day 4, maximum supernatant was removed and the aggregates transferred to 100 mm tissue culture-treated dishes. Differentiation of the cells continued in P19 culture medium which was changed every second day. Cells were harvested on day 12 for western blotting purposes by washing them with PBS, after which they were treated with 240 μℓ lysis buffer, sonicated and stored at –20℃ until analysis.

      6.2.1.2 (B) Assessment of embryoid body formation

Once P19 cells are induced to differentiate, they form aggregates which progressively increase in size. After 4 days of differentiation, once these aggregates are re-plated into tissue culture-treated dishes, they adhere to the surface and grow further to form embryoid bodies which are essential for final differentiation into cardiomyocytes. Under optimal conditions, these embryoid bodies can spontaneously start to contract. Assessing the number of embryoid bodies (or aggregate number) therefore provides a useful measure of differentiation in P19 cell cultures.

On day 6 of differentiation following long-term incubation with TGF-β isoforms, aggregate numbers were determined by counting the amount of embryoid bodies in five fields of view and determining an average number. The experiment was performed in triplicate.

Brightfield images of C2C12 and P19 cells at various stages of differentiation were taken with an Olympus microscope and camera (Olympus CKX 31) at 10x or 20x magnification.

6.2.1.3 Cycloheximide and TGF-β1 treatment

C2C12 cells were plated in 2 mℓ culture medium at a density of 100 000 cells/well in six-well tissue culture-treated plates (day -1). On the following day, the medium was removed, cells washed with PBS and 2 mℓ differentiation medium supplemented with TGF-β1 (5 ng/mℓ) added and compared to control conditions (differentiation medium only) (day 0). After 24 hours (day 1), cells were incubated with cycloheximide (CHX) (50 µM) to inhibit further protein synthesis.

Following CHX incubation for 0 (prior to addition of CHX), 1, 2, 4 and 6 hours, cells were harvested as described in Chapter 5 (*section 5.2.1.2*) with 40 µℓ lysis buffer and stored at -20ºC until later analysis (Langen *et al.*, 2004).

6.2.1.4 Immunofluorescent localisation of MyoD

To determine the cytoplasmic and nuclear localisation of MyoD in control- and TGF-β1-treated differentiating cells and compare it to the distribution of PCNA, C2C12 cells were plated into 8-chambered, cell culture-treated, coverglass units (155411, Lab-Tek, USA) (day -1) as described in Chapter 5 (*section 5.2.1.3*).

After removing the culture medium and rinsing cells with PBS on the following day (day 0), differentiation medium, supplemented with TGF-β1 (5 ng/mℓ), was added to four wells, while the other four wells contained only differentiation medium for comparison to control conditions. Further addition of TGF-β1 to the appropriate cells was repeated on days 1 and 2. Cells were maintained in a humidified incubator at 37ºC, 5% CO_2 and the medium changed every second day. For immunofluorescent staining, the medium was removed, cells rinsed with PBS and fixed in an acetone:methanol solution (1:1) on day 1 and day 5. The chambers were covered and stored at -20ºC until later analysis.

6.2.2 Western Blot Analysis

6.2.2.1 C2C12 and P19 differentiation

Protein analysis of C2C12 and P19 cells were carried out as described in Chapter 3 (*section 3.3.2.2*). Total protein was assessed by the Bradford method to determine equal loading concentrations of protein per sample (*section 3.3.2.1*). To evaluate the expression of cellular markers of differentiation, the C2C12 blots were probed with MyoD, myogenin and myosin heavy chain (MHC) and the P19 blots with connexin-43 and MHC (*section 3.2.2*). Consistency in loading of samples was assessed by immunoblotting for α-tubulin.

The resulting bands were quantified using densitometry (*section 3.3.2.3*). Each sample was evaluated in duplicate and all experiments repeated a minimum of three times.

6.2.2.2 Analysis of MyoD stability

Whole cell lysates were prepared for immunoblotting as described above. 50 µg of protein was loaded and separated on a 10% polyacrylamide gel. MyoD protein abundance was determined by densitometry, the band-intensities measured and normalised so that the absorbance at t=0 was 1. The normalised values were then plotted *versus* time for each time-point to determine the difference in the rate of MyoD degradation between control- and TGF-β1-treated cells. α-Tubulin was again used as loading control. The data are representative of three experiments.

6.2.3 Immunohistochemistry

6.2.3.1 MyoD localisation

Immunofluorescent localisation of MyoD was analysed as described in Chapter 5 (*section 5.2.3*). Briefly, after allowing cells in the 8-chambered units to thaw, they were gently rinsed with PBS and incubated in 5% donkey serum at room temperature. After 20 minutes, the serum was drained off and the cells incubated with either 50 µℓ anti-MyoD (primary antibody) or PBS (control). The cells were left to incubate for 90 minutes at room temperature, after which they were rinsed twice with PBS and incubated for a further 30 minutes in 50 µℓ FITC-conjugated secondary antibody. 100 µℓ Hoechst dye ($^1/_{200}$) was added during the last 10 minutes for nuclear staining. After thoroughly rinsing each chamber with PBS, one drop Fluorescent Mounting Medium (S3023, DAKO) was added, the units covered in a moist chamber and stored in a dark area.

Sections were visualised using the Motorized Inverted System Microscope (Olympus IX81, Imaging Software Cell®) using a 60x oil immersion objective. Photos were taken with a monochromatic camera (F-View Soft Imaging Systems) and a colour overlay applied. MyoD images of the cells were merged with the Hoechst-stained images of the nuclei from the same area. In addition, these images were also merged with the PCNA images (produced in *Chapter 5*, *section 5.2.3*) of the exact area to distinguish co-localisation of these proteins.

6.2.4 Statistical Analysis

Statistical evaluations were made by one-way analysis of variance (ANOVA) and Bonferroni's multiple comparison test using STATISTICA. A student's t-test was used to determine significance in the rate of MyoD degradation between control- and TGF-β1-treated C2C12 cells, as well as in P19 embryoid body numbers between groups. Significant differences were taken at $p < 0.05$. Data are expressed as mean $\pm$ SEM.

6.3 RESULTS

The effect of 24 hour and 72 hour exposure of TGF-β isoforms on differentiating myocytes was assessed in **(A)** skeletal (C2C12) and **(B)** cardiac (P19) cell-lineages. MyoD, myogenin and MHC were used as molecular markers of differentiation in C2C12 cells, whereas connexin-43 and MHC were used to assess differentiation of P19 cells.

In C2C12 cells, additional analysis was performed to determine MyoD stability and localisation in control- and TGF-β1-treated differentiating myoblasts.

In P19 cells, additional analysis of differentiation included assessment of aggregate number.

6.3.1 Assessment of Differentiation in Skeletal Muscle Progenitor Cells under Control- and TGF-β-treated Conditions

Brightfield microscopy revealed that all three isoforms increased the number of myoblasts while decreasing the number of differentiated myotubes formed over 12 days of TGF-β incubation (Figure 6.1). These images indicate a delay in differentiation of C2C12 myoblasts observed following 72 hour treatment with TGF-β isoforms.

Figure 6.1. Typical images of myoblasts (at 10x enlargement) in differentiating C2C12 cells following 72 hour incubation with TGF-β1 (5 ng/mℓ). **(A)** Control- and **(B)** TGF-β1-treated C2C12 cells. Images of TGF-β1-treated cells clearly illustrate an increase in myocyte numbers from day 1, together with reduced myotube formation at day 9 and day 12. TGF-β2 and -β3-treated cells displayed similar images.
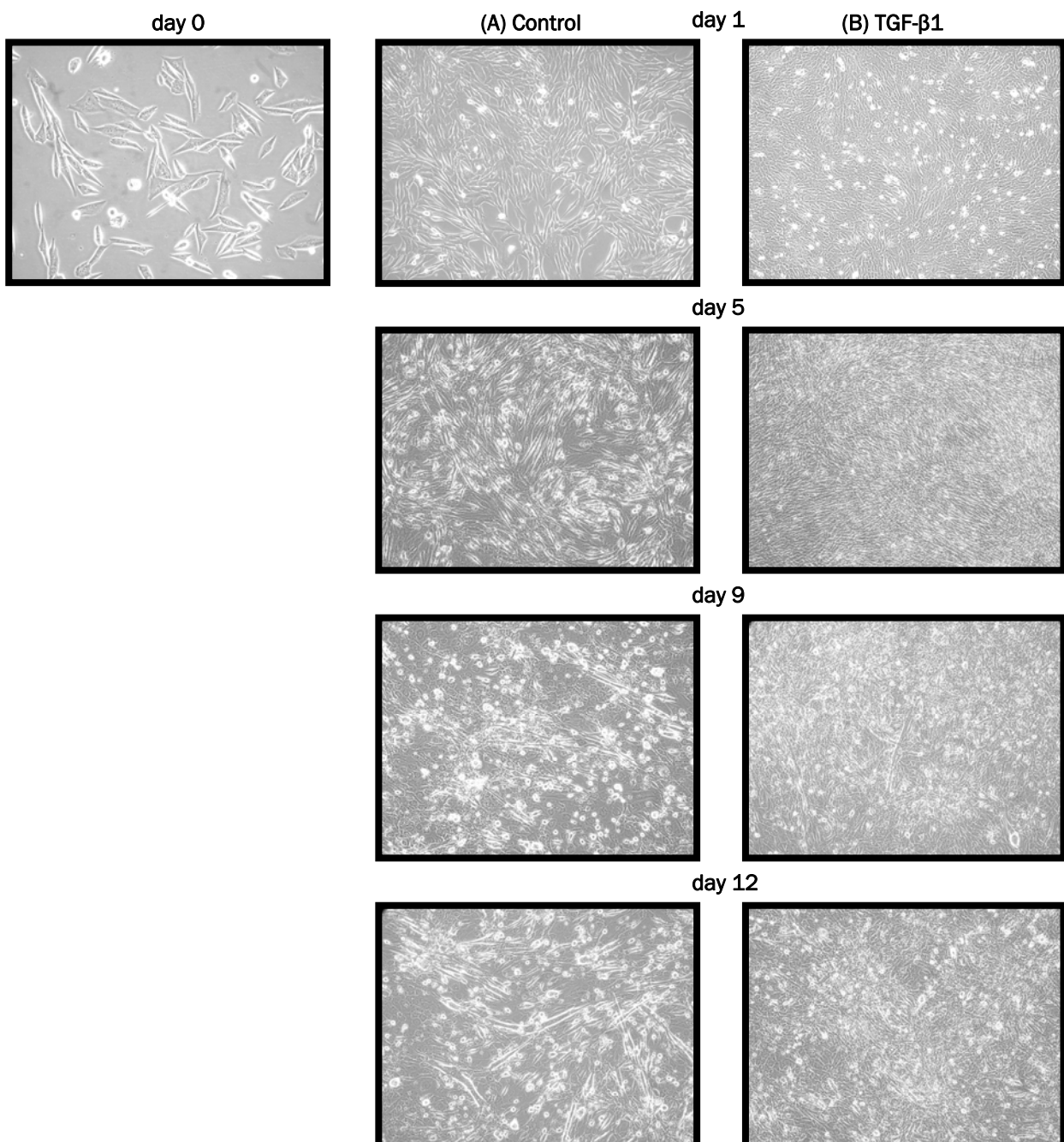
6.3.1.1   Effect of TGF-β isoforms on MyoD expression

It is well known that the expression of MyoD is crucial in the determination of myoblasts to the muscle lineage. Together with Myf-5, MyoD controls cell cycle arrest and withdrawal which is a prerequisite for induction of differentiation into skeletal muscle cells. The expression of MyoD was therefore assessed in response to short- and long-term TGF-β-incubation. Data are expressed as a % of control values.

6.3.1.1 (A) Short-term TGF-β treatment

Analysis of MyoD expression in response to 24 hour treatment with TGF-β1, -β2 and -β3 showed no differences between control- and TGF-β-treated cells at day 1 (Figure 6.2 - i). All three TGF-β isoforms increased the expression of MyoD at day 5 when compared to control conditions, although this was not significant. At day 9, there were again no significant differences between treatment conditions (Figure 6.2 - ii) and expression in the TGF-β-treated cells was reduced compared to day 5 for all isoforms.

6.3.1.1 (B) Long-term TGF-β treatment

Following 72 hour incubation with TGF-β isoforms, there were no significant differences in MyoD expression at either days 5, 9 or 12. By day 9 and day 12, the transcription factor levels tended to be increased in TGF-β-treated compared to control conditions, specifically TGF-β1, however, these increases were not significant (Figure 6.2 - iii).

To identify a potential regulator by which TGF-β isoforms control cell cycle withdrawal and initiation of differentiation, the expression of p21, a protein inducing and maintaining terminal cell cycle withdrawal in muscle cells (Walsh and Perlman, 1997), is illustrated. Failure to enter G_0, which is regulated by p21, would result in cells not being able to exit the cell cycle for differentiation. In response to 72 hour incubation with all three TGF-β isoforms, p21 expression decreased in C2C12 cells, suggesting a reduced number of cells undergoing cell cycle withdrawal (Figure 6.2 - iv).

Figure 6.2. Incubation of C2C12 cells with TGF-β isoforms for either 24 hours or 72 hours displays no significant differences in MyoD expression. MyoD protein levels were assessed by western blot analysis in control- and TGF-β-treated differentiating C2C12 cells: expression after **(i)** one day; following **(ii)** 24 hour and **(iii)** 72 hour incubation with TGF-β1, -β2 and -β3; and **(iv)** p21 expression at day 9 and day 12 following 72 hour incubation with TGF-β isoforms, as indicated. Data are expressed as mean ± SEM; n = 3.
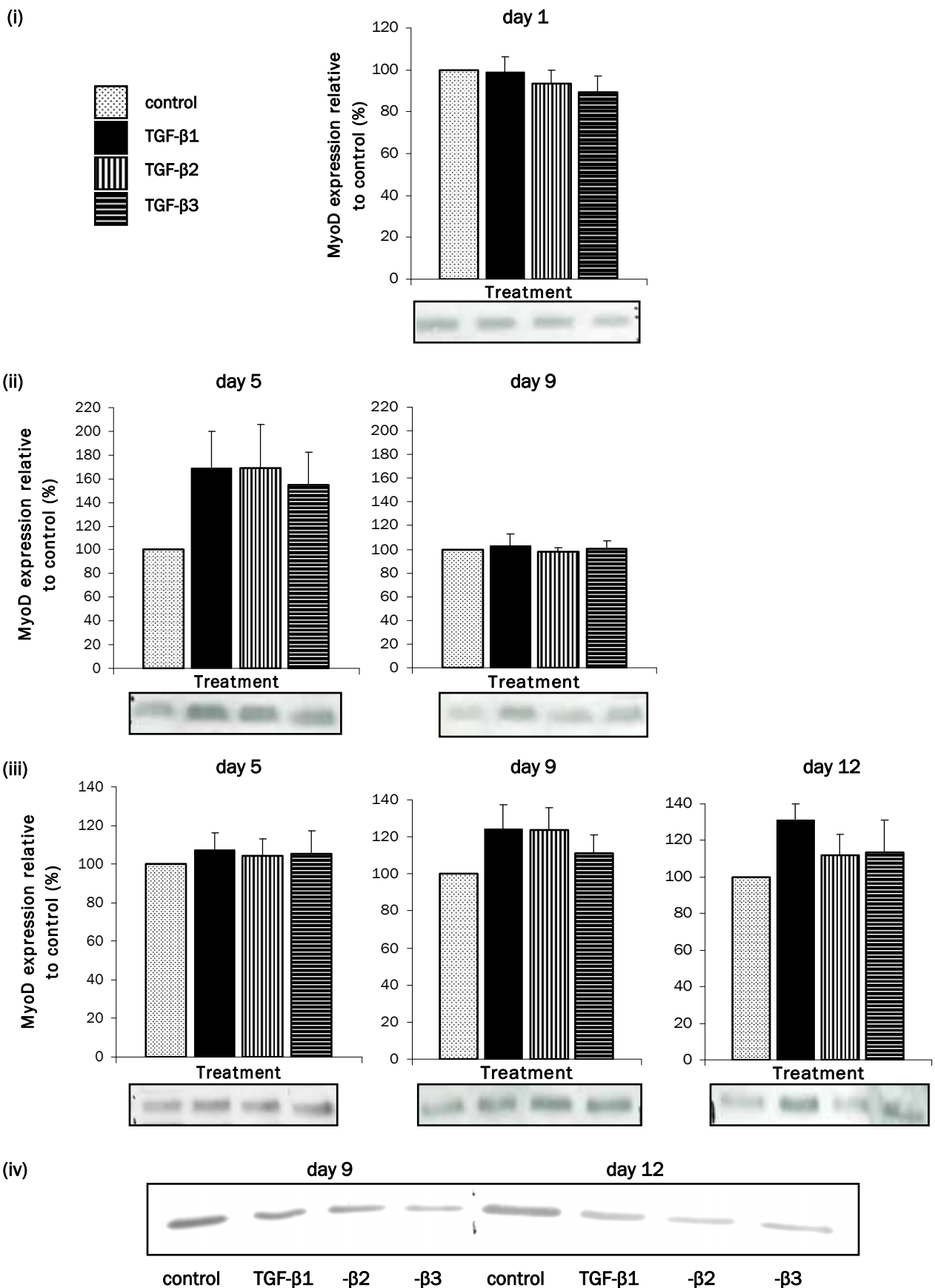
6.3.1.2   Effect of TGF-β isoforms on myogenin expression

As shown in Chapter 4 (*section 4.2.3.2),* myogenin can be used to detect early stages of myogenic commitment. To determine the influence of TGF-β on early stages of myogenesis, the effect of the three isoforms on myogenin expression was evaluated. Data are expressed as a % of control values.

6.3.1.2 (A) Short-term TGF-β treatment

Even though limited myogenin expression is expected following 24 hour induction of differentiation (Figure 6.3 - i), expression was evident and significantly increased in control cells compared to TGF-β-treated conditions which only showed minimal expression at this stage (day 1; $p < 0.01$). However, once the TGF-β signal was eliminated, the cells responded by increasing myogenin expression. By day 5 and day 9, the expression of myogenin was not significantly different between treatments (Figure 6.3 - ii), although of interest is the observation that expression levels in effect increased beyond that of control conditions in the TGF-β-treated cells, possibly compensating for the earlier inhibition.

6.3.1.2 (B) Long-term TGF-β treatment

Following 72 hour incubation, all three TGF-β isoforms significantly decreased myogenin expression at day 5 ($p < 0.05$) when compared to control conditions. However, by day 9 and day 12, expression had equalised with control and there were no significant differences (Figure 6.3 - iii). These results suggest that an extended stimulus of this growth factor is required to delay myoblast differentiation: at day 5 following 24 hour incubation, differentiation had been initiated, whereas following long-term incubation, differentiation was still significantly inhibited. Interestingly, myogenin expression in TGF-β-treated cells was increased to a greater extent at day 9 following short- compared to long-term incubation, which could suggest that the longer the incubation time, the more adversely cells are affected and the slower they are to recover.

Figure 6.3. Incubation of C2C12 cells with TGF-β isoforms significantly delays early differentiation of C2C12 myoblasts. Myogenin protein levels were assessed by western blot analysis in control- and TGF-β-treated differentiating C2C12 cells: expression after **(i)** one day; following **(ii)** 24 hour and **(iii)** 72 hour incubation with TGF-β1, -β2 and -β3. #p < 0.01; *p < 0.05. Data are expressed as mean ± SEM; n = 3.
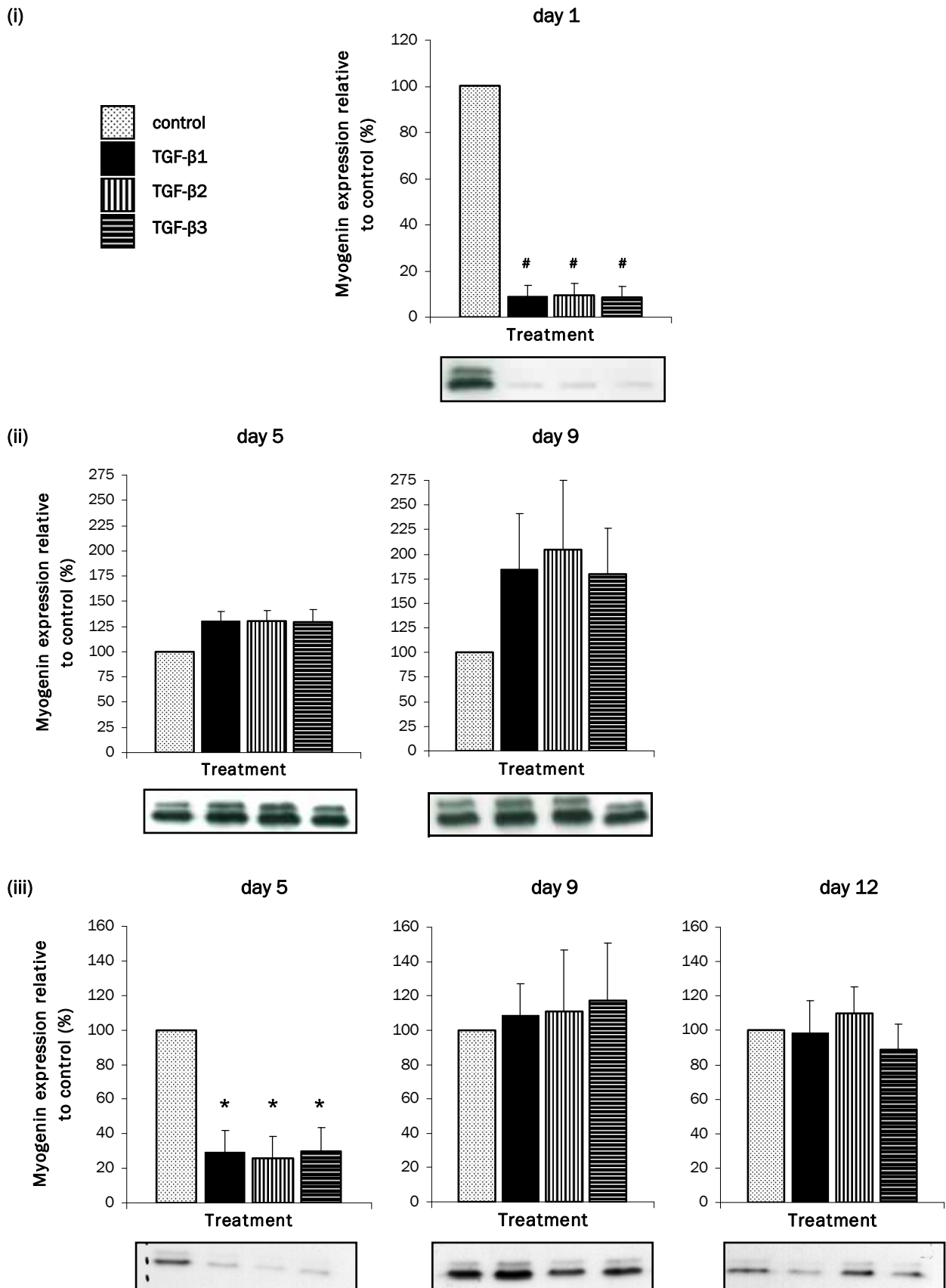
6.3.1.3   Effect of TGF-β isoforms on myosin heavy chain expression

During the later stages of myogenesis, differentiating myoblasts will express the structural protein MHC (Andres and Walsh, 1996). To assess whether the effect of TGF-β isoforms on regulatory myogenin translates into an effect on structural protein expression, MHC protein levels were analysed. Data are expressed as a % of control conditions.

6.3.1.3 (A) Short-term TGF-β treatment

MHC is not expressed in undifferentiated C2C12 cells (day 0, day 1). Following 24 hour incubation with TGF-β isoforms, MHC was expressed at a low level only in control cells at differentiation day 5 (Figure 6.4 - i). By day 9, MHC was strongly expressed in control- and TGF-β-treated cells and no significant differences were observed (Figure 6.4 - i).

6.3.1.3 (B) Long-term TGF-β treatment

Following 72 hour incubation with TGF-β isoforms, the expression of MHC was significantly reduced in all treated cells at both day 9 and day 12 (Figure 6.4 - ii; $p < 0.01$). In these cells, the effect of the isoforms was already evident at day 5, as illustrated on the western blot from this time-point (Figure 6.4 - ii). At day 9, the effect of TGF-β2 (86.4 ± 4.3% lower MHC expression) and -β3 (84.4 ± 5.4%) was greater than that of TGF-β1 (79.1 ± 6.6%), suggesting possible isoform-specific effects, although this was not significant. By day 12, there were no differences between the three isoforms (72.7-74.6% lower MHC expression) and expression tended to be increased compared to day 9.

Taken together, following 72 hour incubation, myogenin expression was reduced in TGF-β-treated cells at early time-points (day 1 and day 5), suggesting a delay in these cells to enter the differentiation pathway. Despite this delay, cells recovered to enter initial stages of differentiation at the later time-points analysed (day 9 and day 12). This effect of TGF-β isoforms on regulatory myogenin translated into an effect on structural protein levels and resulted in the subsequent expression of MHC being significantly reduced at day 9 and day 12 with fewer progenitor cells entering final phases of differentiation. Analysis of additional time-points after day 12 could indicate whether progenitor cells delayed in entering the differentiation programme recover to finally express greater levels of MHC or whether the influence of TGF-β results in permanent inhibition of terminal differentiation.

Figure 6.4. Incubation of differentiating C2C12 cells with TGF-β isoforms significantly decreases terminal differentiation of C2C12 myoblasts. MHC was expressed at day 5 only in control cells, as indicated by western blot analysis following both short- and long-term incubation (this time-point was not analysed): expression following **(i)** 24 hour and **(ii)** 72 hour incubation with TGF-β1, -β2 and -β3. #p < 0.01. Data are expressed as mean ± SEM; n = 3.
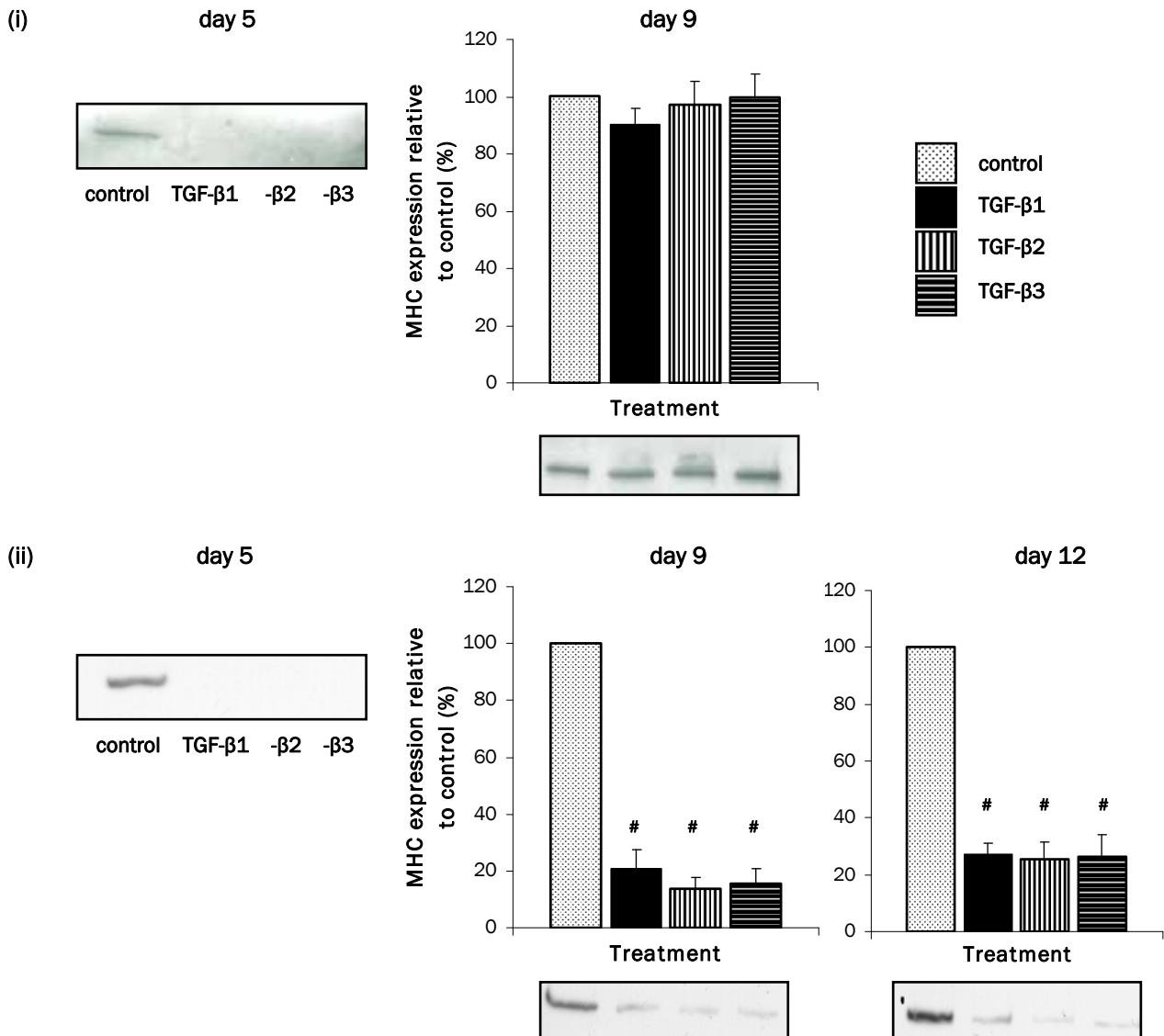
6.3.1.4   Determination of MyoD stability in control- and TGF-β1-treated C2C12 cells

For determination of the stability of endogenous MyoD, cycloheximide (CHX), a protein synthesis inhibitor, was used as described in *section 6.2.1.3*. Compared to control conditions, MyoD degradation occurred much faster in C2C12 cultures differentiated in the presence of TGF-β1. This is illustrated by the reduced intensity of MyoD expression from 60-360 minutes following CHX treatment in TGF-β1-treated cultures (Figure 6.5 A - i) and the corresponding graph (Figure 6.5 B) indicating the rate of MyoD degradation. These results demonstrate an increased rate of MyoD degradation following treatment with TGF-β1 which was significant at 360 minutes (p < 0.05). α-Tubulin was used as loading control (Figure 6.5 A - ii).

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Insufficient MyoD protein levels due to increased degradation could therefore provide a further possible mechanism by which TGF-β isoforms inhibit myogenic differentiation in skeletal myoblasts, as suggested by the early myogenin and MHC expression results. By increasing MyoD stability, myogenic processes could possibly be restored.

Figure 6.5. MyoD protein stability following CHX treatment in control- and TGF-β1-treated C2C12 cells.
(A) Expression of **(i)** MyoD and **(ii)** α-tubulin; and **(B)** TGF-β1 increases the rate of MyoD degradation in CHX-treated cells: band-intensity measured and normalised to t = 0. *p < 0.05. Data are expressed as mean ± SEM; n = 3.
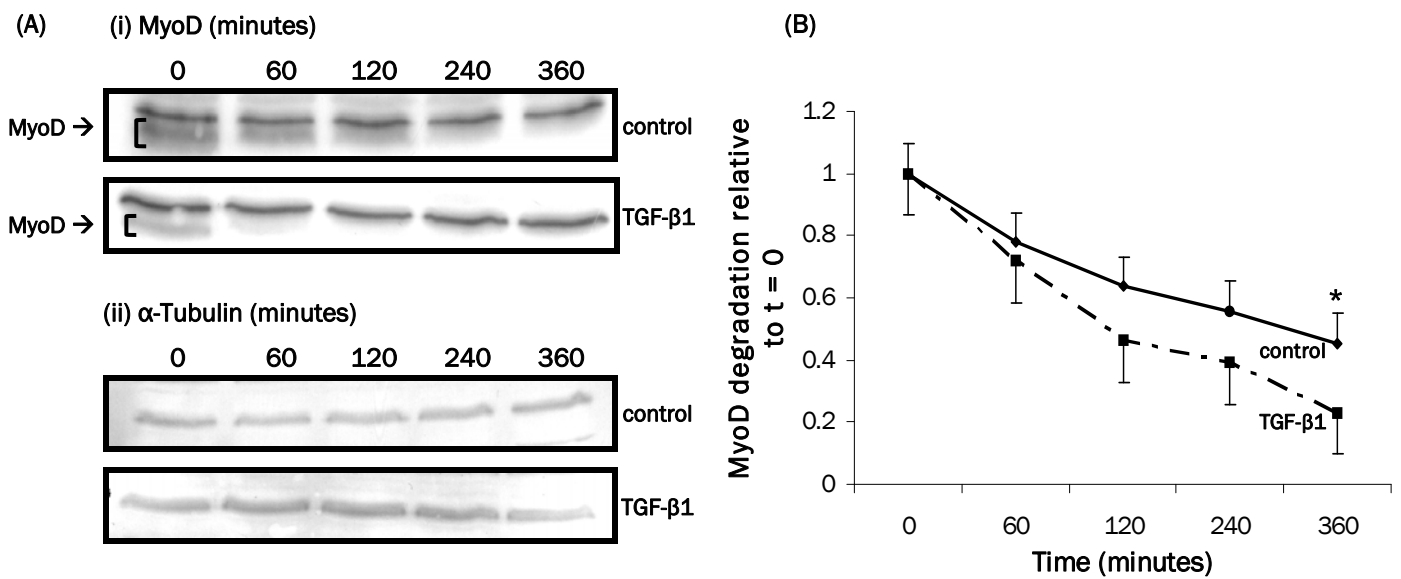
6.3.1.5 Immunofluorescent localisation of MyoD in control- and TGF-β1-treated C2C12 cells

MyoD, a nuclear phosphoprotein localised to the cell nucleus of activated, proliferating myoblasts and differentiating myotubes (Tapscott *et al.*, 1988), is *synthesised* in the cytoplasm and rapidly directed to the nucleus where it resides both free and complexed with DNA (Lingbeck *et al.*, 2003; Lingbeck *et al.*, 2005). Similarly, MyoD is also *degraded* in both the cytoplasm and nucleus (Floyd *et al.*, 2001) by the ubiquitin-proteasome system, its degradation occurring more rapidly in the nucleus than in the cytoplasm (Lingbeck *et al.*, 2003). To determine whether TGF-β alters the localisation of MyoD, thereby influencing and possibly delaying the differentiation of myocytes, C2C12 cells were treated with TGF-β1 for 72 hours and localisation analysed at day 1 and day 5 of differentiation.

Immunofluorescent staining was used to examine changes in MyoD distribution between the cytoplasm and nucleus in control- and TGF-β1-treated C2C12 cells. Control cells at day 1 (Figure 6.6 A - i) displayed increased staining in the nucleus and cytoplasmic areas surrounding the nucleus compared to day 1 TGF-β1-treated cells where positive staining was mostly distributed throughout the cytoplasm (Figure 6.6 B - i). At day 5 in control cells, highly dense, increased positive staining was visible in the nucleus and nuclear envelope (Figure 6.6 A - ii). Interestingly, TGF-β1-treated day 5 myocytes displayed smaller, elongated nuclei with speckled positive staining mainly in the nucleus, less than in control cells, and with limited positive staining also in the cytoplasm (Figure 6.6 B - ii).

Data analysis was not performed on these images, although the features of the control cells suggest higher activity of MyoD in the nucleus of these cells at day 1 and day 5 compared to TGF-β1-treated cells. For the same area of view, more nuclei can also be observed in TGF-β1-treated cells at both days 1 and 5, confirming the increased proliferation of treated cells. The level of nuclear MyoD may contribute to the control of myoblasts to withdraw from the cell cycle and enter stages of differentiation. It has been suggested that a certain level of MyoD must be reached before terminal differentiation can be initiated, and therefore, a reduced amount of MyoD translocated into the nucleus may be rate-limiting in the commitment of myoblasts to enter stages of terminal differentiation (Montarras *et al.*, 1996; Vandromme *et al.*, 1994). Although only two early time-points were analysed, the decrease in nuclear staining of MyoD observed in TGF-β1-treated cells could provide a possible mechanism to explain the reduced terminal differentiation seen in these cells, as demonstrated by the significantly lower myogenin expression at day 1 and day 5 (*section 6.3.1.2)* and MHC expression at days 5 to 12 [*section 6.3.1.3 (B)*].

Although no significant effects on MyoD expression were observed following western blot analysis [*section 6.3.1.1 (B)*], this could be the result of highly variable levels of MyoD detected in asynchronous populations of growing myoblasts (Tapscott *et al.*, 1988; Vandromme *et al.*, 1994).

To illustrate the combined staining of MyoD and PCNA (*Chapter 5, section 5.3.3*) and effect of TGF-β1 treatment on C2C12 cells, co-immunofluorescent staining for MyoD, PCNA and Hoechst is shown in Figure 6.7. Although these images were not analysed, at both day 1 (Figure 6.7 B - i) and day 5 (Figure 6.7 B - ii), TGF-β1-treated cells displayed greater PCNA (red) staining compared to control conditions. Even though this might be the inactive form, PCNA can be extracted from a soluble pool when required for proliferation (Szuts *et al.*, 2005). Nuclear MyoD expression (green) in control cells at day 5 (Figure 6.7 A - ii) is more significant than in TGF-β1-treated cells (Figure 6.7 B - ii). At this stage of myogenesis, observations from these images suggest the possibility that *in vitro* signalling pathways driving proliferation are increased by TGF-β1 while differentiation signalling is suppressed.

Figure 6.6. Immunofluorescent localisation of MyoD (at 60x enlargement) in differentiating C2C12 cells following 72 hour incubation with TGF-β1 (5 ng/mℓ). Typical images of (A) Control- and (B) TGF-β1-treated cells at (i) day 1 and (ii) day 5 of differentiation. n = 1.

(A - i) Control day 1

(B - i) TGF-β1 day 1
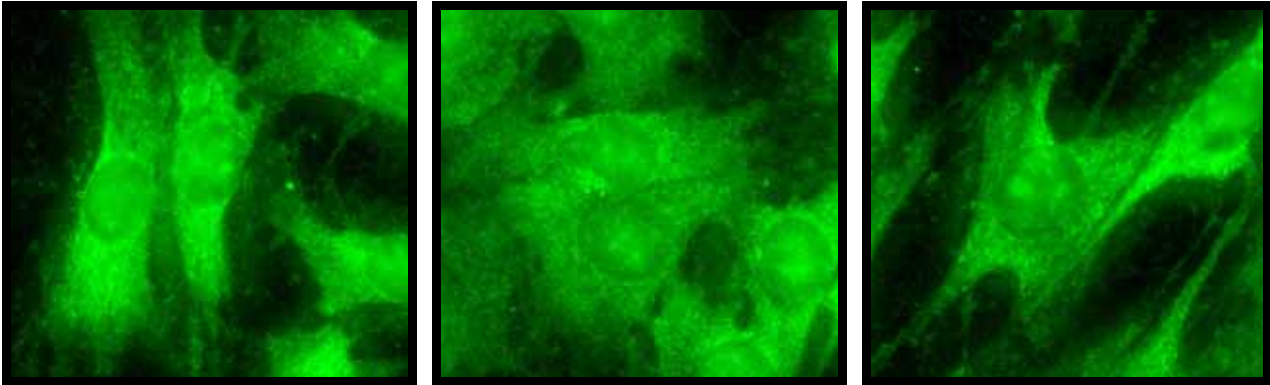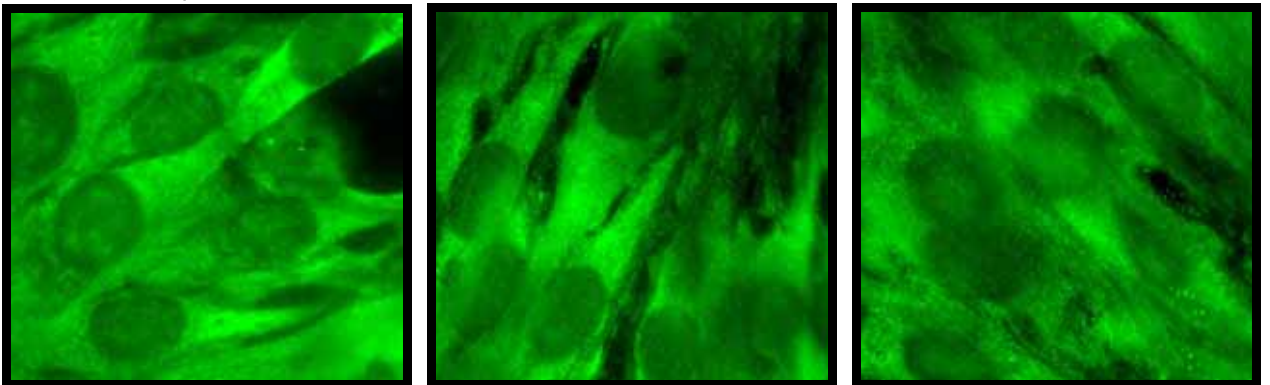
(A - ii) Control day 5
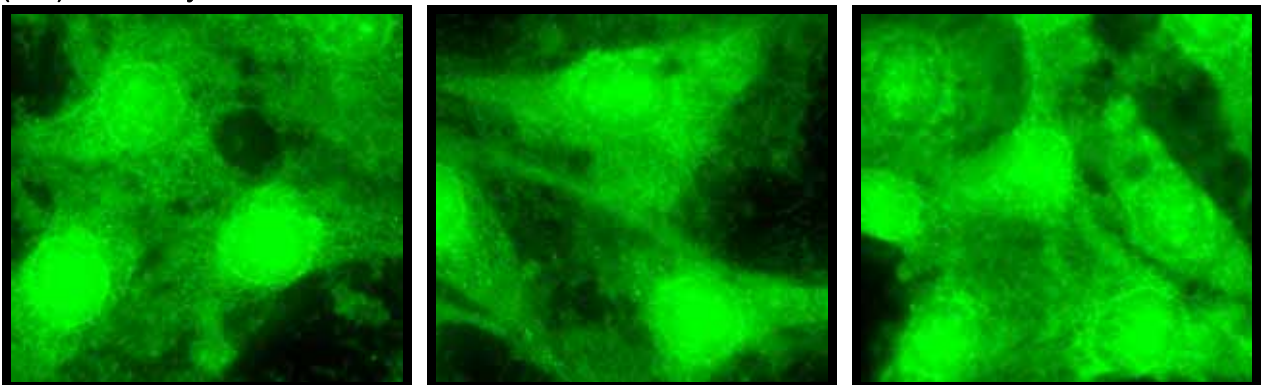
(B - ii) TGF-β1 day 5
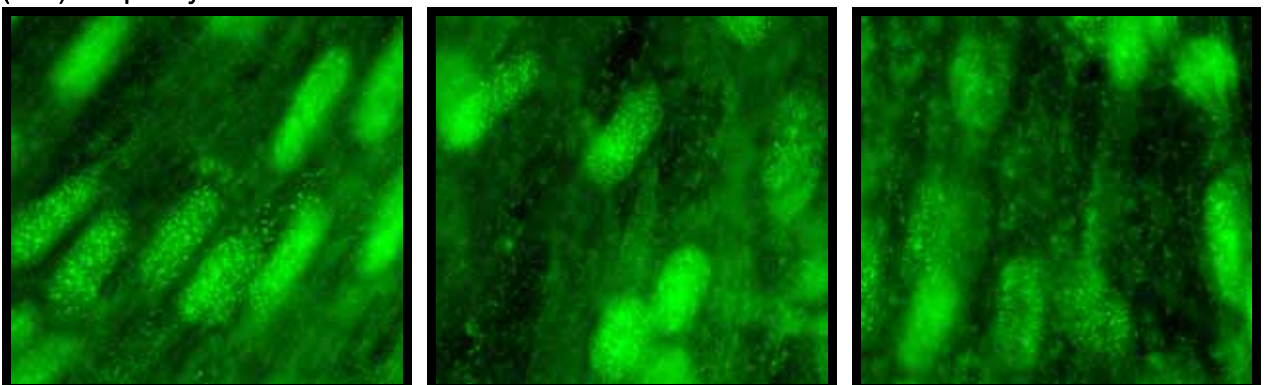
Figure 6.7. Immunofluorescent localisation of MyoD and PCNA (at 60x enlargement) in differentiating C2C12 cells following 72 hour incubation with TGF-β1 (5 ng/mℓ). Merged images of MyoD (green), PCNA (red), and Hoechst (blue) in **(A)** Control- and **(B)** TGF-β1-treated cells at **(i)** day 1 and **(ii)** day 5 of differentiation. n = 1.

(A - i) Control day 1

(B - i) TGF-β1 day 1

(A - ii) Control day 5

(B - ii) TGF-β1 day 5
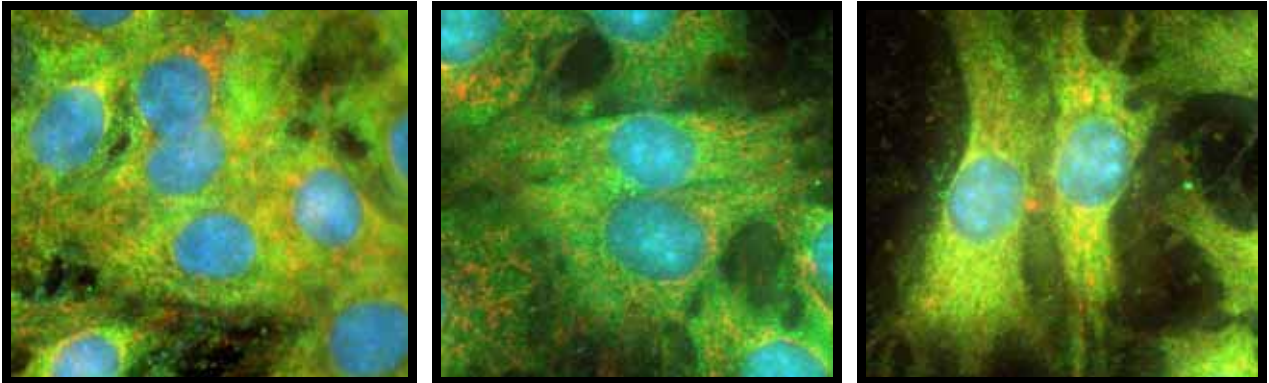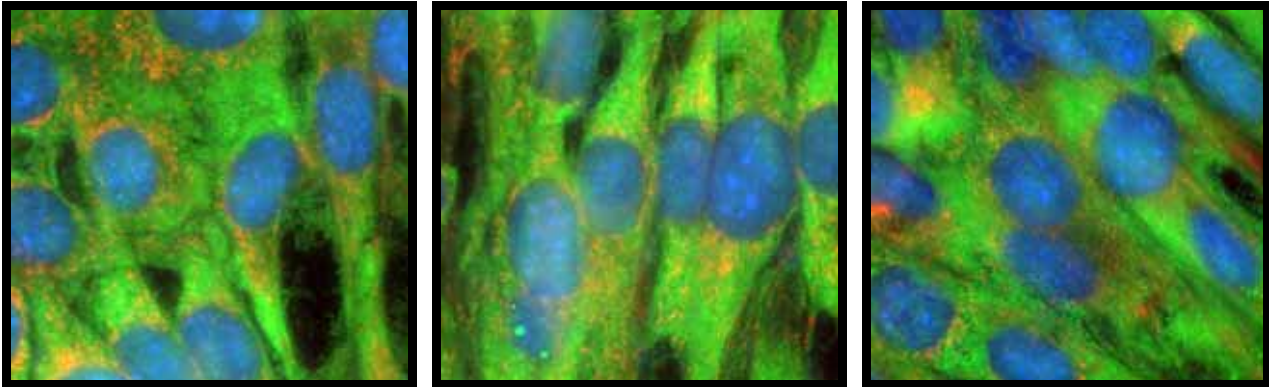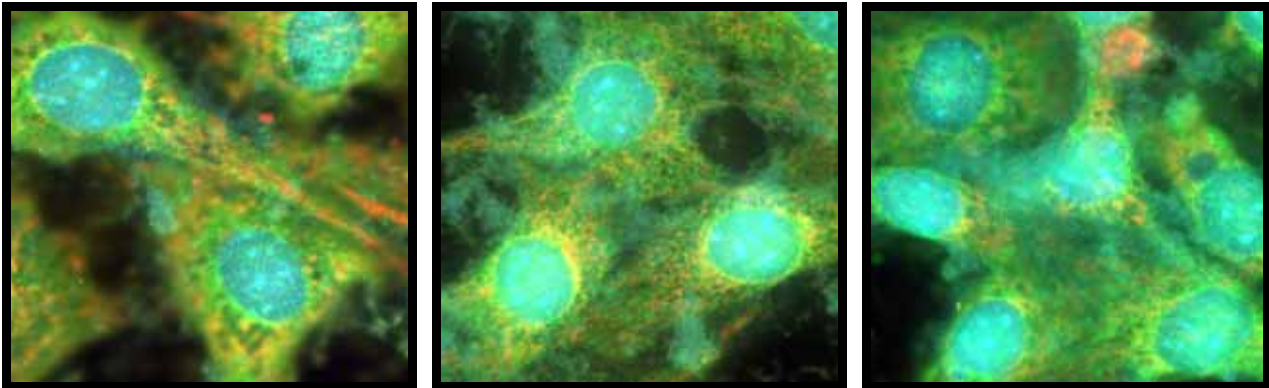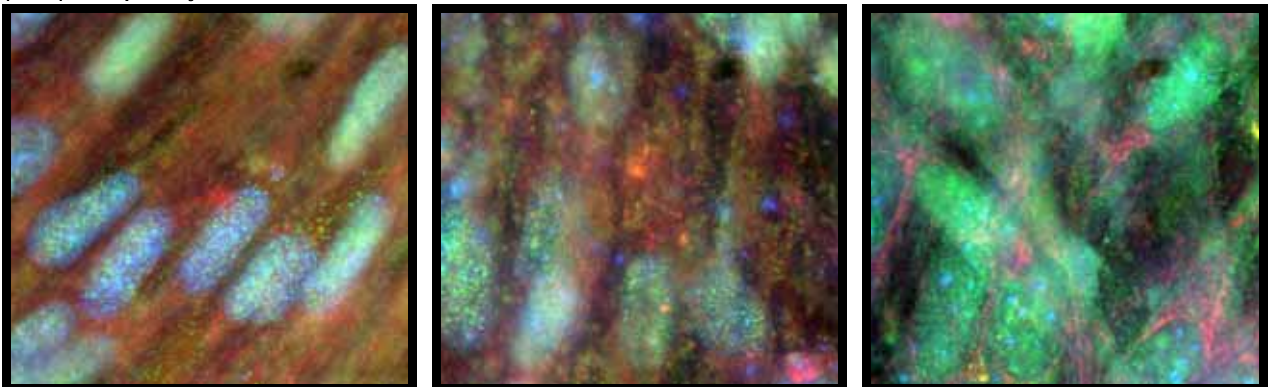
6.3.2 Assessment of Differentiation in Cardiac Muscle Progenitor Cells under Control- and TGF-β-treated Conditions

To compare the effects of TGF-β1, -β2 and -β3 on a model of cardiac progenitor cells, P19 embryonal carcinoma cells, a cell-line routinely used as an *in vitro* model for cardiomyocyte differentiation, were induced to differentiate and selected cardiac myocyte-specific protein expression levels analysed. Embryoid body formation was also determined in differentiating P19 cells.

6.3.2.1 Effect of TGF-β isoforms on connexin-43 expression

The expression of connexin-43 was analysed on day 12 of differentiation following both 24 hour and 72 hour TGF-β treatment. Connexin-43 (which forms gap-junction channels in cardiac tissue) is the dominant connexin isoform expressed by cardiac myocytes. Data are expressed as a % of control.

No significant differences between control- and TGF-β-treated cells were found with respect to connexin-43 protein expression after either 24 hour- (Figure 6.8 - i) or 72 hour (Figure 6.8 - ii) incubation.

Figure 6.8. Incubation of P19 embryonal carcinoma cells with TGF-β isoforms for either 24 hours or 72 hours displays no significant differences in connexin-43 expression. Connexin-43 protein levels were assessed by western blot analysis in control- and TGF-β-treated differentiating P19 cells: expression at day 12 following (i) 24 hour and (ii) 72 hour incubation with TGF-β1, -β2 and -β3. Data are expressed as mean ± SEM; n = 3.
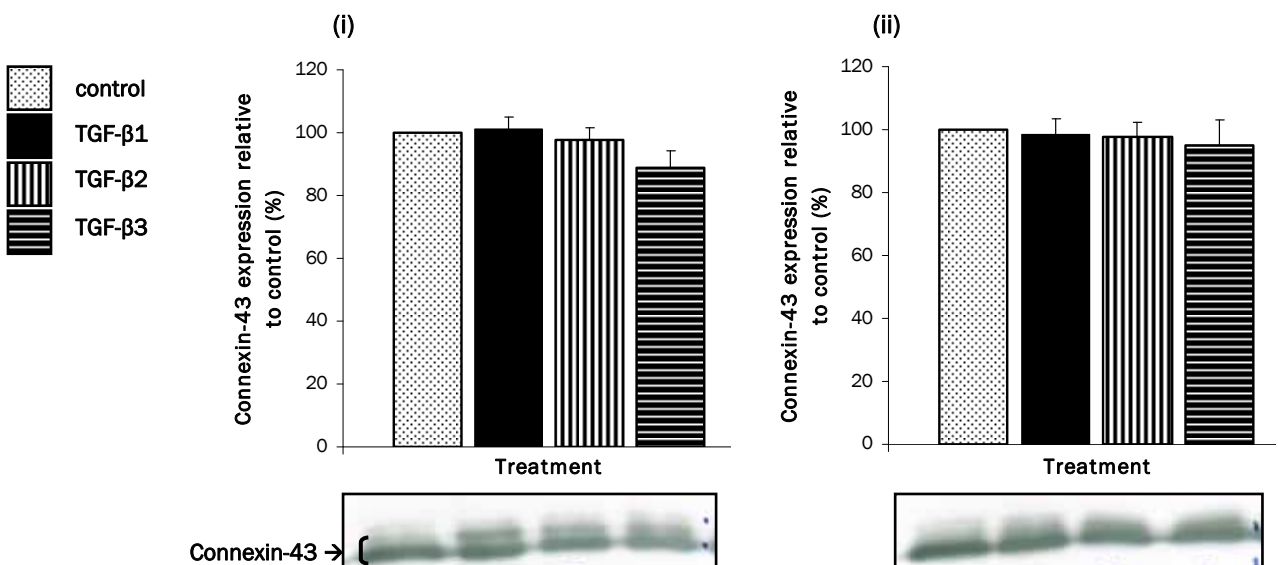
6.3.2.2   Effect of TGF-β isoforms on MHC expression

In addition to connexin-43, MHC was also analysed to determine the effect of TGF-β-isoforms on the differentiation of P19 cells following both short- and long-term incubation. Similarly, no significant differences were seen in the expression of MHC at day 12 of differentiation in response to TGF-β treatment for either 24 hours (Figure 6.9 - i) or 72 hours (Figure 6.9 - ii), for any isoform, relative to control. Even though not significant, TGF-β2 and -β3 did tend to decrease the expression of MHC following 72 hour incubation (Figure 6.9 - ii). A greater sample size may yield significant results (current sample size n = 8).

Figure 6.9. Incubation of P19 embryonal carcinoma cells with TGF-β isoforms for either 24 hours or 72 hours displays no significant differences in MHC expression. MHC protein levels were assessed by western blot analysis in control- and TGF-β-treated differentiating P19 cells: expression at day 12 following **(i)** 24 hour and **(ii)** 72 hour incubation with TGF-β1, -β2 and -β3. Data are expressed as mean ± SEM; n = 3.
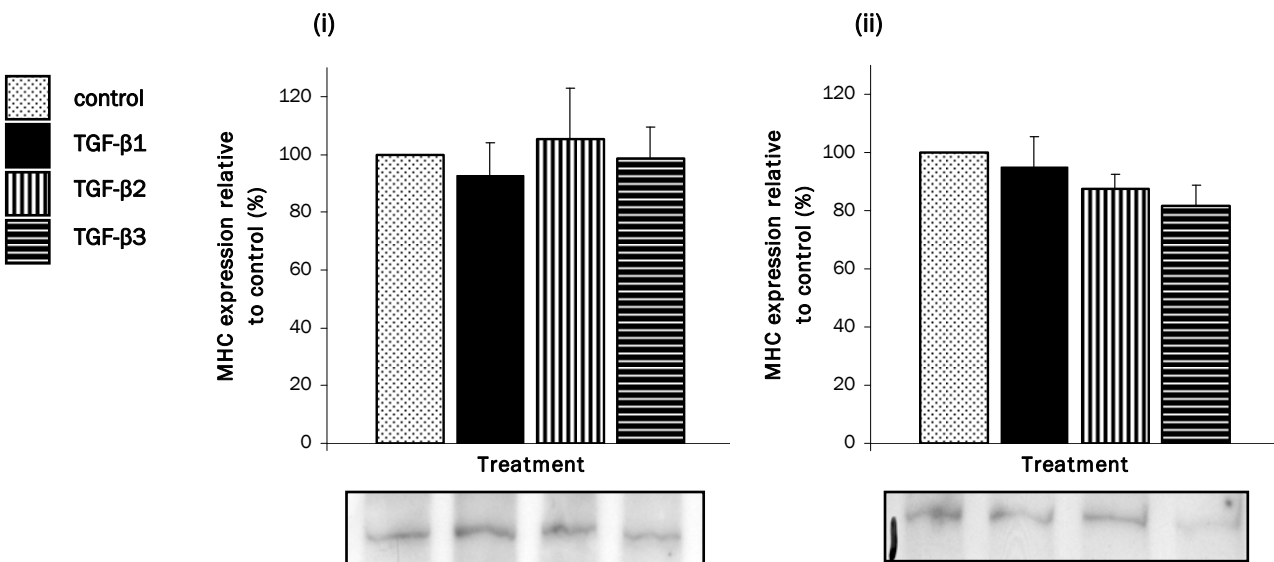
6.3.2.3   Embryoid body formation

The ability of P19 cells to aggregate and form embryoid bodies is required for these cells to undergo myogenesis. Quantification of embryoid body formation is therefore also useful to assess the progress of differentiation in these cells.

In response to 72 hour incubation with TGF-β isoforms, TGF-β1 significantly increased (p < 0.05) the number of embryoid bodies formed at day 6 compared to control conditions (77% increase), and to a lesser extent, also TGF-β2 (31%), although this was not significant (Figure 6.10 - i). TGF-β3 had no effect on embryoid body formation, as illustrated by the images of P19 embryoid bodies at this stage (Figure 6.10 - ii). No effect was observed following 24 hour incubation with the TGF-β isoforms (data not shown).

Figure 6.10. TGF-β1 increases embryoid body formation in differentiating P19 embryonal carcinoma cells relative to control. (i) Embryoid body number at day 6 following 72 hour incubation with TGF-β isoforms; and (ii) typical images (at 20x enlargement) of P19 embryoid bodies at day 6 following 72 hour incubation in (A) Control- and (B-D) TGF-β-treated P19 cells. *p < 0.05. Data are expressed as mean ± SEM; n = 3.

(i)

control
TGF-β1
TGF-β2
TGF-β3

(ii)

(A) Control

(B) TGF-β1

(C) TGF-β2
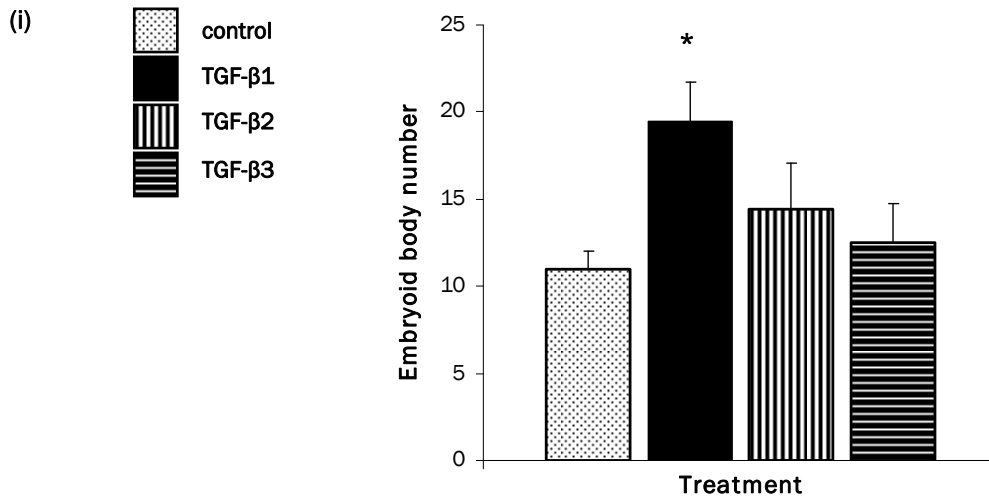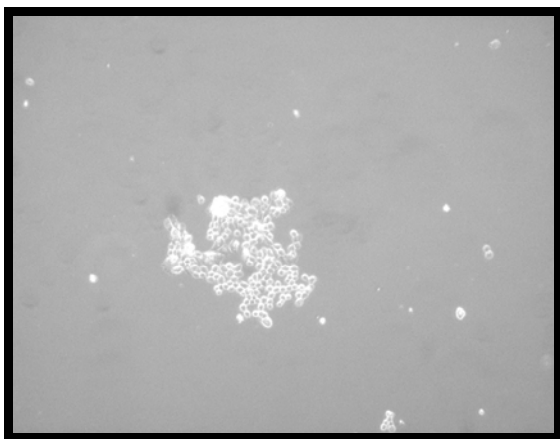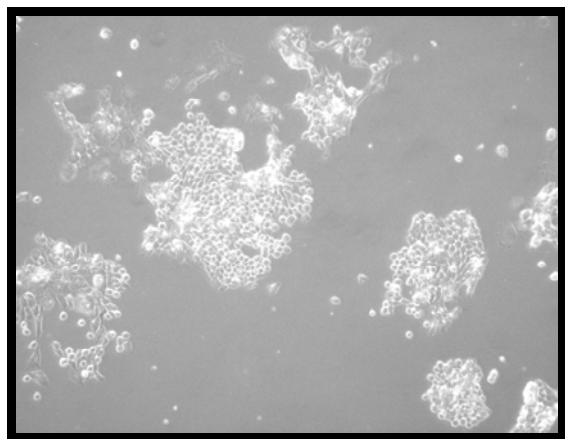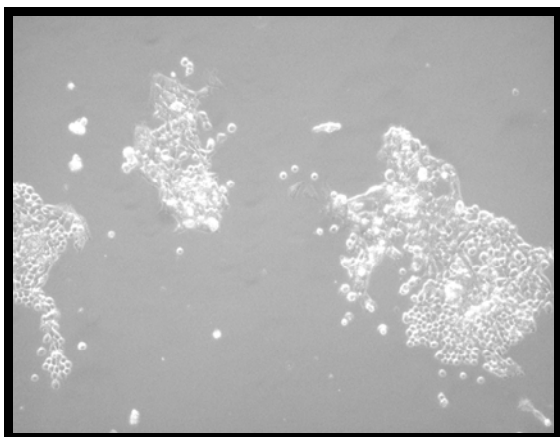
(D) TGF-β3
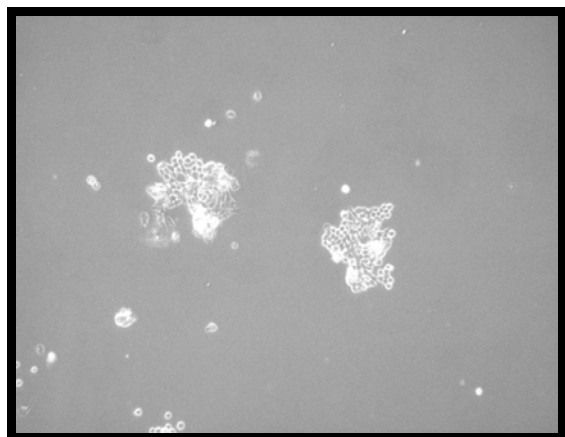
6.4 DISCUSSION

Advances in the use and manipulation of stem and progenitor cells, combined with the prospect of numerous potential clinical applications, have resulted in a rapid increase in research in this field. Specifically, in myogenesis, multiple stem and progenitor cell populations display myogenic potential and have been evaluated for their ability to contribute to both skeletal and cardiac repair mechanisms. The need to identify multiple growth factors which would improve differentiation, homing and migration of these donor populations to the damaged musculature is essential to promote further regeneration.

As discussed (*Chapter 2*), satellite cells are the primary stem cell contributors to skeletal muscle growth, regeneration and repair. Additional sources of myogenic progenitor cells within the muscle include muscle side-population cells, muscle-derived stem cells, somatic stem cells and post-mitotic nuclei. Despite this capacity of skeletal muscle for self-renewal, in a state of disease (such as muscular dystrophy), these local sources is inefficient to contribute to regeneration processes. Other sources of stem cells or muscle progenitors are therefore required from populations outside the muscle; such stem and progenitor cells which have shown the ability to differentiate into the muscle-lineage include embryonic, neural and mesenchymal stem cells, as well as various bone marrow-derived progenitors. By means of cell-based therapies, including myoblast transfer therapy and satellite cell transplantation, as well as transplantation of whole muscle fibers, progenitor cells can be cultivated *in vitro* and delivered to the damaged muscle (Collins *et al.*, 2005; Roberts *et al.*, 1989).

In contrast to skeletal muscle, the adult heart has, until recently, been regarded as a post-mitotic organ where regeneration following injury seemed to be limited. The existence of resident multipotent and primitive cardiac stem cells (Beltrami *et al.*, 2003; Laflamme *et al.*, 2002; Messina *et al.*, 2004; Urbanek *et al.*, 2003), as well as adult heart-derived cardiac progenitor cells (Oh *et al.*, 2004) and cardioblasts (Laugwitz *et al.*, 2005), has changed this notion. However, despite this apparent capacity for post-mitotic growth, the contribution of these cells to repair and renewal processes is limited and not sufficient to result in adequate functional regeneration or the prevention of scar tissue formation following myocardial infarction. As such, progenitor cells from other sources have also been investigated as potential candidates for transplantation strategies and use in clinical trials. These include bone marrow-derived cells, skeletal myoblasts or satellite cells (Drexler *et al.*, 2006; Hagege *et al.*, 2006; Menasche *et al.*, 2006; Price *et al.*, 2007; Schachinger *et al.*, 2006a; Schachinger *et al.*, 2006b; Strauer *et al.*, 2002; Wollert and Drexler, 2005), and embryonic

stem cells (Barberi *et al.*, 2005). Importantly, the micro-environmental changes following injury and the growth factors released affect distinct progenitor cell populations to variable extents, indicating an important area of control and requirement for selection of the progenitor cell type which would result in the most beneficial effects.

In this chapter, the three TGF-β isoforms, TGF-β1, -β2 and -β3, were analysed to determine their effects on differentiation of two progenitor cell types. In the literature, TGF-β has shown to be both a positive and negative regulator of cellular proliferation and differentiation with its effects being cell type specific and dependent on other local factors, with variable effects even within the same cell population (Olson *et al.*, 1986). The *in vivo* expression-pattern of TGF-β during mouse development also indicates the importance of this growth factor in specific events of differentiation (Millan *et al.*, 1991; Pelton *et al.*, 1991), in particular during periods of morphogenesis and remodelling of the mesenchyme (Heine *et al.*, 1987). As mentioned in Chapter 5, the three TGF-β isoforms often exert distinct isoform-specific effects on tissues, however, their individual roles within myogenesis have not been fully established (Ignotz and Massague, 1985; Torti *et al.*, 1989).

In skeletal muscle, TGF-β has shown to both induce myoblast maturation *in vitro*, as well as inhibit differentiation in a dose-dependent manner depending on the serum conditions: treatment of skeletal myoblast cell-lines or primary cultures with TGF-β in low serum conditions inhibits terminal differentiation (Massague *et al.*, 1986), whereas in normal serum conditions, TGF-β induces differentiation of myoblasts (Zentella and Massague, 1992). In cardiac muscle, TGF-β participates in development by enhancing differentiation and hypertrophy in an isoform-specific way (Schultz Jel *et al.*, 2002).

In this chapter, the three TGF-β isoforms were analysed at a concentration (5 ng/mℓ) within the range (2-8 ng/mℓ) used by other investigators. The C2C12 cell-line was used to analyse differentiation of skeletal muscle progenitor cells, whereas the P19 embryonal carcinoma cell-line was used for analysis of cardiomyocyte differentiation.

6.4.1   Differentiation in Skeletal Muscle Progenitor Cells

During early phases of skeletal muscle differentiation, myogenic factors are not *only* required for muscle-specific gene induction, but also for cell cycle control and regulation of the transition from the proliferative phase to cell cycle exit and differentiation.

Early stages of myoblast differentiation are identified by MyoD and Myf-5 which are required for determination to the skeletal muscle lineage, whereas myogenin and MRF4 are expressed during late stages of differentiation (Weintraub, 1993). It has been suggested that a minimal threshold of MyoD protein must be reached before differentiation can take place and as such, slight variations in MyoD expression would change the balance between either continued proliferation or induction of differentiation (Kitzmann *et al.*, 1998; Tapscott *et al.*, 1988). Therefore, MyoD may not be essential for the maintenance of the myoblast-stage of development, but rather act as an *effector* for terminal differentiation in already determined muscle cells (Montarras *et al.*, 1989).

Differentiation of skeletal myoblasts in culture is negatively controlled by serum levels which prevent entry into stages of differentiation until the serum concentration is reduced below a critical threshold. It has been suggested that growth factors in the serum induce signalling pathways which facilitate either proliferation or differentiation. Two such growth factors which have been identified as effective inhibitors of myoblast differentiation in culture include fibroblast growth factor and TGF-β (Olson *et al.*, 1986). Specifically, TGF-β has shown to inhibit the activity of MyoD and myogenin (Florini *et al.*, 1991; Parker, 1995), suggesting a mechanism by which TGF-β could regulate differentiation. TGF-β also induces the expression of cyclins, such as cyclin-D1, which is maximal during the cell cycle G_1-phase and, when over-expressed in proliferative myoblasts, leads to inhibition of MyoD and myogenin activities with subsequent inhibition of skeletal differentiation (Guo and Walsh, 1997), suggestion a further possible inhibitory mechanism of TGF-β.

In the current study, the initial expression of myogenin was down-regulated at day 1 following TGF-β-incubation and also expression at day 5 following 72 hour treatment. Thereafter, all treated cells recovered and showed increased myogenin expression at days 9 and 12. Olson *et al.* (1986) suggested that cellular signals generated by TGF-β are short-lived and require continuous occupancy of the TGF-β-receptors; this is in agreement with observations in this study. Despite these changes in myogenin expression, no accompanying changes in MyoD protein levels were observed after either 24 hour or 72 hour TGF-β treatment. The lack of a

significant effect could however be the result of not using synchronised cells, since the level of MyoD nuclear staining has shown to be highly variable in asynchronous populations of growing myoblasts (Vandromme *et al.*, 1994). Although p21 expression was not analysed in detail, this cell cycle regulator showed decreased expression in response to TGF-β isoforms suggesting an increased presence of myoblasts in the proliferative cycle. Since proliferation and differentiation are assumed to be mutually exclusive in myoblasts (Olson, 1992), TGF-β could be driving proliferation, thereby preventing myoblasts from exiting the cell cycle to enter stages of differentiation.

TGF-β may, similar to TNF-α, exert its inhibitory effect on skeletal muscle differentiation by inducing destabilisation of MyoD protein (Langen *et al.*, 2004). TNF-α has been shown to inhibit myogenesis in C2 myoblasts by increasing proteolysis, possibly by means of a caspase-mediated mechanism (Coletti *et al.*, 2002; Szalay *et al.*, 1997). In the current study, CHX treatment resulted in a reduction of MyoD protein stability in the presence of TGF-β1. As mentioned, it has been suggested that a minimal threshold of MyoD protein must be reached to induce differentiation (Kitzmann *et al.*, 1998; Tapscott *et al.*, 1988) and therefore insufficient MyoD protein levels due to increased degradation by TGF-β could provide a mechanism for the reduced differentiation demonstrated under TGF-β-treated conditions. Immunofluorescent analysis, especially at day 5, illustrated increased nuclear staining of MyoD in control- compared to TGF-β-treated cells. Therefore, MyoD was abundant in the cytoplasm of treated cells as opposed to sufficient levels of functional MyoD in the nucleus under control conditions. The data suggest that, together with the increased proliferation demonstrated in Chapter 5, by reducing MyoD protein stability, TGF-β sustains myoblast proliferation by preventing cell cycle exit and subsequent inhibition of myogenic differentiation.

To determine whether the down-regulation of early myogenin expression and therefore suppression of initial stages of differentiation translates into a decrease in structural protein levels, MHC expression was evaluated. Early (day 5) expression of MHC was reduced following 24 hour TGF-β treatment, after which myoblasts recovered and no further significant differences were observed. Following long-term treatment, however, MHC expression was significantly influenced and limited expression observed as from day 5, indicating that TGF-β isoforms reduced the structural proteins required for functional muscle activity, and as such, also terminal differentiation.

6.4.2 Differentiation in Cardiac Muscle Progenitor Cells

TGF-β has been shown to be involved in cardiac myogenesis by regulating cell growth, differentiation and migration during embryonic development, and inducing cardiac differentiation in embryonic stem cells (Akhurst *et al.*, 1990; Pelton *et al.*, 1991; Potts *et al.*, 1991; Potts and Runyan, 1989). As mentioned (*section 6.1*), TGF-β expression is up-regulated following myocardial infarction (Lefer *et al.*, 1990; Thompson *et al.*, 1988; Weber, 1997) during which the effect of the TGF-β isoforms can be two-fold: whereas TGF-β has shown to induce cardiac differentiation and demonstrate increased expression during cardiac hypertrophy (Deten *et al.*, 2001; Kuwahara *et al.*, 2002), this growth factor also stimulates tissue fibrosis. By increasing fibroblast proliferation and extracellular matrix deposition, while simultaneously reducing the degradation of these components, this structural remodelling could have a further negative effect by reducing stem cell incorporation into the myocardium. The myogenic (Behfar *et al.*, 2002; Singla and Sun, 2005) and protective (Lefer *et al.*, 1990) effects exerted by TGF-β during early phases following myocardial infarction could therefore be undone (Ikeuchi *et al.*, 2004).

The effect of the individual isoforms of TGF-β on cardiac function is unclear (Filvaroff *et al.*, 1994) with its expression being well characterised at the mRNA level in rodent, but not human cardiac tissue. Deten *et al.* showed that the ratio of TGF-β1:β2:β3 changed significantly during the post-infarct repair process, illustrating possible isoform-specific biological effects. TGF-β1 has shown to be the predominant isoform expressed at day 3 following myocardial infarction, after which levels are maintained up to 8 weeks post-infarct (Deten *et al.*, 2001; Sun *et al.*, 2000). This isoform is known to be involved in the initial, acute phase of inflammation, repair and induction of fibrosis following myocardial infarction (Dean *et al.*, 2005) and is less important during the later remodeling phases. It has been suggested that TGF-β1 has a potential role beyond scar-formation such as maintaining physiological functioning of the heart (Azhar *et al.*, 2003). Immediately following infarction (6-24 hours), TGF-β2 is the predominant isoform and is also suggested to play an important role in myocardial remodelling, induction of the foetal gene programme required for cardiac hypertrophy (Jakowlew *et al.*, 1994), as well as stimulation of cardiomyocyte differentiation (Singla and Sun, 2005). TGF-β3, which is involved in early myocardial development, is expressed from day 6 until day 82 post-infarct, with levels being between 2- and 14-fold greater than the other two isoforms during the later developmental stages (Deten *et al.*, 2001). These authors suggested that TGF-β3 could be a target for intervention for the improved healing of myocardial wounds.

Selective knockout of the isoforms have further illustrated how they exert variable effects on the cardiovascular system. Whereas disruption of TGF-β1 causes a diffuse inflammatory disease without cardiac malformations (Kulkarni *et al.*, 1993; Shull *et al.*, 1992), TGF-β2-knockout mice have a range of cardiovascular abnormalities (Molin *et al.*, 2002; Sanford *et al.*, 1997). Mice deficient for TGF-β3 have a defective cardiac phenotype (Kaartinen *et al.*, 1995). It is important to remember, however, that expression of TGF-β isoforms, at mRNA and protein level, include both the latent and active forms and is therefore indicative only of a *potential* difference in function between the three isoforms.

In accordance to the above mentioned studies, Singla and Sun (2005) compared the isoforms in their ability to induce embryoid body formation and subsequent beating of cardiomyocytes using embryonic stem cells. They demonstrated enhanced differentiation following treatment with TGF-β2, confirming the importance of this isoform in cardiac development (Singla and Sun, 2005). In the current chapter, the influence of the TGF-β isoforms on embryoid body formation demonstrated an enhanced effect by TGF-β1 and TGF-β2, with only TGF-β1 numbers being significantly higher than controls. Although it is in contrast to results from Singla and Sun, this could be due to the use of a different cell culture or agent used to induce differentiation. In the above study, embryonic stem cells were used with leukaemia inhibitory factor as differentiating agent, compared to the P19 cells which were cultured and induced to differentiate with DMSO in the described protocol (*section 6.2.1.2*). Also, it has been suggested that progenitor cells lose their differentiation potential with increasing time in culture. Using cells at a high passage number could therefore result in more variable data. Cell cultures at a passage lower than 10 were used in experiments described in this chapter, however, it has been suggested that using cell cultures at a passage greater than 5 could already induce more variable results (Crisostomo *et al.*, 2006).

Both connexin-43 and MHC expression were not significantly affected by either short- or long-term TGF-β treatment. This lack of significant effects could be due to the late time-point analysed (day 12). TGF-β was added for 24 hours or 72 hours and therefore by day 12, the cells may have had time to return to normal differentiation responses and a possible earlier effect overlooked, as demonstrated by the embryoid body formation results which were analysed at day 6. In the C2C12 cell-line, both 24 hour and 72 hour incubation with TGF-β affected the initial stages of differentiation, indicated by the reduced myogenin expression (*section 6.3.1.2*), however, once the TGF-β stimulus was removed, the treated cells recovered and normal differentiation proceeded. Such a delayed response could also have been possible in the differentiating P19 cell culture.

6.5   SUMMARY

In this chapter, the effect of TGF-β on C2C12 myoblasts demonstrated decreased differentiation in an isoform-independent manner. In the skeletal muscle cell-line, MyoD and myogenin induce a program leading to myogenic differentiation and therefore, their suppression by TGF-β isoforms could form the basis of a potential pathway (Figure 6.11) by which muscle cell differentiation is down-regulated by this growth factor. Such a pathway would regulate the induction of MHC and other structural proteins, as demonstrated by the results showing reduced expression of MHC following TGF-β treatment.

In the cardiac cell-line, except for TGF-β1 which increased embryoid body formation, no other significant isoform-specific effects were demonstrated on P19 embryonal carcinoma cells.

Results from this chapter illustrate how two progenitor cell types demonstrate contrasting effects following treatment with TGF-β isoforms, thereby emphasising the need for more information regarding the cellular effect of cytokines and growth factors prior to the selection of suitable transplantation candidates. In this regard, increased TGF-β levels post-infarct could tend to decrease differentiation of skeletal muscle progenitor cells in transplantation procedures. However, the positive influence of selected isoforms on P19 embryoid body formation would suggest that embryonic stem cells, or embryonic-like adult stem cells, may make better candidates than satellite cells for cellular transplantation. Also taking into consideration the effect of TGF-β on wound healing and fibrosis, levels of this growth factor should therefore be manipulated to result in the desired effect.

Figure 6.11. Schematic overview of a suggested mechanism for the inhibitory effect induced by TGF-β isoforms on skeletal muscle differentiation: suppression of MyoD and myogenin expression by TGF-β isoforms down-regulates expression of p21, thereby preventing myoblasts from exiting the cell cycle to enter stages of differentiation. This pathway would also regulate the induction of MHC and other structural proteins, and therefore terminal differentiation processes would consequently be inhibited, as demonstrated by the results indicating reduced expression of MHC.

[Adapted from Andrés and Walsh, 1996; Kitzmann et al., 1998 - with modifications from results generated in Chapter 5 and Chapter 6]
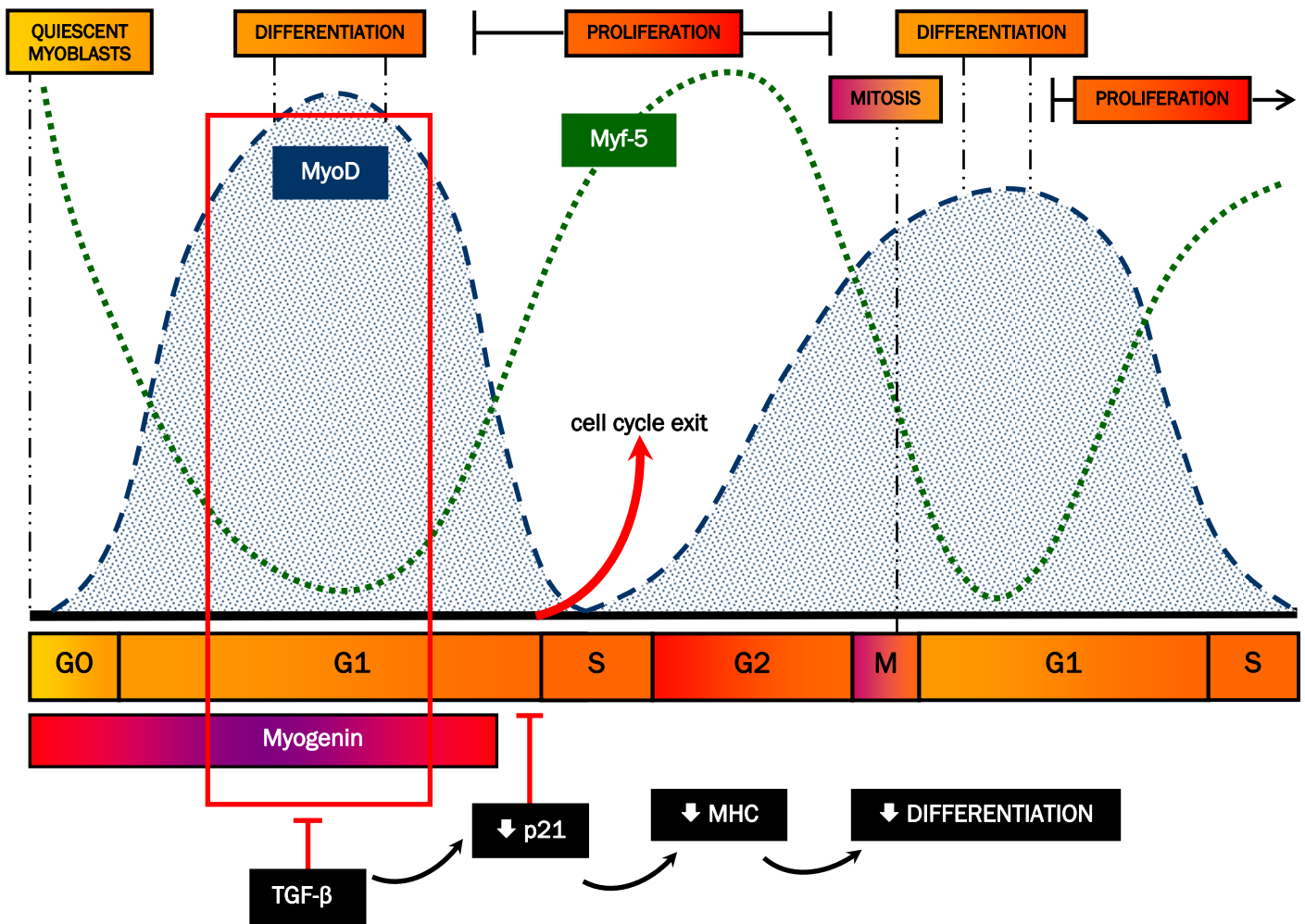
CHAPTER 7

MIGRATION

7.1 INTRODUCTION

Cell migration occurs extensively during embryogenesis and is also essential during adult life in response to tissue damage and infection. Following injury, chemotactic factors are released from the damaged myofibers and inflammatory cells which induce a strong migratory response of myogenic cells. By distinguishing the factors involved in, and the molecular signals required for myoblast recruitment during muscle regeneration and repair processes, strategies can be developed towards improved cell-mediated therapies for muscle diseases. This could enhance the regeneration capacity of diseased or injured tissue by increasing the migration potential of stem and/or progenitor cells, whether within the host or transplanted, to the relevant tissue. The importance of cell migration is also evident in the progression of chronic human illnesses such as cancer, atherosclerosis and inflammatory diseases, which might be restrained if the migration of specific cell types could be controlled.

In both skeletal and cardiac muscle, stem and/or progenitor cells within the resident, injured myofiber, as well as potentially from external fibers migrate to the site of injury following damage. Furthermore, it has been suggested that mobilisation and homing of stem and/or progenitor cells from distant niches to the injured area is also possible. Two phases of cell migration can therefore be distinguished: **(A)** within the damaged area, factors are released which promote *extracellular* signalling, homing and migration of activated cells to the site of injury; *and* **(B)** *intracellular* activation and mobilisation of the cells to migrate and respond to external signals which includes changes in the cytoskeleton and in cell adhesions.

The involvement of various *intra-* and *extracellular signalling* molecules allows for the careful control of the number of cellular responses that have to be co-ordinated during migration (Ridley, 2001). With regards to *intracellular signalling*, transmembrane receptors are stimulated in response to chemotactic factors to initiate and activate effector molecules such as small GTPases, Ca^{2+}-regulated proteins and various protein kinases, to dynamically polarise and activate the cells for migration processes. As an example, during the wound healing response, fibroblasts rapidly develop a polarised morphology to allow migration to the damaged tissue (Fukata *et al.*, 2003). Interestingly, whereas some cell types, such as leukocytes, lymphocytes, fibroblasts and neuronal cells migrate individually, epithelial and

endothelial cells often move in groups or as sheets during stages of wound healing and angiogenesis (Ridley, 2001). *External signals* or *extracellular* cues required for the activation of cell migration include diffusible factors, growth factors, signals on neighbouring cells, signals from the extracellular matrix and/or chemotactic factors released from the damaged myofibers. In addition, macrophages play an important role in regeneration and repair by stimulating the migration process and serving as an additional source of cytokines which act on inflammatory cells, satellite cells or other muscle progenitor cells (Lescaudron *et al.*, 1999; Tidball, 1995).

The essential involvement of various cytokines and growth factors on myogenic migration has been demonstrated in a number of studies. It should, however, be taken into consideration that the chemotactic responses induced by these factors are cell type specific, dependent on the concentration released and also influenced by the environmental conditions. Results from *in vitro* studies should therefore be regarded as *potential* responses which these cytokines and growth factors could induce. In this regard, hepatocyte growth factor (HGF) and selected isoforms of platelet-derived growth factor (PDGF) have displayed strong chemotactic activity on embryonic myoblasts and myogenic cell-lines (Bischoff, 1997; Corti *et al.*, 2001). During stages of skeletal muscle regeneration, it has been shown that macrophages produce PDGF and fibroblast growth factor (FGF) as chemo-attractants to guide mpcs to the area requiring regeneration of old, or formation of new myofibers (Corti *et al.*, 2001). Both vascular endothelial growth factor (VEGF) and insulin-like growth factor-1 (IGF-1) have also been shown to induce migration of myogenic cells (Germani *et al.*, 2003; Suzuki *et al.*, 2000). In cardiac muscle, bone marrow-derived stem cells have shown the ability to be mobilised into the peripheral blood and subsequently migrate towards SDF-1, HGF and LIF gradients following myocardial infarction, suggesting the possibility of these cells to be expanded *in vitro* and be used for therapeutic myocardial regeneration *in vivo* (Jackson *et al.*, 2001; Kucia *et al.*, 2004).

Comparable to its effects on proliferation and differentiation, TGF-β isoforms induce multiple migratory responses depending on the cell type and concentration released. Although TGF-β has shown to be chemotactic for satellite cells and other mpcs (Bischoff, 1997; Robertson *et al.*, 1993), migrating cells from uninjured areas may well reach a point at which the concentration of TGF-β inhibits further migration. Concentrations of TGF-β that may inhibit chemotaxis are comparable to the maximum levels found in wound fluid (Cromack *et al.*, 1987). As an indirect response, TGF-β has also been shown to promote the chemotaxis of macrophages and therefore the subsequent release and effect of additional cytokines (Robertson *et al.*, 1993).

In other cell types, both TGF-β1 and -β2 have been shown to stimulate fibroblast migration in a concentration-dependent manner (Kottler *et al.*, 2005); in carcinoma cells, TGF-β1 has demonstrated significant stimulation of cell migration (Xu *et al.*, 2003). When analysing the migratory effect of TGF-β on osteogenic and chondrogenic precursor cells, all three TGF-β isoforms have demonstrated a dose-dependent chemotactic stimulation of multipotent mesenchymal precursor cells *in vitro*. However, once these cells were treated to express higher levels of osteoblastic gene markers, no significant chemotaxis was evident in response to TGF-β treatment. Finally, TGF-β1, but not TGF-β2, has been shown to significantly inhibit cell migration of bovine endothelial cells (Merwin *et al.*, 1991).

The above mentioned studies clearly illustrate the capacity of this growth factor to exert multiple chemotactic effects depending on environmental conditions, cell type, TGF-β isoform and the concentration released. Therefore, to clarify the migratory response of myogenic cells to TGF-β, the migration-potential of skeletal muscle precursors and embryonic cardiac stem cells was assessed in response to all three isoforms using the C2C12 and P19 cell-lines. IGF-1 was applied as positive control to induce migration.

7.2 METHODS

7.2.1 Migration Assay

To test the effect of TGF-β on cell migration, both C2C12 and P19 cells were cultured and prepared as described in Chapter 4 (*section 4.3.1*). All chemotaxis experiments were carried out using the 8 μm pore size Falcon cell culture inserts together with tissue culture-treated 12-well cell culture companion plates. 50 000 cells were used per well.

7.2.1.1 Chemotactic factors

IGF-1 was used to induce positive chemotactic activity (Suzuki *et al.*, 2000) to which migration results of the three TGF-β isoforms could be compared. The cells were exposed to one of the following treatment conditions:

(i)   standard medium (negative control): DMEM containing 0.1% bovine serum albumin

(ii)   IGF-1 (positive control): standard medium supplemented with 10 ng/mℓ IGF-1

(iii)   positive control medium supplemented with 0.5 ng/mℓ TGF-β1, -β2 or -β3

(iv)   positive control medium supplemented with 5 ng/mℓ TGF-β1, -β2 or -β3

(v)   5 ng/mℓ TGF-β1, -β2 or -β3

After adding 2 mℓ of one of the treatment solutions into the wells of a companion plate, inserts were carefully placed inside the wells. 500 μℓ standard medium containing the cells was then added into the insert and cells allowed to migrate for 7 hours at 37°C, 5% CO_2.

7.2.2 Evaluation of Migration

After incubation, the inserts were taken out of the companion plate and the 500 μℓ media with non-migrated cells discarded. Each insert was then carefully placed on top of a 100 μℓ drop of heated trypsin (37°C) and incubated for a further 10 minutes to allow maximum de-attachment of cells off the underside of the insert-membrane. After this time, each insert was then taken off the trypsin and the underside of the membrane carefully rinsed with 200 μℓ C2C12 or P19 culture medium, allowing this medium with any additional migrated cells to drop back onto the trypsin-cell solution. The C2C12 or P19 culture medium/trypsin solution was then carefully mixed, 30 μℓ samples taken and the required volume placed on a haemocytometer. The number of migrated cells was then counted on the grid of the haemocytometer. Cell counts of six fields were taken per treatment and each treatment run in triplicate. The whole experiment was repeated a minimum of three times for each treatment condition.

7.2.3 Statistical Analysis

Statistical evaluations were made by one-way analysis of variance (ANOVA) and Fisher's multiple comparison test for post-hoc analysis using STATISTICA. Significant differences were taken at $p < 0.05$. All data are expressed as mean $\pm$ SEM.

7.3   RESULTS

To study the chemotactic behaviour of skeletal and cardiac precursor cells in response to growth factors, an *in vitro* migration assay was performed using C2C12 and P19 cells which were exposed to solutions of TGF-β isoforms and/or IGF-1. Using a 12-well chemotaxis system, cells in the insert-solution migrate through a membrane into the well to which chemotactic factors have been added. In preliminary experiments (see *Chapter 4*, *section 4.3.2*), IGF-1 resulted in successful migration of C2C12 cells and was therefore selected as growth factor to induce migration in subsequent migration assays. Treatment solutions included a low and high dosage of TGF-β isoforms added to IGF-1 and a high TGF-β dosage without addition of IGF-1. Results are displayed as the total number of migrated cells.

7.3.1   C2C12 Migration

As shown in Figure 7.1, IGF-1 significantly stimulated migration of C2C12 myoblasts compared to control conditions ($p < 0.01$) with an approximate 6-fold increase in cell migration number, confirming the use of this growth factor as a chemo-attractant for myoblasts. Using IGF-1 as migration agent, ~34.4% of the cells migrated, compared to ~7.1% determined under control conditions. Myoblasts failed to show a significant response with the addition of any TGF-β isoform (5 ng/mℓ) as migration agent, with migration numbers (7.4-11.8% migration) being similar to those determined under control conditions. IGF-1-induced migration numbers were therefore also significantly higher compared to migration following TGF-β treatment ($p < 0.01$).

Figure 7.1. IGF-1 (10 ng/mℓ) significantly stimulates migration of C2C12 myoblasts when compared to control- and TGF-β-treated (5 ng/mℓ) conditions. #$p < 0.01$. Data are expressed as mean ± SEM; n = 3.
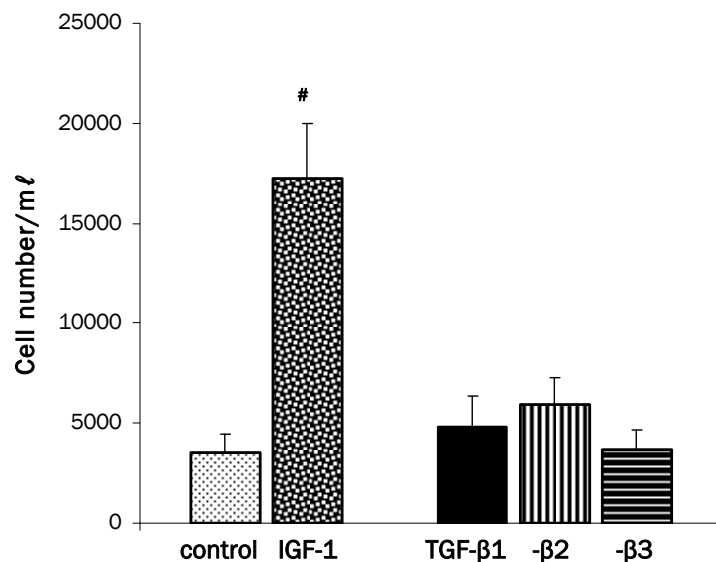
In the presence of TGF-β isoforms, IGF-1-induced migration was reduced, with the largest effect seen in response to TGF-β3 (0.5 ng/mℓ; $p < 0.01$), followed by TGF-β2 (0.5 ng/mℓ; $p < 0.05$) (Figure 7.2). At the higher TGF-β dosage (5 ng/mℓ), migration was also reduced, although not significantly compared to IGF-1-induced migration. Despite the inhibitory effect which the addition of TGF-β isoforms display on IGF-1-induced migration, the number of migrated cells under these conditions was still significantly higher compared to control conditions at both the low (19-24% migrated cells) and high (~26.7% migrated cells) dosage of TGF-β ($p < 0.01$).

Figure 7.2. TGF-β1, -β2 and -β3 reduce IGF-1-induced (10 ng/mℓ) migration of C2C12 myoblasts at low (0.5 ng/mℓ) and high (5 ng/mℓ) dosages. TGF-β2 ($p < 0.05$) and TGF-β3 ($p < 0.01$) at the low dosage were significantly lower than IGF-1-induced migration. Cell migration under control conditions was significantly lower ($p < 0.01$) compared to all treatment conditions. #$p < 0.01$; *$p < 0.05$. Data are expressed as mean ± SEM; n = 3.
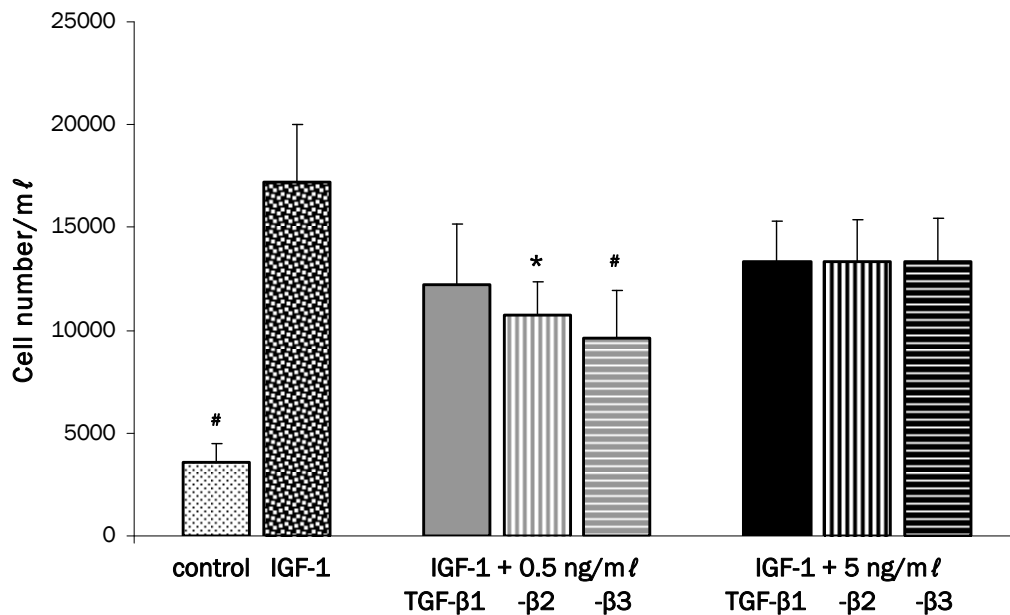
7.3.2   P19 Migration

In comparison with C2C12 cells, cardiac progenitor cells demonstrated limited migration under all treatment conditions. Although IGF-1 induced a significant increase in migration of the P19 cells compared to control conditions (Figure 7.3; $p < 0.01$), the percentage of IGF-1-induced migrated cells was still only ~6.4% of the total amount of seeded cells, compared to less than 1.0% determined under control conditions. Similar to C2C12 cells, TGF-β isoforms (5 ng/mℓ) did not induce migration in the absence of IGF-1, with only 2.2-4.7% of the total amount of seeded cells migrating. Despite the reduced capacity of TGF-β isoforms to stimulate migration, only TGF-β3 resulted in significantly reduced ($p < 0.05$) migration compared to IGF-1 treatment conditions (Figure 7.3).

Figure 7.3. IGF-1 (10 ng/mℓ) significantly stimulates migration of P19 cardiac progenitor cells compared to control- (p < 0.01) and TGF-β3-treated (5 ng/mℓ) (p < 0.05) conditions. #p < 0.01; *p < 0.05. Data are expressed as mean ± SEM; n = 3.
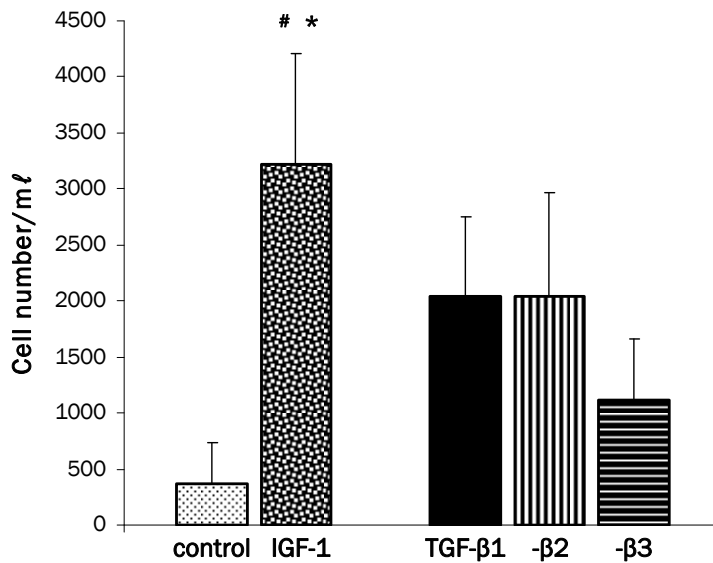
The addition of TGF-β isoforms at both the low and high dosage again reduced the number of IGF-1-induced migrated cells, although only the low dosage of TGF-β1 and -β3 was significant (p < 0.05) (Figure 7.4). At the higher dosage, migration was also reduced, although not significantly. At the low dosage of TGF-β, only 2.2-3% of the cells migrated, whereas 3.3-3.7% cells migrated at the high dosage of TGF-β when added to IGF-1 treatment.

Figure 7.4. TGF-β1, -β2 and -β3 reduce IGF-1-induced (10 ng/mℓ) migration of P19 cardiac progenitor cells at low (0.5 ng/mℓ) and high (5 ng/mℓ) dosages. Only TGF-β1 and -β3 at the low dosage were significantly lower (p < 0.05) compared to IGF-1-induced migration. *p < 0.05. Data are expressed as mean ± SEM; n = 3.
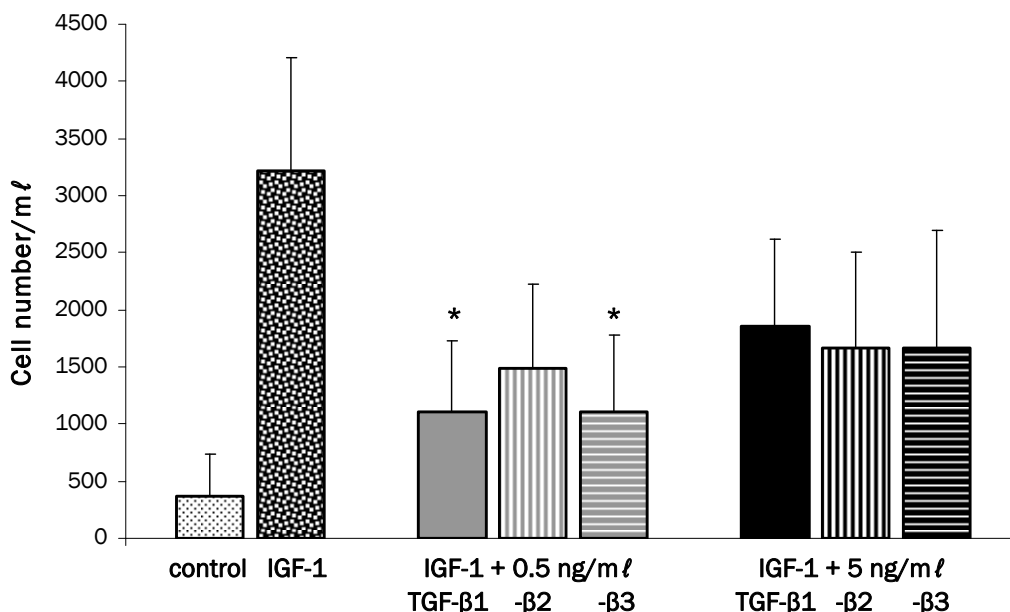
Taken together, this data demonstrates that IGF-1 acts as a chemo-attractant for C2C12 myoblasts, and to a lesser extent for P19 cardiac progenitor cells. Figure 7.5 clearly illustrates the limited migration response of P19 cells to IGF-1 and TGF-β treatment. TGF-β isoforms did not induce migration in either C2C12 or P19 cell-lineages. In the presence of IGF-1-induced migration, TGF-β isoforms decreased C2C12 cell migration to variable degrees. In P19 cells, there was only a minimal increase in cell migration numbers in response to treatment with IGF-1 and therefore a further limited effect with the addition of either dosage of TGF-β isoforms to IGF-1. Even though there were no clear isoform-specific effects for the most part, TGF-β1 and/or -β2 generally displayed greater responsiveness than TGF-β3 to the relevant treatment conditions.

Figure 7.5. Comparative chemotactic response of C2C12 versus P19 cells to IGF-1-induced migration and treatment with TGF-β isoforms relative to the total amount of seeded cells (%).

Data are expressed as mean ± SEM; n = 3.
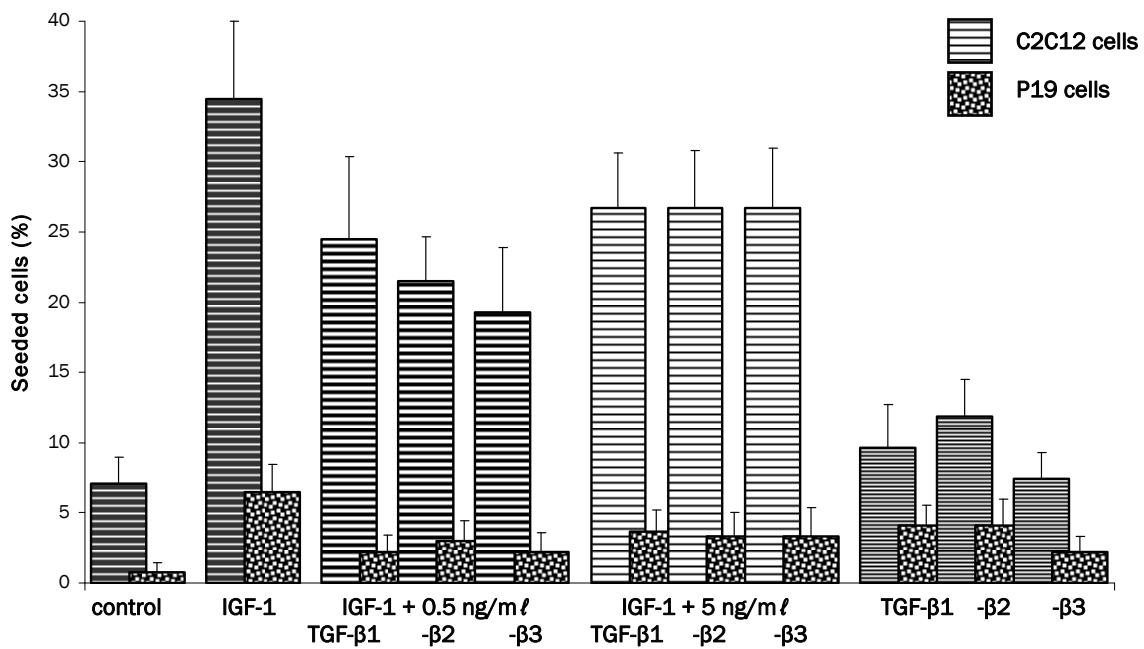
7.4   DISCUSSION

It has been suggested that the migration of muscle stem or progenitor cells during regeneration is regulated by overlapping gradients of several effector molecules released at the site of injury. Furthermore, these molecules may contribute to enhance the dispersion of muscle progenitor cells derived from the host bone marrow, muscle or other tissues which have shown the ability to contribute precursor cells to repair processes. By establishing the signals involved in the recruitment of cells from the circulation, cellular transplantation therapy could be enhanced, which, up to now, has demonstrated low efficiency of muscle integration by the donor cells.

The migration of stem and progenitor cells during embryogenesis is essential for human development. With regards to myogenesis, skeletal muscle progenitor cells migrate from the somite into the developing limb buds where they proliferate and fuse to form primary myotubes (Chevallier *et al.*, 1977). This early migration is possibly induced by signals arising from the mesodermal tissues and is dependent upon attachment of the cells to fibronectin present in the ECM which forms the substratum for migration (Brand-Saberi *et al.*, 1993). It has been suggested that the transcription factor Pax3 is required at this stage for the migration of precursor cells from the somite to the muscle (Daston *et al.*, 1996). As mentioned, in addition to the migration required during early stages of development, in situations of disease or injury during adult life, migration of myogenic cells from nearby viable muscle, or from additional sources of myogenic cells beyond the muscle to the site of injury, is also required and provides an important means of augmenting the population of cells which can participate in regeneration. During such regeneration or inflammatory processes, the damaged muscle produces factors that stimulate the chemotaxis of stem or progenitor cells either from within the muscle (close by or from a distant site within the damaged muscle), or from adjacent muscle fibers (Watt *et al.*, 1994), the latter probably being more restricted (Moens *et al.*, 1996). Macrophages are also attracted to the site of injury, stimulating the release of additional cytokines and growth factors which further induce strong positive chemotactic responses to attract additional myogenic cells (Robertson *et al.*, 1993).

Growth factors which are highly expressed during stages of muscle regeneration include HGF, FGF, PDGF (AB and BB isoforms), EGF, VEGF and IGF-1. In addition, inflammatory cytokines such as TGF-β, TNF-α and IFNγ are also produced by several cell types during the inflammatory response following injury. The chemotactic abilities of these factors display differential effects: research by Corti *et al.* (2001) has shown that HGF significantly increases

migration of myogenic cells at lower rather than higher concentrations; the effect of HGF is greater than the chemotactic response displayed by both FGF and PDGF; EGF has not demonstrated significant effects on migration (Corti *et al.*, 2001); VEGF has shown to induce migration of both skeletal and cardiac myocytes, as well as of smooth muscle and endothelial cells (Grosskreutz *et al.*, 1999). This positive chemotactic ability displayed by VEGF in various cell types, together with its production being enhanced by hypoxic conditions, supports a role for this growth factor in mediating important physiological responses during angiogenesis such as blood vessel formation, and therefore restoration of the vasculature (Zaccagnini *et al.*, 2005). In our initial experiments, VEGF was not consistently able to induce migration and as such, was not selected as growth factor to induce migration in subsequent assays, and therefore warrants further investigation. IGF-1 has shown to significantly contribute to the development, regeneration and migration of skeletal muscle myoblasts and C2C12 cells (Suzuki *et al.*, 2000), as well as cardiac resident stem and progenitor cells (Urbanek *et al.*, 2005; Urbich *et al.*, 2005) and was therefore used as growth factor to induce migration.

Of the cytokines, TNF-α has shown to induce the migration of leukocytes and fibroblasts, however, this cytokine has a limited effect on myoblast migration, similar to IFNγ. Although TNF-α possibly induces migration in a dose-dependent manner, it exerts a toxic effect on myoblasts at higher concentrations, resulting in increased myoblast mortality (Corti *et al.*, 2001). With regards to the TGF-β isoforms, as mentioned above (*section 7.1*), this growth factor exerts multiple effects on myogenic cell migration. Whereas it has demonstrated no chemotactic activity towards C2C12 cells (Suzuki *et al.*, 2002 - unpublished data), it has shown to induce migration of satellite cells (Bischoff, 1997) and other myogenic precursors (Robertson *et al.*, 1993). In cardiac muscle, although TGF-β expression is up-regulated following ischaemic injury, whether it has any significance towards chemotaxis of cardiac progenitor cells, remains to be established. Instead, this increased expression of TGF-β which has been identified in the myocardium during both cardiac hypertrophy and heart failure, has been associated with the development of myocardial fibrosis (Border and Noble, 1994; Hao *et al.*, 2000; Weber, 1997). In this regard, TGF-β has shown to stimulate the migration of fibroblasts (Kottler *et al.*, 2005) rather than cardiac progenitor cells.

The present study was undertaken to determine the *in vitro* chemotactic ability of skeletal and cardiac cells towards TGF-β isoforms with and without the addition of IGF-1. As the positive control, IGF-1 induced migration in both cell-lines, although the potential of the P19 cells to migrate, in all treatment conditions, was greatly diminished. As shown in Figure 7.5, maximum migration of P19 cells, which was under the influence of IGF-1, was only 5-10%.

In the *C2C12* cell-line, TGF-β isoforms displayed no effect on cell migration and migration numbers were similar to those under control conditions. Although no significant isoform-specific effects were displayed, TGF-β3 resulted in the least migratory response. In combination with IGF-1, TGF-β reduced IGF-1-induced migration at both the low and high dosage, with TGF-β2 and -β3 at the low dosage demonstrating significant reductions. Despite this inhibitory effect of TGF-β on cell migration, the influence of IGF-1 was still dominant to result in significantly increased migration numbers compared to control conditions.

P19 cells demonstrated limited responses to all treatment conditions, including IGF-1. Similar to C2C12 cells, treatment with TGF-β3 again showed the greatest inhibitory effect, also at the low dosage in combination with IGF-1. The number of migrated P19 cells was comparable whether treated only with TGF-β isoforms, or TGF-β in combination with IGF-1. Taking into consideration that treatment with IGF-1 also resulted in limited migration of the P19 cells, these results could suggest that the cell type had a greater influence on the migration response than the influence of the TGF-β treatment. P19 cells form non-adhering aggregates in culture (van der Heyden and Defize, 2003) which could have influenced their movement. Also, undifferentiated P19 cells have electrophysiological properties such as inward currents, Na^+/H^+-exchangers and voltage-dependent sodium channels which could possibly interfere or override the action-potentials generated during migration.

The P19 cell-line is often used to induce neurological differentiation when exposed to retinoic acid (Bain *et al.*, 1994). Following four days of differentiation, these neuron-like cells have shown the ability to migrate under the influence of glial-stimulation (Santiago *et al.*, 2005). This response could suggest that P19 cell migration is dependent upon the cells first being induced to differentiate. In the current study, P19 cells were not induced to differentiate into cardiomyocytes with DMSO prior to the migration protocol which could have influenced the outcome. Furthermore, a differentiated mesodermal line also derived from P19 cells has demonstrated a high chemotactic response to PDGF (Liapi *et al.*, 1990), suggesting that varying results could also be due to differences in chemotactic responsiveness of this cell-line to other growth factors.

Although the migration numbers in both the C2C12 and P19 cells were generally higher than control under TGF-β-treated conditions, whether at the concentration of 5 ng/mℓ TGF-β or in combination with IGF-1, it was only significantly higher than control when C2C12 cells were treated with the TGF-β/IGF-1 solutions. These results suggest a general inhibitory effect of TGF-β on myogenic cells, with TGF-β3 having the greatest influence. Although inhibitory effects on C2C12 migration have previously been reported (Suzuki *et al.*, 2000 - unpublished data), others have demonstrated significant, dose-dependent, TGF-β-induced chemotactic activity on primary isolated satellite cells (Bischoff, 1997) and other myogenic precursor cells (Robertson *et al.*, 1993). Differences in results could therefore be due to the use of primary stem or progenitor cells rather than a cell-line, the contribution of other factors in the sample analysed, or the dose applied which varied between studies. In this regard, it has been suggested that, following injury, satellite or myogenic precursor cells migrate from the uninjured area in response to TGF-β and other factors released, but may reach a point at which high concentrations of TGF-β could inhibit further migration (Bischoff, 1997).

Although not analysed, the *in vivo* effect TGF-β exerts on cell migration could be related to the ECM. Factors released from the ECM largely contribute to cellular responses, including migration. One of the activities of TGF-β is control of ECM synthesis and degradation, of which regulation of production and turnover of ECM components is essential for tissue homeostasis and function. Therefore, an indirect mechanism by which TGF-β exerts its effects on cell proliferation, differentiation and migration, is through its capacity to modulate the deposition of ECM components (Verrecchia and Mauviel, 2002), thereby either stimulating or inhibiting the relevant processes. This area is being investigated in further studies *in vitro.*

Growth factors can improve the *in vivo* migration of skeletal myoblasts by modulation of their endogenous proteolytic activity. In this regard, co-injection of IGF-1 and FGF with myoblasts have shown to enhance the migratory capacity of these injected myoblasts (Lafreniere *et al.*, 2004). It was suggested that co-injection of these growth factors increased their proteolytic activities and consequently resulted in breakdown of ECM components which facilitated the migration of transplanted cells through the ECM. TGF-β has shown the opposite effect by stimulating the production of protease inhibitors and inhibiting production of ECM-degrading proteases, thereby preventing enzymatic degradation of the ECM (Laiho *et al.*, 1986; Roberts *et al.*, 1990b). Also, in the myocardium, *connective tissue growth factor* is induced by TGF-β and has shown to be associated with processes underlying fibrosis, specifically fibroblast proliferation, cellular adhesion and ECM synthesis which would obstruct myocyte migration (Grotendorst *et al.*, 1996; Ruperez *et al.*, 2003).

It is therefore possible that pharmacological inhibition of TGF-β could be an effective therapeutic approach to a variety of undesirable fibrotic reactions following skeletal muscle injury or heart failure, as well as to improve the capacity of myocytes to migrate (Chen *et al.*, 2000; Kucich *et al.*, 2001). Several forms of anti-fibrotic therapies have emerged as possible treatment-mechanisms against tissue fibrosis, including anti-TGF-β1 neutralising antibody, endoglin antibody, soluble TGF-β type-II receptor, TGF-β antisense oligonucleotides and Pirfenidone (Lim and Zhu, 2006). As an alternative intervention, attempts have been made to increase the efficiency of transplant therapy by injuring the host muscle before or during myoblast implantation, resulting in the release of the essential growth factors. Although this intervention has increased cell migration in some instances (Vilquin *et al.*, 1995a; Vilquin *et al.*, 1995b), others have shown no effect (Fan *et al.*, 1996b; Rando and Blau, 1994). Whether any of these approaches will yield effective strategies to improve skeletal or cardiac muscle regeneration and repair following injury or disease, remains to be determined.

7.5   SUMMARY

Results in this chapter show that TGF-β itself, at the concentrations utilised, have no effect on the migration of C2C12 myoblasts *or* P19 embryonal carcinoma cells in their undifferentiated state. In the presence of a migratory stimulus such as IGF-1, TGF-β isoforms decrease the induced migration. Despite the limited migratory response demonstrated by the P19 cells, the role of TGF-β warrants further investigation using possibly primary cell cultures. Furthermore, differences in results shown in this chapter compared to other research might suggest re-evaluation of the migration-assay developed, as well as the use of alternative migratory-inducing agents.

CHAPTER 8

FUSION

8.1   INTRODUCTION

The fusion of myoblasts into multinucleated myotubes represents the final stage of terminal differentiation. Two phases of cell fusion can be distinguished (Schulze *et al.*, 2005): induction of the contractile phenotype (such as MHC expression) which is followed by the final fusion process characterised by further steps of inter-myoblast recognition, adhesion, alignment and the actual membrane fusion of differentiated myocytes. These processes are dependent on cell-to-cell and cell-to-extracellular matrix interactions and are regulated by a variety of cell adhesion molecules and growth factors (Cossu *et al.*, 1995; Dickson *et al.*, 1990; Rosen *et al.*, 1992).

By means of this final phase of fusion during myogenesis, myofibers increase in size as a result of the proportional increase in the number of nuclei and cytoplasm within the growing fiber. In the situation of muscle injury, by fusing with the damaged muscle fibers, satellite cells or other myogenic precursor cells provide an extra set of genes required for functional protein synthesis during the repair process (Hill *et al.*, 2003). Importantly, the fusion of myoblasts during development must be carefully controlled if the muscle fibers are to be patterned and sized correctly to result in the formation of a functional syncytium. During stages of both muscle development and repair, formation of mature myofibers can involve either the fusion of myoblasts to form developing myotubes containing a limited number of myonuclei, *or* myoblast-myotube fusion which results in an increase in myotube size and muscle fiber formation, *or* finally, the fusion of myoblasts with resident myofibers to result in repair of the damaged tissue (Park and Chen, 2005). Myotubes rarely fuse with one another *in vivo,* but rather continually absorb myoblasts until the mature state of the muscle is reached (Wigmore *et al.*, 1992; Zhang and McLennan, 1995). Also, the rate at which new nuclei are added to myotubes varies with the stage of muscle development, as does the site of addition of the new nuclei. Whereas myoblasts preferentially fuse with the ends of myotubes or myofibers as they elongate (Aziz and Goldspink, 1974), they will fuse with the middle of the myofiber or myotube during muscle hypertrophy (Zhang and McLennan, 1995).

The formation of mature myofibers can also involve the fusion of host myoblasts with transplanted stem or progenitor cells. This mechanism by which adult stem cells can enhance tissue repair has proven to be of benefit in that other cell populations can be applied to provide additional stem or progenitor cell sources to contribute to regeneration processes. The use of this repair strategy has been demonstrated by the successful transplantation of bone marrow-derived cells into skeletal (Ferrari *et al.*, 1998) and cardiac muscle (Assmus *et al.*, 2002; Strauer *et al.*, 2002), as well as the transfer of skeletal myoblasts (satellite cells) into the infarcted myocardium (Menasche *et al.*, 2001). Interestingly, cell fusion has also been used as an explanation for *transdifferentiation* by research groups which have contradicted this apparent effect in various tissues (Murry *et al.*, 2004): whereas some studies have proven that bone marrow-derived stem cells can transdifferentiate into cardiomyocytes following infarction and acquire a cardiac phenotype (Kajstura *et al.*, 2005; Orlic *et al.*, 2001b; Orlic *et al.*, 2001c), others have provided evidence that such transdifferentiation does not occur, but is rather the consequence of the fusion of circulating bone marrow cells or bone marrow-derived stem cells with the host cardiomyocytes (Alvarez-Dolado *et al.*, 2003; Bittner *et al.*, 1999; Kuramochi *et al.*, 2003; Muller *et al.*, 2002).

During stages of development, injury or disease, stimuli such as neural innervation (Duxson, 1992), growth factors and various effector molecules initiate and regulate the fusion process. Following activation of fusion, intercellular junction structures mediate intercellular adhesion, as well as regulate intracellular cytoskeletal design. Transmembrane proteins such as cadherins, which mediate cell-to-cell interactions in a calcium-dependent manner, are thought to play an essential role in this process (Geiger and Ayalon, 1992). Other factors suggested to be involved in myoblast fusion include members of the immunoglobulin-superfamily (Kang *et al.*, 2002), neural and vascular cell adhesion molecules (Mege *et al.*, 1992; Rosen *et al.*, 1992), β1-integrins and other extracellular matrix receptors (Menko and Boettiger, 1987). Signalling molecules suggested to promote myoblast fusion include growth hormone (Sotiropoulos *et al.*, 2006), the transmembrane-4 superfamily (TM4SF) of proteins such as CD9, CD44 and CD81 (Mylona *et al.*, 2006; Tachibana and Hemler, 1999), and mTOR signalling which specifically controls late-stage fusion (Park and Chen, 2005). In contrast, Rho/ROCK signalling appears to have an inhibitory effect on myoblast fusion (Nishiyama *et al.*, 2004). Of the growth factors, IGF-1 has demonstrated a stimulatory effect on myoblast fusion by means of initial activation of satellite cells which is followed by the later expression of a different splice-variant of IGF-1 to maintain protein synthesis and complete fusion and repair processes (Czifra *et al.*, 2006; Hill *et al.*, 2003). Unfortunately, although myoblast

fusion has extensively been analysed, a significant part of these studies has been performed on *Drosophila* and it still needs to be established to what extent the developmental strategies and essential molecules involved in myoblast fusion are conserved between species (Baylies *et al.*, 1998; Frasch, 1999; Taylor, 2002).

The effect of TGF-β isoforms on myoblast fusion has shown inconsistent results. Although this growth factor has been shown to inhibit myoblast fusion in myogenic cell-lines (Olson *et al.*, 1986), it has also been suggested that the isoforms are essential promoters of myoblast fusion *in vivo* (Filvaroff *et al.*, 1994). *In vitro* results have led to the hypothesis that TGF-β1 controls the onset of myotube formation by suppressing the proliferation and fusion of late-stage myoblasts until primary myogenesis has been completed (Cusella-De Angelis *et al.*, 1994). However, there is no *in vivo* evidence to support this hypothesis. TGF-β2 expression in developing and regenerating muscle has shown to be principally associated with myoblasts and myotubes (McLennan and Koishi, 2002), leading to the suggestion that myotubes release TGF-β2 to stimulate adjacent myoblasts to fuse with them. In addition, the effects exerted by TGF-β isoforms on fusion could also be specific to the skeletal muscle fiber type: whereas TGF-β1 has demonstrated favourable development of fast muscle fibers, this isoform has shown to reduce the fusion of slow muscle fibers (Noirez *et al.*, 2006). The lack of fusion studies comparing the effects of all three TGF-β isoforms could contribute to a suggested significant influence which one isoform induce on myocyte fusion only as a consequence of other isoforms not being analysed.

Stages of proliferation and terminal differentiation in myoblasts are presumed to be mutually exclusive events. As illustrated by the results in Chapter 5, the addition of TGF-β resulted in increased proliferation of C2C12 myoblasts, whereas differentiation of these cells was depressed following treatment with TGF-β (Chapter 6). Although the proliferative expansion of the myoblast population is required to provide sufficient precursor cell numbers for final processes of fusion, phenotypic differentiation is also required and suggested to precede the fusion-stage of myogenesis. To determine how these apparent opposing results would affect final stages of the differentiation programme, the fusion of C2C12 myoblasts was analysed following short- and long-term treatment with TGF-β isoforms.

8.2   METHODS

8.2.1   Cell Culture

To determine the effects of TGF-β isoforms on cell fusion, C2C12 cells were plated onto glass coverslips in six-well tissue culture-treated plates at a density of 50 000 cells/well and treated with TGF-β as described in Chapter 5 (*section 5.2.1.1*). After maintaining C2C12 cells in culture medium, they were induced to differentiate on day 0 and treated with either TGF-β1 or -β2 or -β3 (5 ng/mℓ) for 24 hours and compared to control conditions (differentiation medium only). For long-term TGF-β treatment, cells received differentiation medium supplemented with either TGF-β1 or -β2 or -β3 on days 0, 1 and 2. Cells were fixed and prepared for later immunofluorescent staining on day 5 and day 7. Day 3 was additionally analysed to determine the early effect of TGF-β isoforms following long-term treatment.

8.2.2   Immunohistochemistry

Total nuclear counts were established and antibody staining intensities analysed as described in Chapter 5 (*section 5.2.2.1*). Cells were incubated with anti-M-cadherin (see *Chapter 3, section 3.2.2* for antibody details) as primary antibody and Hoechst dye was added for nuclear determination.

The M-cadherin image of the cells was merged with the Hoechst-stained image of the nuclei from the same cell area to determine bi-nuclear myoblast (2 nuclei per cell) and myotube (3 or more nuclei per cell) stages of differentiation. These myoblast and myotube numbers were added together to quantify cell fusion. The fusion index (Nishiyama *et al.*, 2004; Park and Chen, 2005) was calculated from the ratio (%) of nuclei number in myocytes with two or more nuclei *versus* the total number of nuclei in the field of count (TNC determined in *Chapter 5, section 5.2.2.1*). A minimum of six photos were taken from different regions of each slide. The experiment was performed in triplicate.

8.2.3   Statistical Analysis

Statistical evaluations were made by one-way analysis of variance (ANOVA) and Bonferroni's multiple comparison test using STATISTICA. Significant differences were taken at $p < 0.05$. All data are expressed as mean $\pm$ SEM.

8.3   RESULTS

To investigate the involvement of TGF-β isoforms on myoblast fusion in a skeletal muscle cell-line, C2C12 cells were treated with this growth factor for either 24 hours or 72 hours and analysed on day 5 and day 7, and additionally on day 3 following long-term incubation. To quantify the extent to which the cells were successfully stimulated to fuse, the total amount of bi-nuclear myoblasts and myotubes were determined, as well as the fusion index.

8.3.1   Total Myoblast and Myotube Count

8.3.1.1   Short-term TGF-β treatment

TGF-β isoforms showed limited effects on the total number of bi-nuclear myoblasts plus myotubes following 24 hour incubation (Figure 8.1). The total number of bi-nuclear myoblasts and myotubes was significantly lower at day 5 following treatment with TGF-β2 ($p < 0.01$) and -β3 ($p < 0.05$) compared to control conditions. In addition, the effect of TGF-β2 was also significantly lower than TGF-β1 ($p < 0.05$), suggesting a possible isoform-specific effect. At day 7 of differentiation, the total number of bi-nuclear myoblasts and myotubes was still lower compared to control conditions following TGF-β treatment, however, this effect was not significant for any isoform. This result could be due to the cells recovering following the earlier inhibitory effect of TGF-β isoforms on myoblast and myotube formation which were now able to continue normal differentiation.

8.3.1.2   Long-term TGF-β treatment

Compared to control conditions, all TGF-β isoforms significantly reduced the total bi-nuclear myoblast plus myotube number following 72 hour incubation at all time-points analysed (Figure 8.2; $p < 0.01$). This effect was more pronounced compared to 24 hour treatment. Additionally, the average totals were also lower and no recovery was evident at day 7 compared with that seen after 24 hour incubation. The increased presence of TGF-β isoforms during the initial stages of myocyte development therefore has a possible long-term negative influence on myocyte fusion. No isoform-specific effects were significant, although TGF-β2 and -β3 showed a greater inhibitory influence at day 7.

Figure 8.1. Incubation of C2C12 cells with TGF-β1, -β2 or -β3 for 24 hours has minimal effect on bi-nuclear myoblast and myotube formation. Total myoblast plus myotube numbers were assessed by immunofluorescent staining and image analysis in control- and TGF-β-treated (5 ng/mℓ) differentiating C2C12 cells. TGF-β2 (p < 0.01) and TGF-β3 (p < 0.05) were significantly lower compared to control conditions at day 5. In addition, the effect of TGF-β2 was also significantly lower compared to treatment with TGF-β1 (p < 0.05). *p < 0.05; #p < 0.01. Data are expressed as mean ± SEM; n = 3.
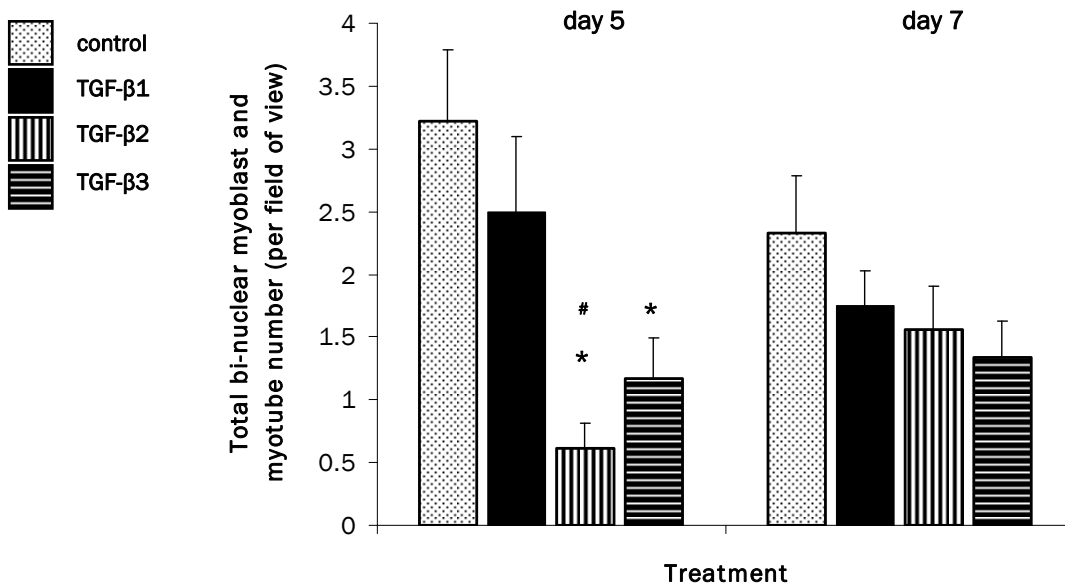
Figure 8.2. Incubation of C2C12 cells with TGF-β1, -β2 or -β3 for 72 hours significantly decreases bi-nuclear myoblast and myotube formation. Total myoblast plus myotube numbers were assessed by immunofluorescent staining and image analysis in control- and TGF-β-treated (5 ng/mℓ) differentiating C2C12 cells. All three TGF-β isoforms significantly decreased total myoblast and myotube numbers at all time-points analysed compared to control conditions. #p < 0.01. Data are expressed as mean ± SEM; n = 3.
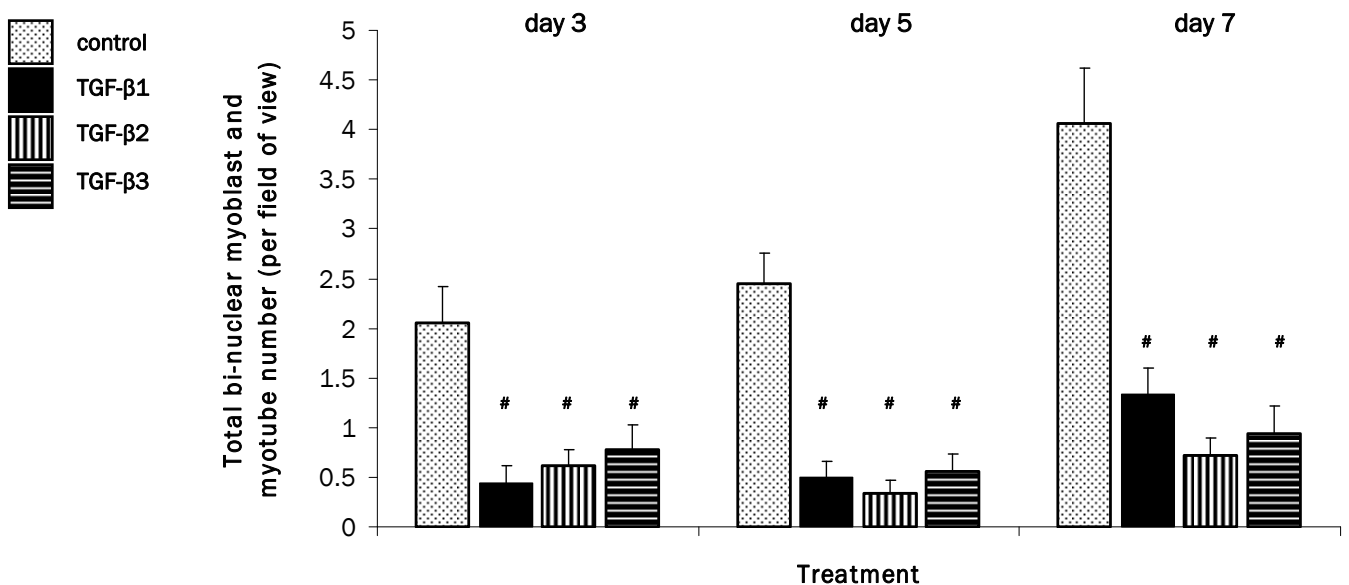
8.3.2　Fusion Index

8.3.2.1　Short-term TGF-β treatment

Following 24 hour incubation, all three TGF-β isoforms resulted in a significantly lower fusion index compared to control conditions at both time-points analysed (Figure 8.3; $p < 0.01$). Although also significant, the effect of TGF-β1 was the least, especially at day 7 ($p < 0.05$). The fusion index under control conditions was 4.4 ± 0.7% and 3.9 ± 0.8% at day 5 and day 7, respectively. In TGF-β-treated conditions, the fusion index ranged from 0.4 ± 0.1% to 1.9 ± 0.5% at day 5, and 1.2 ± 0.3% to 1.6 ± 0.2% at day 7.

8.3.2.2　Long-term TGF-β treatment

The fusion index is calculated from the total amount of nuclei in bi-nuclear myoblasts and myotubes relative to the total amount of nuclei from the same field of view. Therefore, as shown in Chapter 5 (*section 5.3.1*), TGF-β isoforms resulted in significantly higher total nuclear counts, especially following long-term treatment but, as illustrated above, lower total bi-nuclear myoblast plus myotube numbers and therefore also lower nuclei totals in these myocytes. Consequently, the resulting fusion index was greatly reduced ($p < 0.01$) by 6-9-fold in TGF-β-treated conditions at all time-points analysed (Figure 8.4). No isoform-specific effect was seen. In control conditions, the fusion index increased from 2.3 ± 0.4% at day 3 to 6.6 ± 0.9% at day 7. In TGF-β-treated conditions, the fusion index showed limited change, ranging from 0.3 ± 0.1% to 0.5 ± 0.2% at day 3 and increasing only to a range of 0.5 ± 0.1% to 0.9 ± 0.2% at day 7.

Figure 8.3. Incubation of C2C12 cells with TGF-β1, -β2 or -β3 for 24 hours decreases the fusion index. All three isoforms significantly decreased the fusion index (%) in TGF-β-treated (5 ng/mℓ) differentiating C2C12 cells at both time-points analysed compared to control conditions. *p < 0.05; #p < 0.01. Data are expressed as mean ± SEM; n = 3.
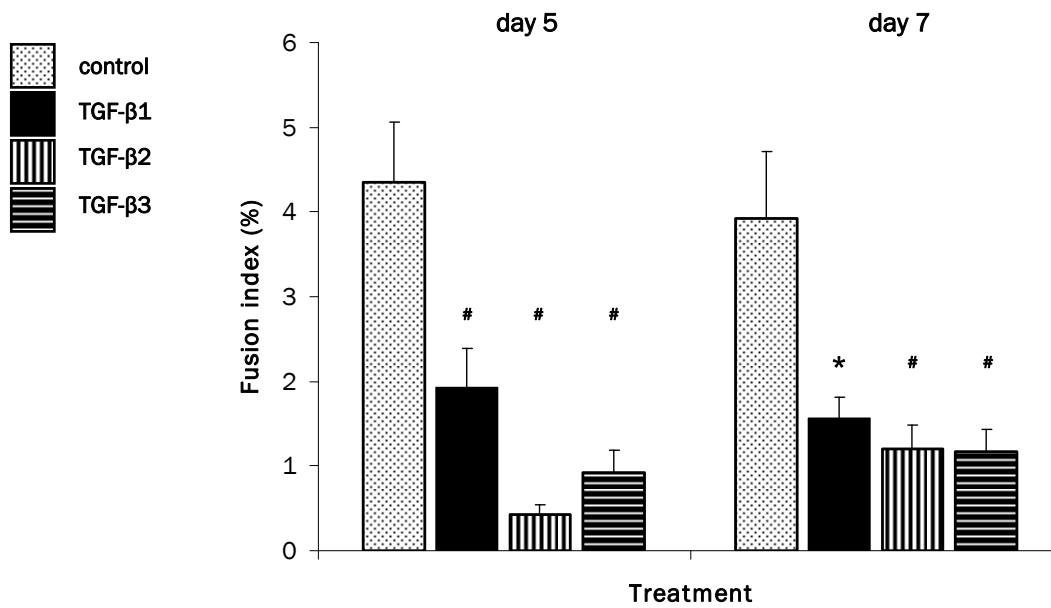
Figure 8.4. Incubation of C2C12 cells with TGF-β1, -β2 or -β3 for 72 hours decreases the fusion index. All three isoforms significantly decreased the fusion index (%) in TGF-β-treated (5 ng/mℓ) differentiating C2C12 cells at all time-points analysed compared to control conditions. #p < 0.01. Data are expressed as mean ± SEM; n = 3.

166.
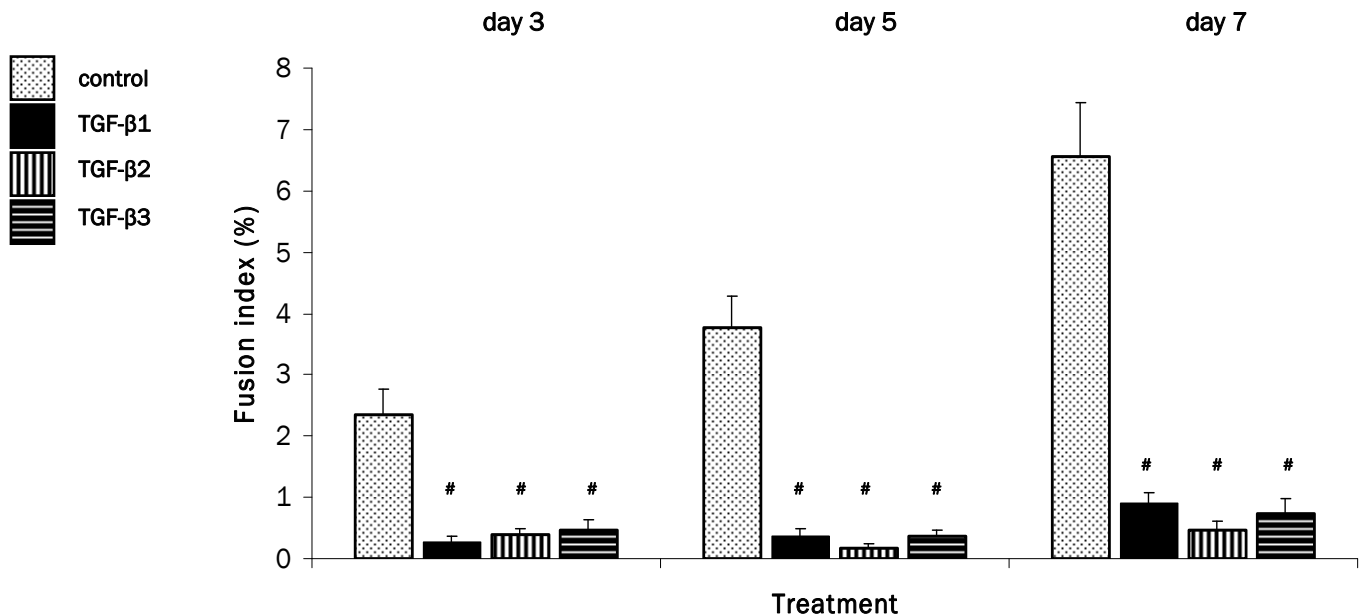
As shown in Figure 8.5, 72 hour TGF-β treatment decreased the number of myotubes formed at day 7 of differentiation despite the significant increase in nuclei number. Taken together, TGF-β isoforms appear to delay muscle cell fusion and the formation of myotubes. As suggested by Andres and Walsh (1996), phenotypic differentiation, indicated by the induction of MHC, precedes cell fusion. In Chapter 6 [*section 6.3.1.3 (B)*], MHC expression was significantly reduced in TGF-β-treated conditions and could therefore imply a mechanism for the subsequent decrease in cell fusion.

Figure 8.5. Effect of incubation with TGF-β isoforms on fusion in differentiating C2C12 cells. Typical images (at 20x enlargement) of **(A)** Control- and **(B)** TGF-β-treated cells at day 7 following 72 hour incubation with TGF-β1. TGF-β-treated cells clearly illustrate an increase in total nuclei number but decrease in myotube formation. Treatment of C2C12 cells with TGF-β2 and -β3 displayed similar images.

(A) Control   **(B) TGF-β1**
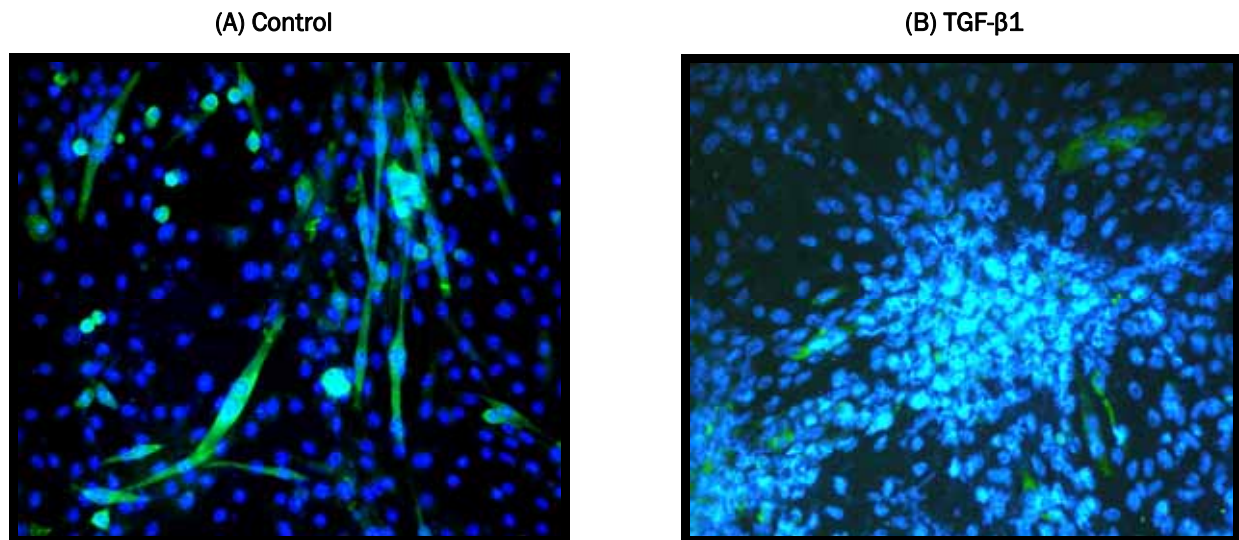
8.4   DISCUSSION

Skeletal muscle development is a highly ordered process of events. Following initial myocyte activation and proliferation, the expression of MyoD, one of the earliest markers of muscle differentiation, initiates the myogenic programme by activating the expression of various transcription factors and muscle-specific genes. Subsequently, myogenin expression increases, followed by cell cycle withdrawal, and phenotypic differentiation characterised by the expression of contractile proteins such as MHC. Finally, cell fusion takes place to form multinucleated, syncytial myotubes (Andres and Walsh, 1996; Chen and Goldhamer, 2003; Rosenblatt *et al.*, 1996). In response to muscle injury, quiescent satellite cells are activated to enter the cell cycle and regenerate a pool of proliferating myogenic precursors, similar to the embryonic myoblasts. Therefore, embryonic myogenesis and adult muscle regeneration have been outlined as comparable events (Chen and Goldhamer, 2003). The ability of two or more cells to fuse is an essential process throughout both development and repair and requires migration, myoblast recognition, alignment, adhesion between cells and finally fusion of the plasma membranes and re-arrangement of cytoplasmic contents. In these series of events, myoblast fusion is required for myofiber growth and completion of terminal differentiation (Mitchell and Pavlath, 2001; Nishiyama *et al.*, 2004).

During myogenic development or regeneration following injury, the need for skeletal muscle growth is controlled by the regulation of myofiber size. Being a syncytium, the myofiber size can be modulated by two distinct mechanisms. In the first mechanism, the cytoplasmic volume associated with individual myonuclei is regulated. This pathway appears to involve regulation of protein synthesis and degradation through PI3K signalling and ubiquitin-ligases, respectively (Schiaffino and Serrano, 2002). Secondly, the number of myonuclei within a myofiber determines the myofiber size: a proportional increase in the number of nuclei and the cytoplasmic volume is required within the growing myofiber (Horsley *et al.*, 2001). By fusing with one another or with an adjacent muscle fiber, myoblasts provide these myonuclei, allowing each nucleus to regulate more cytoplasm for fiber growth and repair (Allen *et al.*, 1999). Similarly, during illness or disease, the muscle atrophies, mostly due to myonuclei which are *lost* possibly through apoptotic mechanisms.

The fusion of myoblasts and subsequent myotube formation is especially controlled by the micro-environmental conditions and influence of circulating effector molecules, proteins and growth factors. Therefore, to determine the *in vitro* effect of one such factor on skeletal myoblast fusion, C2C12 cells were treated with TGF-β isoforms for either 24 hours or 72 hours and the extent of fusion compared to control conditions.

Results following 24 hour treatment showed a lower total number of bi-nucleated myoblasts and myotubes at both day 5 and day 7, although only the effect of TGF-β2 and -β3 at day 5 was significant. This apparent inhibitory effect of TGF-β isoforms on cell fusion was confirmed following 72 hour treatment, which resulted in significantly reduced total numbers of bi-nucleated myoblasts and myotubes at all time-points analysed. Although no isoform-specific results were significant, treatment with TGF-β2 again resulted in the most inhibition at day 5 and day 7. As expected, the total number of bi-nucleated myoblasts and myotubes increased in all conditions from day 3 to day 7 as differentiation progressed, although this increase was less evident in TGF-β-treated cells. Subsequent analysis of the fusion index clearly demonstrated the inhibitory effect of both 24 hour and 72 hour TGF-β treatment on fusion and also suggested a possible isoform-specific effect of TGF-β2. The effect of TGF-β isoforms was most significant following 72 hour treatment, indicating that the constant presence of TGF-β is required for this growth factor to exert its effect and following initial phases of inhibition, the cells are unable to return to normal growth and development even after the signal has been removed.

As mentioned, during embryonic differentiation, mononucleated myoblasts first proliferate then fuse to form myotubes that become innervated and develop into muscle fibers. As shown by the results in Chapter 5, sufficient, increased proliferation of myoblasts resulted following treatment with the TGF-β isoforms. The inhibitory effect which this growth factor exerts on fusion would therefore probably not be as a result of reduced myoblast numbers, but rather as a direct consequence of the inhibitory effect demonstrated by TGF-β isoforms on skeletal muscle differentiation (*Chapter 6*) and/or migration (*Chapter 7*). Alternatively, TGF-β isoforms could influence the regulatory proteins or effector molecules signalling progression of fusion which would result in reduced myoblast activation or generation of a micro-environment unfavourable to fusion processes.

Treatment of myoblasts with TGF-β1 *in vitro* has shown to reduce fusion by decreasing the Ca^{2+}-influx required for myotube formation. Fusion of myoblasts and myotubes involves Ca^{2+}-influx through T-type channels and TGF-β1 has shown to down-regulate the number of these channels in the plasma membrane, resulting in a subsequent decrease in myotube formation. It has been suggested that this T-channel down-regulation by TGF-β1 may be mediated by reduced transcription rather than post-transcriptional modifications of the channels (Avila *et al.*, 2006). It was speculated that by inhibiting myoblast fusion, TGF-β1 might be functioning to ensure regeneration of the original satellite or progenitor cells by preventing irreversible commitment of these cells to myogenesis (Mejia-Luna and Avila, 2004). Such a hypothesis could then explain the increased proliferation demonstrated in Chapter 5. In addition, TGF-β isoforms might be involved in regulating the timing of myoblast fusion during early embryonic development (Olson *et al.*, 1986) which would support this hypothesis: by controlling fusion and commitment to myogenesis, adequate progenitor numbers will first be generated during embryogenesis before irreversible commitment is induced. In this regard, it has been suggested that TGF-β2 regulates when and where myoblasts fuse into myotubes (McLennan and Koishi, 2002). Increased expression of TGF-β3 during stages of both skeletal and cardiac myogenesis also indicates the involvement of this isoform during specific stages of development (Lafyatis *et al.*, 1991). The differential expression of TGF-β isoforms during embryonic development suggests distinct isoform-specific regulation of processes involving tissue development and cellular differentiation (Lafyatis *et al.*, 1991) which remains to be clarified.

In contrast to the well-documented regulatory effects of the MRFs on cell cycle control and differentiation, the molecular mechanisms known to regulate myoblast fusion are limited (Dworak and Sink, 2002; Horsley and Pavlath, 2004; Taylor, 2002). In addition to the factors suggested to be involved in myoblast fusion mentioned above (*section 8.1*), the cytokine IL-4 has been identified as a molecular signal which control myoblast fusion with myotubes through activation of the signalling pathway involving the nuclear factor of activated T-cells (NFAT)-family of transcription factors (Crabtree and Olson, 2002; Horsley *et al.*, 2001). The transcription factor NFATc2 controls myoblast fusion at a specific stage of myogenesis after the initial formation of myotubes and is required for further cell growth. NFAT proteins regulate the expression of many secreted cytokines, including IL-4. Although muscle cells lacking IL-4 form normally, they are reduced in size and myonuclear numbers. It has been suggested that following the initial fusion of myoblasts into myotubes, these newly formed myotubes secrete IL-4 to interact with IL-4α-receptors present on surrounding myoblasts, thereby activating further steps of cell fusion and acting as a myoblast-recruitment factor for

addition of more myonuclei and subsequent increases in myotube size (Schulze *et al.*, 2005). This report of IL-4 release by a non-immune cell (myotube) (Horsley *et al.*, 2001) suggests that cytokines secreted during the immune response immediately following muscle injury may contribute additional functions during the muscle regeneration process such as myoblast proliferation (Hawke and Garry, 2001) and myoblast fusion with subsequent muscle growth. However, this effect of IL-4, together with IL-13 and TNF-α, induces the release of TGF-β from macrophages, resulting in collagen deposition and fibrosis (Fichtner-Feigl *et al.*, 2006). IL signalling therefore requires careful regulation to contribute to increased fusion without the detrimental effects of fibrosis during stages of repair. These responses also suggest, together with the inhibitory results displayed by TGF-β on muscle differentiation and fusion, that when regeneration is required, the release of TGF-β is primarily directed towards modulation of wound healing and fibrosis, rather than contributing to the processes of muscle growth.

A further possible mechanism by which TGF-β isoforms may influence cell fusion, could be by reducing the expression of the cell-adhesion protein, M-cadherin. It has been suggested that M-cadherin mediates myoblast interaction to function as a molecular link between satellite cells, myoblasts and damaged muscle fibers (Irintchev *et al.*, 1994; Zeschnigk *et al.*, 1995), thereby playing an important role in terminal muscle differentiation and repair mechanisms. The preferential expression of M-cadherin during myogenesis and muscle regeneration has suggested that this protein may be involved in myoblast fusion and the regulation of skeletal muscle morphogenesis (Kaufmann *et al.*, 1999b; Moore and Walsh, 1993), particularly regarding the alignment of myoblasts to form and expand developing myotubes (Cifuentes-Diaz *et al.*, 1995). It has been suggested that M-cadherin interacts with microtubules to keep the myoblasts aligned during fusion processes (Kaufmann *et al.*, 1999a). It is possible that TGF-β exerts its effect by interfering with M-cadherin-mediated cell-to-cell interaction and adhesion and therefore is a mechanism of TGF-β-control to investigate in future research.

8.5   SUMMARY

The ability to influence the process of myoblast fusion with mature muscle fibers in response to trauma or injury during adult life could be of great therapeutic value. Since engineered myoblasts can be induced to fuse with mature muscle, skeletal muscle has become a prime target for gene and transplantation therapy and as such has provided a model for clinical applications in other tissues and adult organs (Blau *et al.*, 1993; Miller and Boyce, 1995). In this respect, results presented in this chapter provide some valuable insight into mechanisms that modulate this fusion process.

CHAPTER 9

SUMMARY AND CONCLUSIONS

9.1   STEM CELL RESEARCH

Advances in the knowledge of both embryonic and adult stem cell systems have resulted in the successful use of these cells in transplantation therapeutics. In order to be of clinical use, the relevant cells must be easily obtained, upon isolation remain capable of differentiating into the required cell-lineage and, once transplanted, engraft into the host-tissue to restore and/or improve function. Despite the initial excitement regarding the potential use of these cells, several research studies and clinical trials have demonstrated variable results. A greater understanding of stem and progenitor cell activation, signalling and involvement in growth and repair, as well as the profile of growth factor expression during these processes, is essential in establishing more effective cell-based therapies and transplantation strategies. Estimates of the amount of muscle formed from given numbers of transplanted stem cells indicate very low levels of efficiency: it is estimated that only 10-20 mg of muscle (containing 3×10^5 myonuclei) can be obtained from a graft containing 5×10^5 myogenic cells, therefore less than the transplanted number (Partridge, 2002). As such, it is essential to determine stimuli that would produce highly proliferative environments which will result in sufficient cell masses without risking tumour-like overgrowth (Murry *et al.*, 2002). Careful interaction between stem and progenitor cells and growth factors are therefore required to enable controlled proliferation and subsequent differentiation into the required tissue.

Since engineered myoblasts have shown the ability to fuse with mature muscle, *skeletal muscle* has become a prime target for gene and transplantation therapy and as such has provided a model for clinical applications in other adult organs. In *cardiac tissue*, the myocardium has always been regarded as a post-mitotic organ although a series of recent studies have indicated the existence of stem-like cells in the heart, as well as the contribution of extracardiac stem cells to promote at least partial reconstitution of the myocardium following an ischaemic insult. Despite the success seen in research, as well as in clinical trials, further optimisation of stem cell incorporation into the damaged tissue is required.

A group of cytokines which has been shown to be involved in various cellular events is the TGF-β-superfamily. Specifically, the isoforms of TGF-β are up-regulated post-injury and play an important role in regeneration processes, as well as during growth and development. Therefore, in the work discussed, the three TGF-β isoforms were analysed to determine their effect on myogenic growth processes in skeletal myoblasts and embryonic-like cardiac progenitor cells.

At the start of this thesis, a significant amount of time was spent establishing protocols which would be most suitable to generate valid and reliable data, taking into consideration the laboratory techniques, equipment and skills available. Although protocols were set up to best analyse the effect of a growth factor (TGF-β isoforms) on processes of myogenic development, there will always be other relevant growth factors, cell types, treatment-concentrations, time-points and antibodies to analyse, together with different or new assays to apply. As studies progressed, more questions became apparent, as well as the realisation that a protocol could be improved or an assay done differently. These questions and limitations however remain to be included in future investigations.

9.2   SUMMARY OF RESULTS

Figure 9.1 is a schematic overview of the *in vivo* responses of TGF-β from which aims for this thesis were determined, and the subsequent *in vitro* results generated.

(A) *In vivo*, skeletal muscle damage, disease or myocardial infarction results in an inflammatory response and the release of inflammatory factors, cytokines, growth factors and several other mediators of wound healing, including TGF-β, PDGF, fibroblasts and macrophages which contribute to the subsequent repair process and produce a micro-environment suitable for enhanced tissue regeneration.

(B) *In vitro*, experiments have demonstrated the influence of several growth factors, such as TGF-β, on myogenesis. As such, the effect of the three isoforms of TGF-β was investigated on progenitor cell recruitment and differentiation into skeletal and cardiac cell-lineages. To simulate *in vivo* conditions and induce differentiation, C2C12 cells were deprived of serum and P19 cells treated with DMSO, and either the TGF-β1, -β2 or -β3 isoform added.

(C) Following the inflammatory response *in vivo*, changes in the environmental conditions produce signals for the activation and mobilisation of satellite and/or other stem cells within the damaged tissue, as well as non-resident stem cells, to aid in the regenerative process. With regards to TGF-β, its isoforms bind to cell membrane receptors and activate signal transduction to the nucleus via the Smad-pathway which subsequently regulates gene transcription to illicit desired responses depending on cell type and other environmental stimuli (see Figure 2.6 and Figure 6.11).

(D) Once activated, stem or progenitor cells migrate or home to the injured area and enter stages of the cell cycle, and, depending on the stimulus, either follow pathways of proliferation to form precursor cells and expand the myogenic cell population, or undergo myogenic differentiation.

(E) Alternatively, cells undergo self-renewal and return to quiescence to replenish the satellite or stem cell pool to contribute to future muscle repair processes. Myogenic transcription factors are essential for optimal control of these processes. Furthermore, specifically in the case of regeneration, proliferation will occur, followed by terminal differentiation and fusion of myoblasts to the damaged myofibers for repair, or to each other for new myofiber formation and muscle growth.

(F) Results described in this thesis suggest an inhibitory effect of TGF-β isoforms on C2C12 cell differentiation and migration, but enhancement of proliferation. In P19 cells, even though migration was strongly inhibited, differentiation could be improved depending on the selected TGF-β isoform.

Figure 9.1. Schematic overview of key processes involved in skeletal and cardiac muscle regeneration, the contribution of stem cells and integration of the TGF-β signalling pathway, as incorporated into this thesis.

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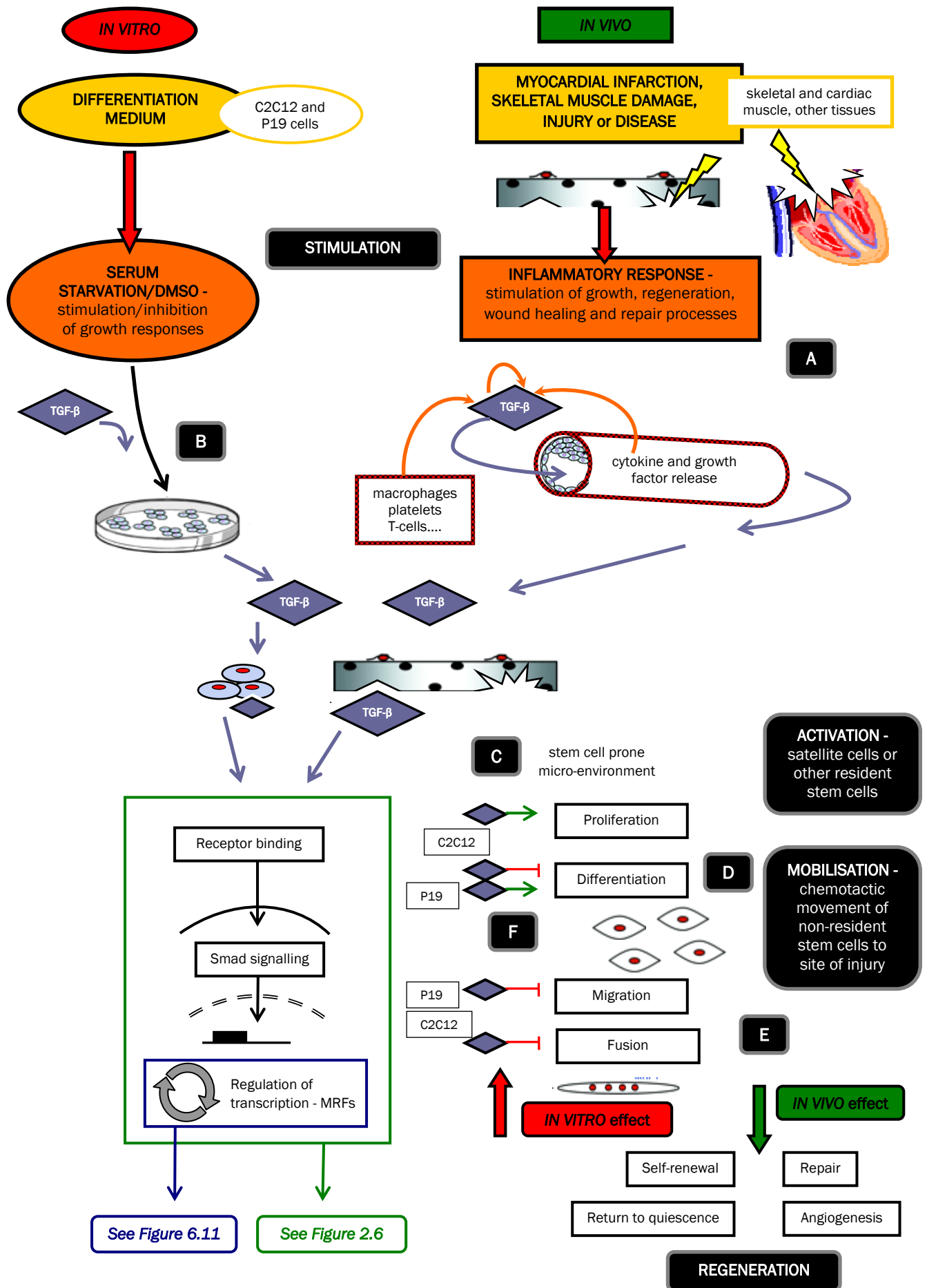
9.2.1 Research Limitations and Recommendations for Future Studies

In *Chapter 5*, results have shown that, under conditions where differentiation would take place, all three isoforms of TGF-β increased proliferation of C2C12 cells in culture conditions. Although PCNA expression was not significantly different between treatments, changes in cellular localisation of this protein were evident and could have contributed to the decision of the cell to differentiate or continue proliferation. Decreased differentiation of C2C12 myoblasts was indeed demonstrated following treatment with TGF-β in an isoform-independent manner (*Chapter 6*). Suppression of the myogenic regulatory factor myogenin, as well as the structural protein MHC, could suggest a possible mechanism for the inhibitory effect of TGF-β on myogenic differentiation in skeletal myoblasts. It would be of interest to analyse additional signalling proteins, myogenic regulatory factors or cell cycle regulators to more closely establish areas of TGF-β-control and gain insight into mechanisms by which this growth factor controls processes of proliferation and differentiation. In addition, TGF-β receptor expression is an important area of control to investigate in future studies. The TGF-β receptors could exert isoform-specific responses and as briefly mentioned in Chapter 5, two effects of TGF-β, stimulation or inhibition of myoblast proliferation and differentiation, could be mediated by the specificity of a TGF-β isoform binding to a selected receptor.

In these chapters, the western blot assay and selected protein markers were used to distinguish between phases of cell growth towards terminal differentiation. Whole cell lysates were used in these assays for protein determination. It was clear from the localisation experiments, which were only performed at a later stage when new equipment became available, that nuclear extraction and analysis of nuclear proteins separate from the cytoplasmic fraction could have produced significant results, specifically with regards to analysis of MyoD and PCNA.

In contrast to C2C12 differentiation, no inhibitory effects of TGF-β isoforms were evident from western blot results following differentiation of P19 embryonal carcinoma cells. TGF-β1 treatment did, however, significantly increase embryoid body formation during early (day 6) stages of differentiation. Unfortunately, only one late time-point (day 12) was analysed by western blotting and therefore possible early effects of TGF-β treatment could have been overlooked in this assay, as suggested by the embryoid body formation results. Future protocols should therefore include additional analysis of both early time-points by western blot analysis, as well as later time-points for embryoid body formation.

Myoblast fusion (*Chapter 8*) was significantly reduced in TGF-β-treated C2C12 cells which could have been the direct result of the inhibitory effects produced by the TGF-β isoforms on differentiation and migration (*Chapter 7*). It is also possible that TGF-β exerts its effect on fusion by interfering with M-cadherin-mediated cell-to-cell interaction and adhesion. Analysis of M-cadherin expression as a possible mechanism of TGF-β-control is an area to investigate in future research, possibly together with immunohistochemical analysis of TGF-β isoform expression in skeletal and cardiac muscle following injury or infarction. Knowledge regarding the specific localisation and time of TGF-β isoform expression could give a clearer indication of isoform-specific contributions to processes of repair and regeneration.

TGF-β isoforms also resulted in the inhibition of P19 cell migration, the effect being much greater than in the C2C12 cells. Despite the limited migratory responses demonstrated by these cell-lineages, the role of TGF-β warrants further investigation using different cell populations, possibly including primary cell cultures, or inducing differentiation *before* implementing the migration assay. Differences between results from this work compared to other research might also suggest re-evaluation of the migration protocol developed, together with the use of alternative migratory-inducing agents such as VEGF, HGF and PDGF.

Given that cytokines exert very distinct effects depending on the cell type, environmental conditions and the active circulating concentration, it will be important to continue the current analysis to include other dosages of TGF-β and preferably primary cell cultures. Research results have shown that due to the multi-functionality of this growth factor, the actions of TGF-β in isolated cell systems *in vitro*, compared to a similar environment *in vivo*, may still differ markedly. Many such results indicate that the nature of the TGF-β-action is dependent on cell-to-cell contact, the presence or absence of other molecules found in the ECM, as well as the presence or absence of other cells and the factors which they secrete. These influences may amplify or modify the actions of TGF-β and therefore the *in vivo* analysis of the TGF-β isoforms is essential to establish the actions of this growth factor with greater accuracy.

9.2.2   Practical Significance of Results

In vitro addition of TGF-β demonstrated greatly increased proliferation in the skeletal muscle cell-line. This characteristic could be of use to expand myogenic cell populations for clinical application *in vivo.* Long-term treatment did however result in detrimental effects on terminal differentiation and therefore, should such a strategy be employed, TGF-β application over the short-term, which did not show late-stage decreased differentiation, would rather be applied.

Both proliferation and migration of stem and progenitor cells are required for effective regeneration of the injured tissue. It has been suggested that motility is suppressed in proliferating cells, consequently decreasing the efficiency of directed migration. In the work described, TGF-β has been shown to promote proliferation with limited effect on migration. This growth factor therefore needs to be carefully co-ordinated to result in the desired response. Under conditions where migration or homing of stem or progenitor cells to an injured area is required, inhibition of TGF-β could be of greater therapeutic benefit to enforce migration rather than proliferation.

TGF-β has shown the potential to enhance differentiation in cardiac tissue. Results in *Chapter 6* indicated such a capacity particularly for TGF-β1 and to a lesser extent for TGF-β2, which has also been demonstrated by others (Lim and Zhu, 2006; Singla and Sun, 2005). TGF-β is up-regulated in response to myocardial overload and injury. In this situation, although TGF-β contributes to cardiomyocyte hypertrophy, it also induces the synthesis and deposition of ECM proteins to contribute to structural cardiac remodelling. As such, over-expression of TGF-β would result in tissue fibrosis. The application of natural TGF-β inhibitors such as decorin or neutralising antibodies could therefore be applied in combination with TGF-β-release to result in cardiac remodelling without the detrimental effects of fibrosis.

An apparent contradictory influence of TGF-β on cells of the immune system, both stimulatory and inhibitory, is partly the result of the differential effects of TGF-β on resting and activated cells: in *general*, resting, immature cells are *stimulated* by TGF-β, whereas an activated population of the same cell group might be *inhibited* (Wahl, 1994). If this is also true for resting stem cells (i.e. quiescent cells), the application of TGF-β could be a mechanism to prevent uncontrolled proliferation often seen in activated stem cells which results in tumour progression.

9.3   STEM CELL OBSTACLES AND LIMITATIONS

Despite the progress in the field of stem cell therapy, many questions still remain unanswered and clinical trials need to be designed to address these issues. This will require integration of all biological disciplines involved, including the use of increasingly powerful molecular biological tools. Advanced strategies already in use include cell replacement therapy, therapeutic cloning, tissue engineering and genetic manipulation (gene therapy).

In vitro research and the success of stem cell use in animal models have demonstrated the principle and capacity to which human embryonic and adult stem cells can potentially be applied as a regenerative source for transplantation therapies. However, before human stem cells can be clinically applied, several concerns need to be overcome. In brief, these include:

(i) immunological incompatibility associated with the use of human embryonic stem cells for tissue regeneration results in rejection of mismatched grafts. Possible solutions could include immunosuppressive drugs, genetic alteration of human embryonic stem cells to develop a "universal" donor, or therapeutic cloning;

(ii) prolonged cultivation results in genetic and epigenetic modifications and therefore the degree to which these cells remain genetically stable during long-term culture needs to be determined; *and*

(iii) it has been shown that undifferentiated, early embryonic stem cells commonly generate teratomas or teratocarcinomas following transplantation. Although tumour formation might not be a problem over the short-term, strategies to eliminate tumorigenic cells are required to ensure safety during long-term stem cell therapy.

In addition, besides the ethical issues involving embryonic stem cells, factors limiting progress in this research field which need to be addressed, include:

(i) greater efficiency in adult and embryonic stem cell isolation;

(ii) greater survival and subsequent migration from the site of injection;

(iii) low rate of engraftment of cells in ischaemic tissue;

(iv) establishment of human stem cell lines and the use of animal-free products in culturing methods to decrease the risk of contamination; *and*

(v) the need for greater standardisation of techniques and procedures to allow accurate reproduction of studies.

Therefore, important issues to consider for future research and therapeutic applications include **(A)** the type of cell for optimal results; **(B)** the number of cells required for effective transplantation; **(C)** the method of administration; **(D)** the need to keep cells where they are administered (cell retention); *and* **(E)** clinically relevant end-points (at which stage do results strongly predict improvements in symptoms or mortality?).

Contrasting responses to treatment with TGF-β isoforms displayed by the two progenitor cell populations used in this thesis, emphasise the need for greater understanding regarding the cellular effects of cytokines and other factors to enable the selection of suitable candidates for cellular transplantation strategies.

Immunofluorescent localisation: negative control merged image of PCNA and MyoD (at 60x enlargement) in differentiating C2C12 cells following 72 hour incubation with TGF-β1. This image illustrates the absence of non-specific antibody-binding for the immunofluorescent images of PCNA (Chapter 5) and MyoD (Chapter 6).
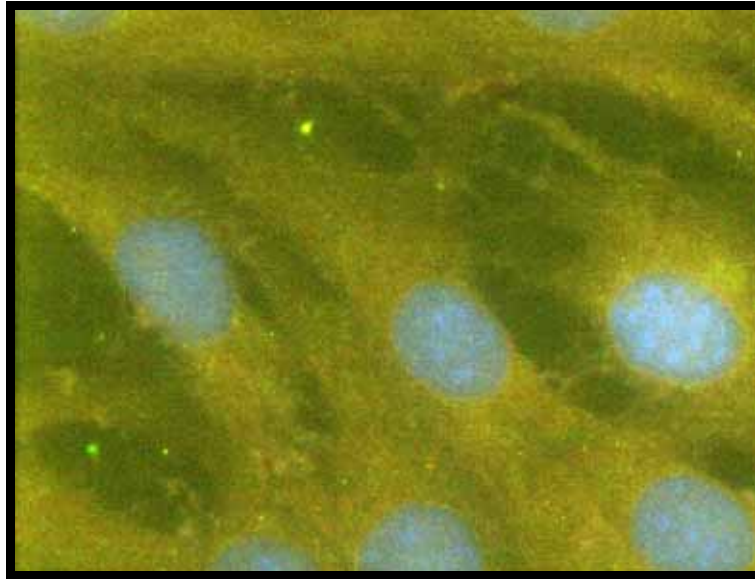
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