The osteogenic potential of adult human skeletal muscle cells: 
New insights into heterotopic ossification after central nervous system trauma

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School of Surgery and Pathology 
School of Anatomy and Human Biology

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Submitted in 2006
“This one goes out to the one I love…” – R.E.M.

To Tammy, my Matey, who understood why and was willing to sacrifice even more than I did: thanks for being there even when we couldn’t touch.
Abstract

Heterotopic ossification (HO) is a pathological condition defined by the development of bone within soft tissues. It is particularly common following trauma to the central nervous system (CNS). The pathogenesis of this phenomenon is unknown, although a serum-mediated osteoinductive factor has been proposed. *In vitro* studies have shown that serum from subjects following traumatic brain injury (TBI) and traumatic spinal cord injury (SCI) has mitogenic and osteogenic effects. The osteogenic effect of cerebrospinal fluid (CSF) after TBI has also been explored in one study. As yet, no study has specifically investigated the effect of serum or CSF on skeletal muscle cells even though skeletal muscle is a common site of HO. Thus, the ability of skeletal muscle cells to produce bone in response to serum or CSF from patients following CNS trauma is unclear. This study attempted to answer the question of whether cells from skeletal muscle adopt an osteoblastic phenotype when exposed to serum and CSF following TBI and SCI.

Blood was collected from 29 patients with severe TBI and 14 patients with SCI at 6, 24 and 72 hours after injury, as well as from 10 control subjects. CSF was collected from 6 patients with TBI, on one occasion following injury, and 29 control patients. Skeletal muscle was obtained from 38 adult orthopaedic patients. Primary skeletal muscle cell cultures were established and then characterised using light microscopic, immunohistochemical and reverse transcriptase polymerase chain reaction techniques. The proliferation rates of the skeletal muscle cells after exposure to serum and CSF from the CNS trauma patients was measured using a cell assay and compared to the controls. The osteoblastic differentiation of the skeletal muscle cells was tested by measurement of osterix protein expression, alkaline phosphatase (ALP) activity and Villanueva bone stain.

The primary skeletal muscle cell cultures consisted of two main morphologies: desmin-positive multinucleated myotubes, and vimentin-positive spindle-shaped mesenchymal cells, some of which showed ALP positivity. All muscle cell populations strongly expressed ALP mRNA and weakly expressed core binding factor alpha-1, but had absent osterix expression. Although MyoD mRNA
expression was absent, mRNA for myosin heavy chain IIa, a marker of mature myotubes, was detected. All serum-treated muscle cell populations showed evidence of osterix expression after one week. Furthermore, cells treated with serum in the presence of osteogenic culture additives showed an increase in ALP activity and development of bone nodules from the mesenchymal cell subpopulation, which were not present in the negative controls and the CSF-treated cells. Only serum from patients with TBI induced a significant increase in the rate of proliferation of these muscle cells compared to the normal control.

These results show that cell populations with a predominantly mesenchymal phenotype and possessing at least myogenic and osteogenic capabilities exist within adult human skeletal muscle and have the capability to be directly involved in the development of HO. Human serum, but not CSF, can support the osteoblastic differentiation of these cells, however, only serum from patients with TBI collected within the first three days after injury also accelerates their proliferation. This suggests the presence of humoral factors following TBI that may stimulate the expansion of mesenchymal cells and early osteoprogenitors within skeletal muscle. An increased rate of proliferation may be required to expand the pool of osteoinducible cells within skeletal muscle thereby overcoming the influences that normally prevent HO. The lack of a proliferative effect of serum from SCI patients suggests that the underlying aetiology of HO in these patients may be different to that following TBI. The lack of an effect of CSF from patients with CNS trauma on the proliferation or differentiation of skeletal muscle cells possibly suggests that the damaged CNS may not be the origin of putative osteogenic factors. Much work lies ahead to better define the identity and source of factors leading to HO within skeletal muscle following CNS trauma. A better understanding of this condition may result in a reduction in morbidity and societal financial burden. In addition, if this study serves as a stimulus for the isolation of a novel serum growth factor from TBI patients, untold numbers of patients with fractures and other bone disorders could benefit.
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And to the patients, those unfortunate ones of the past and present, who have given so much to help the luckier ones of the future. I owe you a debt that I will spend my career repaying.
I hereby declare that the work presented in this thesis is entirely my own, except where the contributions of others have been acknowledged.

_____________________________
Andrew Toffoli

October 2006
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<td>↑</td>
<td>increase</td>
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<td>↓</td>
<td>decrease</td>
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<td>ALP</td>
<td>alkaline phosphatase</td>
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<td>ANOVA</td>
<td>analysis of variance</td>
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<td>ATP</td>
<td>adenosine triphosphate</td>
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<td>BBB</td>
<td>blood-brain barrier</td>
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<td>bHLH</td>
<td>basic helix-loop-helix</td>
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<td>(r)(h)BMP</td>
<td>(recombinant) (human) bone morphogenetic protein</td>
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<td>BMPR</td>
<td>bone morphogenetic protein receptor</td>
</tr>
<tr>
<td>BMSC</td>
<td>bone marrow stromal cells</td>
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<td>bp</td>
<td>base pairs</td>
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<td>BrdU</td>
<td>5-bromo-2-deoxyuridine</td>
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<td>Cbfa</td>
<td>core binding factor alpha</td>
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<td>CD</td>
<td>cluster differentiation</td>
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<td>CNS</td>
<td>central nervous system</td>
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<td>COX</td>
<td>cyclo-oxygenase</td>
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<td>CRP</td>
<td>C-reactive protein</td>
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<td>CSF</td>
<td>cerebrospinal fluid</td>
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<td>DAPI</td>
<td>4´, 6-diamidino-2-phenylindol-dihydrochloride</td>
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<td>DMEM/F-12</td>
<td>Dulbecco’s modified essential medium/Ham’s nutrient mixture F-12</td>
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<td>(c)DNA</td>
<td>(complimentary) deoxyribonucleic acid</td>
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<td>ECL</td>
<td>enhanced chemiluminescence</td>
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<td>(b)FGF</td>
<td>basic fibroblast growth factor</td>
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<td>foetal rat calvarial</td>
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<tr>
<td>GCS</td>
<td>Glasgow Coma Scale</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GH</td>
<td>growth hormone</td>
</tr>
<tr>
<td>hFOB</td>
<td>human foetal osteoblasts</td>
</tr>
<tr>
<td>HLA</td>
<td>human leukocyte antigens</td>
</tr>
<tr>
<td>HO</td>
<td>heterotopic ossification</td>
</tr>
<tr>
<td>hOB</td>
<td>human osteoblasts</td>
</tr>
<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
</tr>
<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IMN</td>
<td>intramedullary nail</td>
</tr>
<tr>
<td>kDa</td>
<td>kiloDaltons</td>
</tr>
<tr>
<td>LDL</td>
<td>low density lipoprotein</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>MRF</td>
<td>myogenic regulatory factor</td>
</tr>
<tr>
<td>MSC</td>
<td>mesenchymal stem cell</td>
</tr>
<tr>
<td>MTS</td>
<td>3-(4,5-dimethylthiazol-s-y1)-5-(3-carboxymethoxyphenol) -2-(4-sulfonphenyl)-2H-tetrazolium, inner salt</td>
</tr>
<tr>
<td>MyHC</td>
<td>myosin heavy chain</td>
</tr>
<tr>
<td>MyoD</td>
<td>myogenic determination factor</td>
</tr>
<tr>
<td>n/a</td>
<td>not applicable</td>
</tr>
<tr>
<td>NFL</td>
<td>neurofilament protein</td>
</tr>
<tr>
<td>ORIF</td>
<td>open reduction internal fixation</td>
</tr>
<tr>
<td>Osx(-S/L)</td>
<td>osterix(-short/long splice variant)</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCIP</td>
<td>propeptide of type I procollagen</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>POD</td>
<td>peroxidase</td>
</tr>
<tr>
<td>PPAR</td>
<td>peroxisome proliferative activated receptor</td>
</tr>
<tr>
<td>PTH</td>
<td>parathyroid hormone</td>
</tr>
<tr>
<td>RANKL</td>
<td>receptor activator of nuclear factor kappa beta ligand</td>
</tr>
<tr>
<td>(m)RNA</td>
<td>(messenger) ribonucleic acid</td>
</tr>
<tr>
<td>R.P.H.</td>
<td>Royal Perth Hospital</td>
</tr>
<tr>
<td>(RT-)PCR</td>
<td>(reverse transcriptase-)polymerase chain reaction</td>
</tr>
<tr>
<td>SAH</td>
<td>subarachnoid haemorrhage</td>
</tr>
<tr>
<td>SCI</td>
<td>spinal cord injury</td>
</tr>
<tr>
<td>SCID</td>
<td>severe combined immunodeficient</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SDH</td>
<td>subdural haemorrhage</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate-poly acrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>sp</td>
<td>side population cells</td>
</tr>
<tr>
<td>SPSS</td>
<td>Statistical Package for the Social Sciences</td>
</tr>
<tr>
<td>TBI</td>
<td>traumatic brain injury</td>
</tr>
<tr>
<td>TBS-T</td>
<td>tris-buffered saline tween-20</td>
</tr>
<tr>
<td>TEMED</td>
<td>tetramethyl-ethylenediamine</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>THR</td>
<td>total hip replacement</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
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</tr>
<tr>
<td>TSH</td>
<td>thyroid stimulating hormone</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>WB</td>
<td>western blot</td>
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</table>
Chapter 1: Introduction

Heterotopic ossification (HO) is the formation of mature bone within tissues that do not normally exhibit ossification (Pape et al. 2004). In general, HO is asymptomatic, but it can cause pain and result in severe functional limitation (Garland 1988; Sawyer et al. 1991; Subbarao and Garrison 1999). HO is commonly found in patients following central nervous system (CNS) trauma (Mendelson et al. 1975; Sazbon et al. 1981; Garland et al. 1989; Wittenberg et al. 1992; Banovac and Gonzalez 1997; Flin et al. 2002). It has also been recognised in non-traumatic CNS conditions, such as infections, tumours and cerebrovascular accidents, but the frequency of HO in these cases is much less than following trauma (Taly et al. 1999; Taly et al. 2001; McKinley et al. 2002; van Kuijk et al. 2002). The connection of these conditions by means of some form of CNS damage hints at a central mechanism for the development of HO in these patients.

HO requires stimulation of mesenchymal cell recruitment, proliferation and differentiation, followed by osteoprogenitor maturation and osteoblast activation (Marusic et al. 1999). One pleiotropic factor or a combination of factors is required to produce this cascade of cellular events. Systemic humoral factors, possibly released from CNS tissue following trauma, have been implicated in the development of HO by several in vitro studies that have demonstrated the mitogenic and osteogenic effect of serum from CNS trauma subjects (Bidner et al. 1990; Kurer et al. 1992; Klein et al. 1999; Boes et al. 2006). The one published study on the osteogenic effect of cerebrospinal fluid (CSF) after TBI concluded that CSF is intrinsically able to support osteogenesis (Klein et al. 1999). These investigations used models of osteoblastic cells, mesenchymal cells and fibroblasts but none reviewed the effect of serum and CSF on cells derived from skeletal muscle.

In vivo evidence suggests that skeletal muscle is a preferred source of HO in comparison to other soft tissues sites (Yoshida et al. 1998; Musgrave et al. 2000; Okubo et al. 2000), perhaps due to its blood supply and the presence of viable stem cells capable of osteogenic differentiation (Fujimura et al. 2001).
Cells isolated from adult human skeletal muscle can differentiate into multiple lineages, including osteoblastic cells, in vitro (Williams et al. 1999; Alessandri et al. 2004). These skeletal muscle cells can also form bone when reimplanted into animals (Musgrave et al. 2002; Mastrogiacomo et al. 2005; Sun et al. 2005). Histological analysis of heterotopic bone deposits also supports the central role of mesenchymal cells from skeletal muscle connective tissue (Damanski 1961; Urist 1965; Craven and Urist 1971; van Kuijk et al. 2002). Thus, it is plausible that skeletal muscle cells could be involved in the pathogenesis of HO.

For the first time, this in vitro study investigated the effects of serum and CSF from patients following traumatic brain injury (TBI) or spinal cord injury (SCI) on cells derived from adult human skeletal muscle in an attempt to mimic the fundamental processes underlying the HO observed in these patients. The hypothesis for this study was that skeletal muscle cells exposed to serum and CSF from patients with CNS trauma would proliferate and undergo osteoblastic differentiation.
Chapter 2: Literature Review

The following chapter is a review of the literature pertinent to the topic of heterotopic ossification following injury of the central nervous system in trauma patients. The intentions of this chapter are to illustrate the plausibility of the current investigations and to introduce terminology and information that will be used later.

The first section explains the events of ossification, including fracture healing and heterotopic ossification. The markers of osteoblastogenesis and ossification are also introduced. The second section provides detailed information about heterotopic ossification, including current knowledge on its pathogenesis, with an emphasis on centrally mediated mechanisms.

A discussion of normal muscle formation and important markers of this process takes place in the third section. The idea that muscle formation probably has multiple cellular origins is explored in this section, whilst section four expands on the presence and functionality of cells with osteogenic potential within adult human skeletal muscle.

Finally, section five includes a review of the epidemiology of central nervous system trauma with a description of relevant central nervous system anatomy. It also contains a summary of changes in a variety of markers in the serum and cerebrospinal of patients with central nervous system trauma and attempts to relate these changes to accelerated osteogenesis.
2.1 Principles of bone formation

2.1.1 Bone: a unique tissue

Bone is a connective tissue composed primarily of a calcium hydroxyapatite matrix interspersed with type I collagen fibres that together provide a unique combination of strength and elastile properties (Seeman and Delmas 2006). Bone is important for movement, the protection of viscera, haematopoiesis and calcium-phosphate homeostasis (Ryoo et al. 2006). Anatomically, the bony skeleton is composed of the axial skeleton – skull, vertebrae, ribs, sternum – and the appendicular skeleton, which includes the limbs and their respective girdles. A histological perspective of a typical long bone reveals the presence of several bone layers, each with their own characteristic appearance and function (Figure 1). Cortical bone consists of numerous concentric layers of lamellar bone surrounding a small artery, vein, nerve and lymph vessel (Haversian canal). Red bone marrow resides within the medullary cavity and functions as a centre of haematopoiesis and a source of osteoprogenitors. Yellow bone marrow, consisting of fat and connective tissue, replaces the red bone marrow over time. The ends (metaphyses and epiphyses) of a long bone have thinner cortical walls and are formed internally by spongy (cancellous) bone, which is constructed of trabecular bone (Downey and Siegel 2006). The periosteal envelope lining the cortical bone provides neurovascular supply to the bone and houses osteoprogenitor cells that assist in bone repair. The endosteum on the internal surface of the cortical bone contributes osteoprogenitor cells that are predominantly involved in lengthening of the long bone (Seeman and Delmas 2006).
Clinically, diseases of bone can affect its structural or functional characteristics, such as mineral density, stiffness, elasticity, the rate of bone remodelling or the location of bone formation (Seeman and Delmas 2006). For instance, osteoporosis is the prototypical disease of decreased bone mineral density. The genetic disorder fibrodysplasia ossificans progressiva is a member of the group of conditions defined by bone formation at abnormal sites. There is also an association between trauma to the central nervous system (CNS) and heterotopic deposition of bone within skeletal muscle.
2.1.2 Definition of ossification
Ossification is the formation of bone. In normal adult humans, bone is continually being remodelled to provide optimal biomechanical support. Following disruption of the structural integrity of a bone, a special case of ossification – fracture healing – is responsible for repair of the damaged bone. Heterotopic ossification (HO), the formation of bone outside the skeleton, resembles normal mature bone so it is important to analyse the events of normal ossification and fracture healing to better understand the mechanisms by which HO may occur in response to CNS trauma.

2.1.3 Cells involved in ossification
Bone homeostasis is maintained through the balance between osteoblasts and osteoclasts. Bone resorption is mediated by osteoclasts, which are found only in bone but are derived from circulating mononuclear haematopoietic cells originating in the bone marrow (Karsenty and Wagner 2002; Boyle et al. 2003). The bone matrix is formed by functionally mature osteoblasts. Osteocytes, which are osteoblasts that have become entrapped within the bony matrix, are also important in bone metabolism through intercellular signaling and monitoring of mechanico-chemical stimuli in the local microenvironment (Knothe Tate et al. 2004; Hartmann 2006).

2.1.3.1 Osteoblast development
Osteoblast development occurs in sequential stages, involving recruitment, proliferation, lineage commitment, extracellular matrix maturation and mineralisation (Lian et al. 2004). During these stages, cells initially proliferate then differentiate into more specialised forms, with expression of key osteoblastic markers and the ultimate development of a characteristic mineralised matrix. The most undifferentiated cell in osteoblast development is the mesenchymal stem cell. These cells are derived from the embryonic mesoderm and are the common progenitors of osteoblasts, chondrocytes, adipocytes and muscle cells (Yamaguchi et al. 2000) (Figure 2). They have been identified in virtually all tissues of the adult organism and, amongst other cells, serve as potential sources of osteogenesis within skeletal muscle (Young and Black 2004; da Silva Meirelles et al. 2006).
Figure 2: Mesenchymal stem cells are present in virtually all adult tissues. They are a common progenitor for mesodermal-derived tissues, including bone and muscle. Lineage-specific transcription factors and signalling molecules mediate their differentiation into specialised cells (Tuan 2004).

Initially, mesenchymal stem cells proliferate and form cellular condensations. Subsequently, they differentiate into osteoprogenitor cells, which are spindle-shaped cells with significant proliferative abilities but limited self-renewal (Aubin 1998; Heng et al. 2004). Some osteoprogenitors are constitutively osteogenic, whilst a distinct population – the inducible osteoprogenitors – require an osteoinductive stimulus to progress further into the osteoblastic lineage (Turksen and Aubin 1991). The next cell stage is called the preosteoblast stage, which is distinguished from the osteoprogenitor stage by limited proliferation but
increased specificity of osteoblastic markers (Aubin 2001; Nakashima et al. 2002). Mature osteoblasts form from pre-osteoblasts that develop the ability to secrete a mineralised extracellular matrix. Aside from matrix production, this stage is defined by a cuboidal morphology, post-proliferative behaviour and strong expression of osteoblastic markers (Candeliere et al. 2001). However, all mature osteoblasts are not identical despite homogenous morphology and matrix secretion. They are heterogenous with respect to detectable osteoblastic markers. In an in vivo study of fetal rat calvarial cells, only alkaline phosphatase and the parathyroid hormone receptor were found on all cells. Expression of other typical osteoblast markers was dependent on the stage of development of the cell and its location within bone (Candeliere et al. 2001). Eventually, the mature osteoblasts are encased in the bony matrix and become osteocytes or, alternatively, quiescent bone-lining cells (Aubin 1998). The completion of the cycle is manifested by osteoblast apoptosis (Aubin 2001) (Figure 3).

Figure 3: The complex process of osteoblastogenesis. Multipotential mesenchymal stem cells undergo a stepwise progression through the multiple cell types of the osteoblastic lineage (Aubin 2001).
2.1.3.2 Other cells
The local bone microenvironment contains many other cell types that are required for the nutrition and innervation of bone and which may directly influence ossification. Blood vessels lined by endothelial cells and pericytes are essential in supplying bone with adequate blood flow and, hence, oxygen, minerals and proteins for ossification. In addition, the endothelium, through the production of angiogenic and osteogenic factors, can directly mediate ossification (Bouletreau et al. 2002). Pericytes also potentially contribute to ossification by acting as a source of osteoprogenitor cells (Levy et al. 2001; Collett and Canfield 2005; Tavian et al. 2005). The importance of neurotransmitters at the bone surface is suggested by the presence of neural synapses within bone (Spencer et al. 2004). In particular, the periosteum has high sensory innervation, which helps to explain the significant pain associated with fractures that disrupt this layer of bone. Bone also has a close relationship with neighbouring soft tissues, such as the connective tissue and skeletal muscle, through migration of stem and osteoprogenitor cells from neighbouring soft tissues to fracture sites during bone formation and repair (Einhorn 1998; Gerstenfeld et al. 2003).

2.1.4 Pathways to bone formation: signalling pathways and extracellular factors
2.1.4.1 Bone morphogenetic proteins
The most well described factors in ossification are the bone morphogenetic proteins (BMPs). This family of proteins is a member of the transforming growth factor (TGF) β superfamily through conservation of cysteine residues and overall structure (Wozney et al. 1988). BMPs are ubiquitous, pleiotropic extracellular proteins involved in cellular signal transmission during cell and organism development. They are most recognised for their roles in bone formation, although they also play pivotal roles in other domains. BMPs are central to the development of bone and are the most promising factors for improving bone healing (Termaat et al. 2005). They offer the function of osteoinduction, which is stimulation of the host tissue to produce de novo bone (Cheng et al. 2003). BMPs play a central role in the entrance of mesenchymal cells into the osteogenic lineage (Wozney, 2002). Intriguingly, individual BMPs are capable of initiating the entire cascade of osteogenesis in vitro and in vivo,
at least in animal models, thus imitating the effect of unpurified demineralised bone matrix (Wozney 2002). This suggests a great deal of redundancy with regard to this function (Ripamonti 2006).

BMPs alter gene expression through initiation of intracellular cascades that result in alteration of gene expression (Figure 4). These cascades begin with binding of the BMP to its respective receptors on the cell surface. These type I and type II membrane receptor serine/threonine kinase receptors are phosphorylated after BMP binding. The phosphorylated receptor complex then activates intracellular messenger molecules called receptor Smads (Smad 1, 5 and 8). Smads are approximately 500 amino acid length proteins so called because of their sequence similarity to the insect proteins ‘Sma’ and ‘Mad’. After activation, the receptor smads are bound by the mediator Smad (Smad 4) and then enter the nucleus where they bind directly to DNA to alter gene transcription (Massague et al. 2005). This cascade ultimately results in the upregulation of osteoblast-specific genes, such as core binding factor alpha 1, alkaline phosphatase and osteocalcin, with elaboration of the bone tissue phenotype. The significance of other BMP pathways not involving Smad signalling is beginning to come to light. These involve the intracellular mediators mitogen-activated protein kinase (MAPK), p38 and c—Jun N-terminal kinase (Valcourt and Moustakas 2005). Multiple extracellular and intracellular mechanisms modulate BMP-mediated signalling (Dattatreyamurty et al. 2001; Termaat et al. 2005; Valcourt and Moustakas 2005; Yanagita 2005). As elucidated by genetic studies in human cases of fibrodysplasia ossificans progressiva, disruption of the various mechanisms and pathways involved in BMP signalling can lead to the development of HO (de la Pena et al. 2005; Shore et al. 2006).
Figure 4: The smad-dependent BMP signalling pathway. Binding of BMPs to type 1 and type 2 membrane receptor serine/threonine kinases initiates a cascade of intracellular events involving Smads 1, 4, 5 and 8. A system of antagonistic mechanisms, including Noggin, Smurfs and Tob, regulates this pathway. Dysregulation of this pathway may be important in the development of heterotopic ossification (Katagiri and Takahashi 2002).

2.1.4.2 Other extracellular signalling factors in bone formation

BMPs represent one of many groups of extracellular signalling factors that are involved in the regulation of ossification. The Wnt family is a group of glycosylated lipid-modified proteins that, like BMPs, are ubiquitous, having many functions throughout development and into post-natal life (Nakashima et al. 2005). Wnts and the related intracellular molecule β-catenin are known to be involved in embryonic osteogenesis and have recently been implicated in post-natal bone formation. The function of both osteoblasts and osteoclasts are affected by these factors (Holmen et al. 2005). Mice lacking β-catenin have
increased osteoclast number and osteopenia, while a deficiency in the Wnt co-receptor LRP-5 causes decreased bone mass secondary to a reduction in osteoblast number and function (Kato et al. 2002; Holmen et al. 2005). Humans with a mutated LRP-5 gene have osteoporosis or high bone mass depending on the functional effect of the mutation (Hartmann 2006). The Wnt/β-catenin system mediates its pro-ossification effects through a variety of mechanisms. It has been shown to increase the sensitization of mesenchymal precursor cells to osteogenic factors, such as BMPs, by a method dependent on the transcription factors Lef and Tcf but independent of Core binding factor alpha 1 (Mbalaviele et al. 2005). It also blocks differentiation of mesenchymal cells to alternate lineages, and reduces osteoclast formation by increasing osteoprotegerin levels (Krishnan et al. 2006). However, BMPs have recently been shown to inhibit the Wnt-mediated proliferation of bone marrow stromal cells by a Smad1-dependent mechanism (Liu et al. 2006). Furthermore, Wnt3a over-expression in myoblastic cells results in impaired inhibition of myotube formation by BMP-2, suggesting a complex interplay between these two signaling pathways (Nakashima et al. 2005).

A number of other hormones, growth factors and cytokines add to the complexity of the control of ossification in the adult. Key factors include TGFβ, platelet-derived growth factor (PDGF), growth hormone, insulin-like growth factors (IGFs), fibroblast growth factors (FGFs), oestrogen, parathyroid hormone, calcitonin, thyroxine and interleukins 1 and 6 (Morley et al. 2005; Westerhuis et al. 2005; Dimitriou et al. 2006). Their specific roles include chemotaxis, differentiation and proliferation of key cells involved in the ossification process, such as mesenchymal cells, osteoprogenitors, osteoblasts, osteoclasts and inflammatory cells (Morley et al. 2005; Dimitriou et al. 2006). However, as opposed to BMPs, these factors are incapable of stimulation of the entire process of ossification alone (Khouri et al. 1996; Chaudhary et al. 2004). Some factors, such as corticosteroids, BMP antagonists and different forms of FGFs and IGFs have inhibitory roles in the maintenance of bone mass (Morley et al. 2005; Dimitriou et al. 2006). However, the exact methods by which these factors cooperate in the local environment to coordinate ossification are not completely understood (Barnes et al. 1999).
2.1.5 Markers of osteoblast development and ossification

During osteoblast development, cell stages are characterised by specific phenotypes, marker expression and functions. These characteristics can be used to monitor the differentiation state of the cell in culture (Table 1).

<table>
<thead>
<tr>
<th>Transcription factors</th>
<th>Early</th>
<th>Late</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Core binding factor alpha 1</td>
<td>Osterix</td>
</tr>
<tr>
<td>Phenotype markers</td>
<td>Alkaline phosphatase</td>
<td>Osteocalcin</td>
</tr>
<tr>
<td></td>
<td>Type I Collagen</td>
<td>Mineralisation</td>
</tr>
</tbody>
</table>

Table 1: Bone formation requires the development of mature osteoblasts from precursor cells. This process can be monitored by the measurement of several key transcription factors and phenotype markers that are characteristically expressed at early or late stages of osteoblast development.

2.1.5.1 Early markers

Alkaline phosphatase (ALP) is an early marker of bone formation. ALP exists in several isoforms, namely germ cell, placental, intestinal and tissue non-specific types, and is present in high serum concentrations in a number of conditions unrelated to bone pathology, such as bile duct disease (Le Du and Millan 2002). However, high expression of the tissue non-specific form of ALP is a key trait of osteoblastic cells (Aubin 1998). ALP is required for the release of inorganic phosphate into the bony matrix (Le Du and Millan 2002) but it is also a relatively early marker of the osteoblastic lineage, being present in osteoprogenitor cells, suggesting that it may also have other functions (Bosse 1997; Mastrogiacomo et al. 2005). It can be used to assess the osteogenic potential of clonal populations of osteoprogenitors (Purpura et al. 2003). In addition, the expression of ALP generally correlates well with the development of HO in vivo (Wildburger, Zarkovic, Dobnig et al. 1994; Bosse 1997; Rigaux et al. 2005). Interestingly, it has also been found in human capillaries, tendons and intramuscular connective tissue (Grim and Carlson 1990). The other important early phenotypical marker of osteoblastogenesis is type I collagen, which is first present in osteoprogenitors (Zhang et al. 2003).

Core binding factor alpha 1 (Cbfa1) (also known as Runx-2 and AML-3) is the earliest and best-understood marker of the osteoblastic lineage (Karsenty et al. 1999). It is essential in the differentiation of mesenchymal cells into pre-osteoblasts (Nakashima et al. 2002). Cbfa1 is primarily found in bone, within
committed cells of the osteoblastic lineage as well as pre-hypertrophic and hypertrophic chondrocytes, but it can also be found in much lower amounts in T-cell lines, the thymus and testes (Ogawa et al. 2000; Lian and Stein 2003). It is also detectable in non-osseous mesenchymal cells of neonatal rats and fetal humans (Banerjee et al. 2001; Bronckers et al. 2005), perhaps providing a general indication of cell plasticity (Hegyi et al. 2003). Haploinsufficiency of this gene in humans causes the autosomal dominant disease cleidocranial dysplasia (Karsenty and Wagner 2002). Mice possessing a homozygous mutation of Cbfa-1 show no evidence of ossification due to a lack of mature osteoblasts (Komori et al. 1997). Conversely, forced overexpression of Cbfa1 in an in vitro model results in the osteoblastic differentiation of myoblasts (Gersbach et al. 2004). Binding elements for Cbfa1 are present in important osteoblast function-related genes, including osteocalcin, osteopontin and type I collagen, indicating a regulatory capacity of Cbfa1 on these genes (Ducy et al. 1997). It appears to act as a scaffold for other proteins that regulate the transcription of lineage determining genes (Lian et al. 2004). Its transcriptional activity is increased by several osteogenic factors, particularly BMP-2, through binding to their respective membrane receptors and activation of either Smad or MAPK intracellular pathways (Nakashima and de Crombrugghe 2003).

2.1.5.2 Late markers

As osteoblast development progresses, later markers of differentiation become expressed. These markers are heterogeneously expressed, being dependent on the specific function, location and developmental stage of the cell (Zhang et al. 2003). Osteocalcin is the most abundant non-collagenous protein present in the osteoblast-derived matrix. It is produced only by osteoblasts and is widely used as a marker of the mineralisation phase of osteoblast development (Ducy et al. 1996). Rather than just being a passive molecule secreted during matrix development, osteocalcin provides a feedback mechanism so as to negatively regulate mineralisation and osteoblast maturation (Trentz et al. 2005). To highlight this, osteocalcin deficient mice have increased bone mass (Ducy et al. 1996). Other markers of the mineralisation stage of ossification include bone sialoprotein, osteopontin and osteonectin. Furthermore, mineralisation itself is assessable by specific stains, such as the Villanueva, von Kossa and Alizarin red methods, which exploit the calcified nature of the extracellular matrix.
Osterix (Osx) is a zinc-finger transcription factor essential for osteoblast maturation, particularly the mineralisation stage. It is even more specific to the osteoblastic lineage than Cbfa1, being found only within the bone matrix, the endosteum and periosteum post-natally (Nakashima and de Crombrugghe 2003). Osx-null mice embryos completely lack ossification although chondrogenesis is unaffected, in keeping with arrested osteoblast maturation prior to mineralisation (Nakashima et al. 2002). Putative osteoprogenitor cells in Osx-null mice retain the potential to differentiate into chondrocytes, suggesting a common progenitor of osteoblasts and chondrocytes. In fact, Osx acts as a negative regulator of the master chondrogenic transcription factor Sox-9 (Nakashima and de Crombrugghe 2003). In other words, Osx commits potential osteoprogenitor cells to become mature osteoblasts and, by doing so, prevents their differentiation into an alternative lineage (Tai et al. 2005). However, fibroblasts that have been modified to express Osx show enhanced proliferation and expression of osteopontin, but not other markers of osteoblastogenesis, nor bone matrix production (Kim et al. 2006). This highlights the temporal role of Osx later in osteoblast development and the requirement for other factors, such as Cbfa1, for full elaboration of the osteoblast phenotype. The absence of Osx in Cbfa1-null mice and the presence of normal Cbfa1 levels in Osx-null mice indicate that Osx operates downstream of Cbfa1. However, Cbfa1 does not upregulate Osx in a myoblastic cell line (Nakashima et al. 2002; Celil et al. 2005). Like Cbfa1, Osx expression is regulated by BMPs, Smads and MAPK, amongst other factors (Celil et al. 2005).

2.1.5.3 Other transcription factors
Several other transcription factors, such as distal-less 5 (Dlx-5), Msx-2 and Inhibitor of differentiation-1 (Id-1) are involved in the regulation of ossification (Figure 5). Dlx-5 is expressed within osteoblastic cells during the mineralisation stage of ossification (Ryoo et al. 1997). Suppression of expression results in craniofacial defects and decreased periosteal thickness. Mice lacking Dlx-5 show no alteration in their expression of Cbfa1, however they have delayed expression of osteocalcin (Nakashima and de Crombrugghe 2003). BMPs require Dlx-5 to induce expression of Osx and the development of ossification at ectopic sites (Valcourt and Moustakas 2005). Another homeodomain-containing factor is Msx-2, so called because of its relationship to the
Drosophila gene msh. It is important in the early stages of ossification, especially in the cranial bones (Ducy 2000; Komori 2006). Although prevalent in osteoprogenitor cells, it is also associated with downregulation of Cbfa1 and osteocalcin expression and is itself downregulated during osteoblast maturation (Ryoo et al. 1997; Ducy 2000). The transcriptional regulator Id-1 is upregulated during BMP signalling and can block differentiation of progenitor cells to non-osteoblastic lineages by binding to helix-loop-helix transcription factors (Peng et al. 2004; Valcourt and Moustakas 2005). Other transcription factors, such as the basic helix-loop-helix family members Twist and Dermo-1, and the zinc finger containing factors Krox-20 and Sp3, have also recently been implicated in the control of ossification (Nakashima and de Crombrugghe 2003; Komori 2006).

Figure 5: Several transcription factors coordinate the differentiation of mesenchymal cells into mature osteoblasts and can be used to monitor the progress of osteoblast development (Stains and Civitelli 2003).
2.1.6 Endochondral and intramembranous ossification

Although ossification ultimately occurs through the singular process of osteoblastic secretion of a bony matrix, there are two mechanisms for the development of this matrix: intramembranous and endochondral ossification. Intramembranous ossification is the process that generates the flat bones of the skull and clavicle within the embryo (Aguila and Rowe 2005). During this mechanism of ossification, mesenchymal stem cells directly differentiate into osteoblastic cells without a preceding cartilage scaffold (Dimitriou et al. 2005). Within the embryo, endochondral ossification produces the majority of bones, including the long bones. This process involves mesenchymal stem cell differentiation into both chondrogenic and osteogenic lineages. Initially, stem cells differentiate into chondrogenic cells, resulting in the deposition of a cartilage matrix. This is followed by differentiation of additional stem cells, this time to form osteoblastic cells that replace the cartilage scaffold with bone (Aguila and Rowe 2005; Dimitriou et al. 2005). The degree of endochondral and membranous ossification that occurs is dependent on cell availability, the relative amounts of inducing factors, and local stimuli, such as micro-motion and tissue oxygenation (McKinley 2003; Termaat et al. 2005).

2.1.7 Ossification in special contexts

2.1.7.1 Fracture healing

When the structural integrity of a bone is breached and a fracture occurs, ossification is required for healing. The process of fracture healing has been called a ‘replay’ and a ‘recapitulation’ of ossification during embryonic development (Nakashima et al. 2005; Termaat et al. 2005). It has also been likened to wound healing (Einhorn 2005). It involves ossification across a fracture line so as to re-establish the integrity of the damaged bone. Based principally on animal models, significant understanding of this complex process has been attained. The cells involved in fracture healing are located in the cortex, periosteum, bone marrow and surrounding soft tissues (Einhorn 2005). Clinical and laboratory evidence has clarified important physiological factors that contribute to the microenvironment that fosters fracture healing: viable bone, nutrition, oxygen supply, growth factors, acid-base balance and immobility at the fracture site (Pape et al. 2001; Termaat et al. 2005). At the molecular level, the balance between BMPs and their antagonists is crucial in
determining the success of the healing process (Dimitriou et al. 2006). Non-unions are associated with a downregulation of osteogenic BMPs (Niikura et al. 2006). Other factors, such as TGFβ, interleukins, IGFs, FGFs, PDGF, VEGF and a family of inhibitory factors are essential in the facilitation of ossification by stimulation of chemotaxis, proliferation, neoangiogenesis and differentiation (Barnes et al. 1999; Phillips 2005; Dimitriou et al. 2006; Tsiridis and Giannoudis 2006).

Fracture healing can be divided into primary or secondary forms:

1. Primary bone healing only occurs when the fractured bone ends are reduced to near anatomical dimensions and there is minimal movement at the fracture site, such as when rigid internal fixation is used (Einhorn 2005). Ossification can then proceed directly across the fracture line by way of intramembranous ossification. Initially, osteoclasts form microscopic burrows across the fracture that are subsequently filled with bone through the differentiation of stem cells into osteoblastic cells (McKinley 2003). The bony cortices, and more specifically endothelial cells and perivascular mesenchymal cells, are predominantly involved in primary fracture healing. Subsequently, there is little periosteal reaction and, therefore, little callus (Dimitriou et al. 2005).

2. Secondary healing is the much more common form of fracture healing. As opposed to primary healing, this is promoted by micro-movement at the fracture site, separation of fracture fragments and bleeding into the local area, and is associated with prominent callus formation. Conservative management and surgical fixation by intramedullary or external devices predispose to secondary fracture healing because these allow movement at the fracture site (Einhorn 2005). This form of fracture healing is divided into several continuous and overlapping phases that describe cellular and molecular events. The initial stages involve local bleeding with haematoma formation and inflammation, with secretion of cytokines and growth factors that induce chemotaxis and proliferation of mesenchymal and osteoprogenitor cells (Morley et al. 2005; McKinley 2003). These events are followed by the invasion and activation of chondrogenic and osteogenic cells that facilitate the repair stage (Phillips 2005). Both membranous and endochondral ossification occur during this form of
healing, with stem cells originating from the skeletal muscle, bone marrow, periosteum (Einhorn 1998; Gerstenfeld et al. 2003), and the vascular wall (Bouletreau et al. 2002) contributing to callus formation. Endochondral ossification occurs predominantly in the relative hypoxia and instability adjacent to the fracture site (McKinley 2003). Membranous ossification is found away from the fracture within the periosteal area and the surrounding soft tissue, constituting ‘hard callus’ that is visible early on plain radiographs (Phillips 2005; McKinley 2003). Solid union in humans usually takes in excess of six months to occur, and is accompanied by the ongoing process of remodeling of the fracture site to allow recovery of bone integrity (Morley et al. 2005; Phillips 2005).

2.1.7.2 Heterotopic ossification
Heterotopic ossification (HO) is the formation of bone within tissues, such as skeletal muscle, that do not normally exhibit ossification (Pape et al. 2004) (Figure 6). Histologically and radiologically, HO resembles mature bone and is distinct from simple calcification by nature of its osteoblastic capacity (Cope 1990). An orderly progression from soft tissue environment to fully mature bone occurs during HO. The initial detectable anomaly is an increased vascularity within soft tissues. Next, collagen fibres interspersed with small foci of calcification are found (Keenan and Haider 1996). Extracellular matrix, comprised primarily of type I collagen, then forms and becomes mineralised rapidly often after only three to four weeks (Banovac and Gonzalez 1997; Sawyer et al. 1991). After six to twelve months, organised trabecular bone is evident (Puzas et al. 1989). If allowed to develop to full maturity, the bone becomes histologically similar to normal skeletal bone, complete with Haversian canals, blood vessels and bone marrow (Damanski 1961; Friedenstein et al. 1968; Wang et al. 2004). However, heterotopic bone lacks a periosteal layer (Wlodarski 1989). In addition, a significantly elevated rate of bone remodelling compared with normal bone has been described in deposits of HO. Both osteoblast and osteoclast number are increased in the order of 50% to 300% compared to normal sites of bone formation (Puzas et al. 1989). The balance of bone turnover in HO is shifted from a steady-state situation towards increased osteoblast activity (Handschin et al. 2006). Bone resorption may also be impaired (Andermahr et al. 2006).
Figure 6: X-ray of heterotopic ossification within the skeletal muscle (arrows) of a patient with a femoral fracture and severe traumatic brain injury
2.2 Heterotopic ossification

2.2.1 History
In the 17th century, Patin described a patient who had “turned to wood”, probably due to fibrodysplasia ossificans progressiva, a rare genetic disorder involving widespread deposition of heterotopic bone (Goncalves et al. 2005). Riedel described soft tissue bone deposits following spinal trauma in the German literature of 1883 (Damanski 1961). However, it was not until the mass casualties of World War I and the advent of X-ray technology that a significant number of cases of extra-skeletal ossification following central nervous system (CNS) trauma were described (Dejerne and Ceiller 1918). In Dejerine & Ceillie’s 1918 paper, heterotopic bone was given the French name “para-ostéoarthropathies”. The prevalence of this condition increased over the next decades as the survival rates of brain and spinal cord injured patients improved. More recently, there has been a sequence of papers that have used titles such as “ossifying peri-articular myositis” (Comarr et al. 1962), “hyperplastic callus” (Glenn et al. 1973), “myositis ossificans” (Bellamy and Brower 1974), and “peri-articular new bone formation” (Mendelson et al. 1975) to describe the phenomenon now most commonly known as “heterotopic ossification” (Garland et al. 1980).

A renaissance of investigation into heterotopic ossification (HO) came after the discoveries of Professor Marshall Urist in the 1960s. Through a series of pioneering experiments, Urist outlined the osteoinductive potential of demineralised bone implanted in skeletal muscle (Urist 1965; Urist et al. 1967; Urist et al. 1968). This research eventually led to the isolation of bone morphogenetic proteins (BMPs). In the last decade, as the interest in the powerfully osteogenic BMPs has escalated, the attention paid to understanding the pathogenesis of HO has dwindled. Surprisingly little has been added to the knowledge of the pathogenesis of HO since the early experiments. This is both a tribute to the early researchers and a signal to modern researchers that much more remains to be understood.
2.2.2 Principles of heterotopic ossification

For heterotopic bone to form, three ingredients are required (Chalmers et al. 1975):

1. A cell capable of producing bone
2. An osteoinductive factor; and
3. An environment in which osteoinduction is sustained

These criteria were developed after the observations that skeletal muscle facilitated osteoinduction by decalcified bone, whereas spleen, liver and kidney were inhibitive.

2.2.2.1 A cell capable of producing bone

Mesenchymal stem cells are the primary cells involved in HO, although distinct populations of skeletal muscle derived stem cells and migrated osteoblastic cells may also play a role (Sawyer et al. 1991; Pape et al. 2004; Eghbali-Fatourechi et al. 2005; Sata et al. 2005). In 1965, Urist identified young connective tissue cells within skeletal muscle that could form bone by multiplication and differentiation into osteoblasts (Urist 1965). This early concept of a skeletal muscle cell capable of ossification has been reinforced by modern methods, which have revealed the osteogenic potentials of diverse populations of skeletal muscle cells. Indeed, the availability of osteogenic cells is unlikely to be the rate–determining step in HO in vivo (Sawyer et al. 1991). Rather, the presence of osteoinductive factors and the local environment are likely to control the degree of bone formation.

2.2.2.2 An osteoinductive factor

HO requires stimulation of mesenchymal cell recruitment, proliferation and differentiation, followed by osteoprogenitor maturation and osteoblast activation (Marusic et al. 1999). Ultimately, at least one factor must be present at the sites of HO to produce this cascade of cellular events. Many factors can operate on the local level to mediate the development of HO, especially those implicated in fracture healing and bone remodelling, such as BMPs. A role for systemic factors has been suggested by several in vitro studies that have demonstrated the mitogenic and osteogenic effect of serum from CNS trauma subjects (Boes et al. 2006; Kurer et al. 1992; Bidner et al. 1990). Several systemic factors involved in the ossification process, including hormones, growth factors and
cytokines, are present in abnormal concentrations following CNS trauma. A combination of these factors acting together or a novel factor is likely to be responsible for HO in this context (Goff and Reichard 2006).

2.2.2.3 **Skeletal muscle as a preferred environment for heterotopic ossification**

In addition to housing cells that are capable of forming heterotopic bone, skeletal muscle is also able to support the osteoinductive process. It has been shown repeatedly that mature bone forms and persists within skeletal muscle *in vivo* upon introduction of adequate osteogenic stimuli (Urist 1965; Musgrave et al. 2002; Kang et al. 2004). In fact, a number of experiments involving ectopic induction of ossification by BMPs have revealed that skeletal muscle is a preferred source of HO in comparison to other soft tissues sites. Yoshida et al. (1998) compared the osteogenic response after implantation of BMP-2 into subcutaneous tissue or skeletal muscle of twenty rats. After a week, ALP and calcium levels were significantly higher at the intramuscular site. Another study by the same group compared the osteoinduction of BMP implantation within fat tissue, subcutaneous tissue and at inter- and intra-muscular sites. Again, the intramuscular group had the highest levels of BMP-mediated osteogenesis (Okubo et al. 2000). Adenoviral transduction of skeletal muscle derived cells with BMP-2 is also preferred for ossification compared to cells from other soft tissues (Musgrave et al. 2000). The diversity of the osteogenic response of each tissue site is a function of its blood supply and the presence of viable stem cells capable of osteogenic differentiation in response to osteogenic stimuli (Fujimura et al. 2001). Other factors within soft tissues, such as the calcium content of the neighbouring skeleton, cardiovascular alterations leading to oedema and disrupted acid-base balance also play a role (Major et al. 1980). Following neurological injury, sensory loss, lengthy periods of iatrogenic paralysis or physical therapy may inadvertently lead to muscle trauma that might insidiously promote ossification (Sobus et al. 1993; Snoecx et al. 1995). Furthermore, many patients with CNS trauma are managed with artificial hyperventilation to reduce intracranial pressure. This results in respiratory alkalosis, which predisposes to bone formation. Microvascular changes and alteration of local blood flow have also been implicated to precipitate HO by creating abnormal electrolyte and protein gradients and reducing tissue oxygenation (Comarr et al. 1962; Fujimura et al. 2001; Lotta et al. 2001).
2.2.3 Epidemiology

2.2.3.1 Incidence and location in traumatic brain and spinal cord injury

Most studies report an incidence of HO with traumatic brain injury (TBI) or spinal cord injury (SCI) of between 15 and 40%, with SCI patients having a slightly greater overall incidence compared to TBI patients. However, the range of published incidences of HO is large (Table 2), which is mainly related to discrepancies in the method of diagnosis and management of HO across different institutions. For example, the definition of HO is sometimes defined radiologically and sometimes symptomatically. HO is usually asymptomatic (Sawyer et al. 1991), so studies that employ intensive investigations to discover cases will significantly overestimate the clinically important incidence of this condition. Also, some studies only consider HO at sites without concurrent fractures. Lastly, differences in the routine measures taken to prevent HO alter its prevalence. Despite these differences in measured incidences of HO between centres, it is important to note that even the lower ends of the published ranges (5% for TBI patients and 20% for SCI patients) represent a significant level of morbidity. HO can cause pain and reduced range of motion of joints. At the worst extreme, it results in severe functional limitation (8-10% of cases) and even complete bony ankylosis in 5% of cases (Subbarao and Garrison 1999; Sawyer et al. 1991; Garland 1988). Interestingly, just as patients with TBI and SCI share disparate rates of HO, they also vary with respect to the sites of occurrence of the bone deposits. TBI patients typically develop HO within the soft tissues surrounding the hip, shoulder, elbow and knee, and generally in anterolateral, inferomedial and posterior aspects of these joints. Conversely, SCI patients most commonly have involvement around the hip, knee, pelvis and thigh, within the anteromedial plane, and they are typically only affected at regions below the level of paralysis (Garland 1988; Garland et al. 1980).
### Injury Incidence Selected studies Locations affected

<table>
<thead>
<tr>
<th>Injury</th>
<th>Incidence</th>
<th>Selected studies</th>
<th>Locations affected</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBI</td>
<td>5-73.3%</td>
<td>Flin et al. 2002; Garland et al. 1982; Sazbon et al. 1981; Garland et al. 1980; Mendelson et al. 1975</td>
<td>Hip &gt; shoulder &gt; elbow &gt; knee</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Anterolateral, inferomedial and posterior aspects of these joints</td>
</tr>
<tr>
<td>SCI</td>
<td>16-48.7%</td>
<td>McKinley et al. 2002; Banovac and Gonzalez 1997; Wittenberg et al. 1992; Garland et al. 1989; Lal et al. 1989; Garland 1988; Dejerine and Ceillier, 1918</td>
<td>Hip &gt; knee &gt; pelvis &gt; thigh &gt; upper limb</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Anteromedial aspect</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Below level of paralysis</td>
</tr>
</tbody>
</table>

Table 2: Incidence and location of heterotopic ossification in association with traumatic brain injury (TBI) and spinal cord injury (SCI)

#### 2.2.3.2 Associated neurological conditions: evidence for a central mechanism?

A number of conditions are associated with the development of HO. Based on these associated conditions, HO can be categorised into neurogenic, post-traumatic, genetic or “reactive” (McCarthy and Sundaram 2005). Within the neurogenic group, HO can be further classified into those cases associated with traumatic CNS injury or non-traumatic encephalomyelopathies, such as infections, tumours and cerebrovascular accidents (Table 3). The connection of these conditions through some form of CNS damage hints at a central mechanism for HO development in these patients. Interestingly, HO associated with non-traumatic causes of CNS damage occurs much less frequently (about 4-15% incidence) than those following trauma (McKinley 2002; van Kuijk et al 2002; Taly et al. 2001; Taly et al. 1999).

### Condition Selected studies

<table>
<thead>
<tr>
<th>Condition</th>
<th>Selected studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myelitis</td>
<td>(Taly et al. 1999)</td>
</tr>
<tr>
<td>Encephalitis</td>
<td>(An et al. 1987)</td>
</tr>
<tr>
<td>Tabes dorsalis</td>
<td>(Rava 1966)</td>
</tr>
<tr>
<td>Stroke</td>
<td>(Chua and Kong 2003)</td>
</tr>
<tr>
<td>Anoxic brain damage</td>
<td>(Chua and Kong 2003)</td>
</tr>
<tr>
<td>Malignant neuroleptic syndrome</td>
<td>(Peylan et al. 1987)</td>
</tr>
<tr>
<td>Cerebral cysticercosis</td>
<td>(Spencer and Ganpath 1988)</td>
</tr>
<tr>
<td>Epidural abscess</td>
<td>(Radt 1970)</td>
</tr>
<tr>
<td>CNS tumours</td>
<td>(Roberts 1968)</td>
</tr>
</tbody>
</table>

Table 3: Evidence for a central mechanism? Non-traumatic central nervous system conditions associated with heterotopic ossification
2.2.3.3 Effect of age and gender

HO is relatively uncommon in children, regardless of the aetiology. After SCI, children have rates of HO of approximately 3.3% (Garland et al. 1989), rising to 17% if total body bone scans are used for diagnosis (Sobus et al. 1993). Children also have delayed onset of HO and different patterns of localisation of their ectopic bone compared to adults. The ectopic bone deposits also have a much greater tendency to resorb spontaneously in children. There appears to be no bias towards the development of HO following CNS trauma with respect to gender (Scher 1976; Lal et al. 1989; Flin et al. 2002), suggesting that hormonal differences between males and females do not play significant roles in this process.

2.2.3.4 Genetic predisposition

The interplay between genetic and acquired predisposition to enhanced ossification is likely to ultimately dictate the level of observable HO (Garland 1988). Approximately 70% of bone mass variability in humans is attributable to genetic variation. Furthermore, pure inbred mice of different breeds have grossly different responses to an osteoinductive stimulus within their skeletal muscle (Marusic et al. 1999). The importance of genetic factors in the development of HO in humans can be better appreciated by a review of the rare and serious diseases fibrodysplasia ossificans progressiva (FOP) and progressive osseous heteroplasia (POH). The histological manifestation of these genetic conditions is widespread severe connective tissue inflammation, intra-muscular oedema and angiogenic fibrous collections, which lead to exuberant HO in soft tissues throughout the body. Clinically, patients suffer significant morbidity due to pain and difficulty with mobility and early death usually secondary to pneumonia. The pathogenesis of these conditions involves dysfunctional regulation of ossification signalling pathways. Patients with FOP have elevated BMP-4 and decreased BMP antagonist levels within lymphocytes and lesional cells (de la Pena et al. 2005) due to a genetic defect in a type I BMP receptor (Shore et al. 2006). POH is caused by abnormalities in the G protein signalling pathways, especially those involving GNAS1. This causes disordered mesenchymal cell differentiation into bone (Kaplan and Shore 2000). The influence of genetics on the development of HO can also be seen in the increased prevalence of HO in patients who have a past history of HO (Sawyer
et al. 1991), severe osteoarthritis (Ahrengart 1991) and in the contralateral hip following hip arthroplasty (DeLee et al. 1976). These observations may be partly due to an underlying genetic disposition to systemic ossification. That is, the threshold for formation of HO may be lower in some patients due to genetic differences, perhaps such as those controlling BMP signalling, like in FOP. Motivated by similarities to ankylosing spondylitis, some have proposed a genetic link between HO and expression of certain human leukocyte antigens (HLA) (Chantraine and Minaire 1981; Larson et al. 1981). A genetic predisposition would also help to explain why certain patients do not develop HO despite possessing many risk factors, such as severe CNS trauma.

2.3 Myogenesis and muscle regeneration

2.3.1 Introduction
Skeletal muscle comprises approximately forty percent of the body and, through its contractile properties, is essential for locomotion (Guyton and Hall 1996). Skeletal muscle, like cardiac muscle, is a form of striated muscle, so called because of the microscopic appearance of lines (striations) running along myofibrils within each myofibre (elongated, multinucleated muscle cell) (Figure 7). In skeletal muscle, dark striations, formed by A bands, alternate with light striations, which are called I bands. A bands are formed by the protein myosin and I bands contain actin and tropomyosin filaments, which are anchored to Z disks. The distance between two Z disks represents one contractile unit and is known as a sarcomere. In the middle of each sarcomere, the M band fixes the myosin proteins. According to the sliding filament hypothesis, skeletal muscle contraction involves the binding of myosin and actin, resulting in the hydrolysis of adenosine triphosphate (ATP), the conversion of chemical energy into mechanical energy and the movement of the filaments relative to each other, thus shortening the muscle. Unlike cardiac and smooth muscle, skeletal muscle is a voluntary muscle, meaning that its contractions are under deliberate control, via motor neurons. Skeletal muscle is ensheathed in layers of connective tissue, called the endomysium, perimysium and epimysium, which contain its innervation and blood supply. Skeletal muscles are typically attached to the periosteum of bones at two ends, at least, by strong connective tissue links called tendons (Guyton and Hall 1996).
Primary muscle diseases such as muscular dystrophy, a condition defined by reduced amounts of the cytoskeletal protein dystrophin, significantly impair skeletal muscle function and result in severe disability. HO is commonly found within skeletal muscle, suggesting that the skeletal muscle environment has a role in its development. The close ontological link between muscle progenitors and bone progenitors, due to their common mesodermal origins, implies a connection between these two cell types that might be deranged pathologically but manipulated therapeutically. By understanding the molecular mechanisms controlling the development of skeletal muscle, more clues into the pathogenesis of HO may be revealed.
2.3.2 Definition of skeletal myogenesis
Skeletal myogenesis is the formation of skeletal muscle. It occurs during the embryonic phase of development and continues into post-natal life to facilitate regeneration of damaged skeletal muscle.

2.3.3 Markers of myogenesis
Through analysis of the effects of gene mutations in animals, the roles of muscle specific markers - the myogenic regulatory factors (MRFs) - during embryonic myogenesis have been elucidated. The MRFs consist of a group of four basic helix-loop-helix (bHLH) transcription factors that control differentiation of cells into the myogenic lineages: MyoD, Myf5, myogenin and Mrf4. They differ from other proteins containing a bHLH region by possession of a muscle-specific motif that facilitates activation of muscle-specific genes (Berkes and Tapscott 2005). All four MRFs are individually capable of activation of myogenesis in mesenchymal cells (Aurade et al. 1994). Indeed, their individual roles in myogenesis overlap but each activates a specific set of genes (Chanoine et al. 2004).

2.3.3.1 Determination factors
Myf5 and MyoD represent myogenic determination factors (Megeney and Rudnicki 1995). They are responsible for the commitment of progenitor cells to the myogenic lineage (Berkes and Tapscott 2005). Mice lacking expression of both factors generally do not develop skeletal muscle (Rudnicki et al. 1993). Instead, putative myogenic cells adopt alternative fates (Tajbakhsh et al. 1996). MyoD functions partly by upregulation of p21, with the permanent exit of cells from the cell cycle. It also has a role in elaboration of genes associated with the structure of muscle cells (Berkes and Tapscott, 2005).

2.3.3.2 Differentiation factors
Specialisation of the skeletal muscle phenotype is achieved by the terminal differentiation of committed myogenic precursor cells (Zammit and Beauchamp 2001). The transcription factor myogenin is required for this terminal differentiation and is thus considered to be a myogenic differentiation factor (Perry and Rudnicki 2000; Ohkawa et al. 2006). Its expression follows the specification of the precursor cell to the myogenic lineage by MyoD and Myf5.
Mrf4 possesses functions of both a determination and a differentiation factor, with evidence that Mrf4 is responsible for skeletal myogenesis in novel forms of Myf5/MyoD double negative mice (Kassar-Duchossoy et al. 2004; Berkes and Tapscott 2005).

2.3.3.3 Myosin heavy chains
The myosin heavy chains (MyHC) are a family of proteins that are present in terminally differentiated muscle cells. Adult skeletal muscle contains four different isoforms, while developmental and regenerating muscles contain a further two. All six isoforms are found on chromosome 17 (Weiss et al. 1999). The functional properties of muscle fibres are dependent on the contribution of the different isoforms (Beylkin et al. 2006). The myosin heavy chains are commonly used as markers of the fully mature skeletal muscle phenotype. For corroboration, evidence of multinucleated myotubes stained by another, less specific myogenic marker, the cytoskeletal protein desmin, is often used (O'Brien et al. 2002; Baj et al. 2005).

2.3.4 Embryonic skeletal myogenesis
Skeletal myogenesis within the embryo involves several coordinated steps that ensure appropriate cell orientation and differentiation (Figure 8). Skeletal muscle develops from somites, which are condensations of mesodermal tissue laying either side of the neural tube and notochord. The dermatomyotome, which constitutes the dorsal part of the somite, is the site of origin of cells that will ultimately form the back and limb muscles (Bailey et al. 2001). Myoblastic cells from the hypaxial portion of this area break off (delaminate) in response to local cues and migrate to their respective sites in the developing limb buds. Expression of Pax-3 is required at this time for the transcription of the tyrosine kinase receptor c-met, which binds to the migratory factor hepatocyte growth factor (Dietrich et al. 1999; Francis-West et al. 2003). Subsequently, these progenitor cells increase in number and begin to express MyoD and Myf5 (Buckingham et al. 2003). Subsequently, terminal differentiation occurs, with expression of myogenin, Mrf4 and the MyHC proteins. The fusion of myoblasts creates terminally differentiated multinucleated myotubes, which mature to form functional skeletal muscle fibres after innervation (Grounds et al. 2002; Francis-West et al. 2003). On embryonic day 7 in the chick, a second wave of
myogenesis occurs, resulting in the formation of secondary fibres that surround
the primary fibres and constitute most of the skeletal muscle present at birth
(Buckingham et al. 2003; Francis-West et al. 2003).

Figure 8: Embryonic myogenesis in the limb involves several coordinated steps
that ensure appropriate cell orientation and differentiation. Muscle precursor
cells originate in the hypaxial dermatomyotome, migrate to the limb and
undergo myogenic differentiation under the control of an array of transcription
factors and signalling molecules (Buckingham et al. 2003).

2.3.5 Satellite cells and post-natal muscle regeneration
Post-natal muscle regeneration in humans resembles the process of
myogenesis in the embryo. A “conserved transcriptional hierarchy” (Parker et al.
2003) involving the MRFs in muscle progenitor cells is central to the generation
of the skeletal muscle phenotype (Buckingham et al. 2003; Bailey et al. 2001).
However, there are notable differences between embryonic and post-natal
myogenesis (Figure 9). For example, the factors responsible for signalling the
initiation of the myogenic processes in each phase are different. In the embryo,
signalling molecules such as Wnts, Shh and Noggin are required for myogenic
differentiation, whilst adult muscle is stimulated to regenerate by damage to
muscle fibres (Bailey et al. 2001). MyoD is also more important in the adult as
Myf5 more effectively compensates its functions in the embryo (Parker et al.
2003). Furthermore, Knapp et al (2006) recently outlined a role for myogenin in
adult growth distinct from its embryonic functions (Knapp et al. 2006).
Figure 9: Comparison of embryonic myogenesis and adult muscle regeneration. Conservation of regulatory transcription factors results in development of the skeletal muscle phenotype during both stages of development. However, notable differences between the two processes include different initiating stimuli and different levels of importance of MRF expression (Bailey et al. 2001).

Satellite cells are spindle-shaped cells that lie outside the sarcolemma (plasma membrane) and beneath the basal lamina of skeletal muscle fibres. Their morphology and location are considered the most reliable indicators of cell phenotype (Grounds et al. 2002; McKinnell et al. 2005). Resting satellite cells are mitotically quiescent, with minimal transcriptional activity (Baj et al. 2005). They constitute approximately 2-5% of all sublaminar nuclei in adult skeletal muscle (Asakura et al. 2001). Although there is not one set of markers unique to satellite cells, they are most commonly reported to express Pax-7, c-met, desmin, Myf5+/-, m-cadherin+/-, CD34+/-, CD45-, Sca-, Bcl-2 +/-, syndecan-3+ and syndecan-4+ (Cornelison et al. 2001; Zammit and Beauchamp 2001; O'Brien et al. 2002; Charge and Rudnicki 2004; Baj et al. 2005; Sherwood and Wagers 2006). The variability of marker expression may indicate the heterogeneity of function of this population (Wagers and Conboy 2005). Another indication of their heterogeneity is that although most satellite cells in the limbs originate from the hypaxial dermatomyotome, some may arise during development of the vascular system (De Angelis et al. 1999; Schiendi et al. 2006).
Satellite cells are the predominant cell type responsible for skeletal muscle repair in humans post-natally (Collins et al. 2005). Upon stimulation by muscle damage, such as trauma or exercise, satellite cells are awoken from their quiescent state and proliferate. Activation is initially associated with upregulation of Myf5 and MyoD, which subsequently leads to differentiation of the satellite cells into myoblasts. Myogenin and MRF4 expression then occurs before any evidence of the MyHCs and creatine kinase (Seale and Rudnicki 2000; Baj et al. 2005). The myoblasts ultimately fuse with existing fibres to effect muscle regeneration (McKinnell et al. 2005).

2.3.6 Novel sources of muscle regeneration

Although only satellite cells are likely to play significant physiological roles in muscle regeneration in vivo, myoblastic cells may also be derived from other cells within and outside skeletal muscle (Grounds et al. 2002; Asakura 2003; Collins 2006) (Figure 10).

2.3.6.1 Sources of myoblasts outside skeletal muscle

- Bone marrow derived cells

The bone marrow compartment contains mesenchymal and hematopoietic stem cells, both of which have multilineage potential (Goldring et al. 2002). Circulating cells originating in the bone marrow compartment can form myoblastic cells during muscle regeneration. In the special circumstance following irradiation injury to skeletal muscle, cells derived from adult bone marrow can adopt sublaminal positions and incorporate into myofibres (Ferrari et al. 1998; LaBarge and Blau 2002; Camargo et al. 2003; Corbel et al. 2003; Dreyfus et al. 2004; Long et al. 2005). However, the degree of engraftment is generally low, especially in normal conditions, implying that these cells may only be important as satellite cell substitutes in “extreme” cases not encountered physiologically (McKinnell et al. 2005; Dreyfus et al. 2004; Camargo et al. 2003).

2.3.6.2 Alternative sources of myoblasts within skeletal muscle

- Dedifferentiation of myofibre nuclei

Another possibility for the origin of skeletal muscle precursor cells is the post-mitotic nuclei of the muscle fibre itself (Grounds et al. 2002). Although the exact
situations in which this dedifferentiation may occur and which factors could mediate its progress in vivo are not known, ciliary neurotrophic factor has been shown to be capable of initiating dedifferentiation of myotubes in human cells in vitro, with a concurrent decrease in expression of MRFs and attainment of multilineage differentiation capacity (Chen et al. 2005). The observation that a neurotrophic factor is capable of restoration of multipotent progenitor cells in skeletal muscle suggests a possible mechanism for the generation of cells with osteogenic potential following CNS trauma.

- **Stem cells within skeletal muscle**

  The relatively constant number of satellite cells within adult muscle despite constant episodes of regeneration implies a source of self-renewal (Asakura 2003). A heterogenous population of skeletal muscle cells distinct from satellite cells has been implicated in this function. These putative skeletal muscle stem cells can be extracted from skeletal muscle in a more purified form using flow cytometry or culture techniques. They may be involved in myogenesis by acting as progenitors of satellite cells or by differentiating into myoblasts directly (Deasy et al. 2001; Zammit and Beauchamp 2001; Chen and Goldhamer 2003). Another progenitor, the mesenchymal stem cell, present in the interstitial connective tissue of skeletal muscle, can also generate myoblasts (Grounds 1999).
Figure 10: Cells from within and outside skeletal muscle are capable of forming myoblasts during post-natal muscle regeneration. Satellite cells, expressing Pax-7 when quiescent and the MRFs when activated, are predominantly responsible for muscle repair. However, other sources of cells, including skeletal muscle stem cells, circulating bone marrow stem cells and dedifferentiated myonuclei also have myogenic potential (Charge and Rudnicki 2004).

2.4 The osteogenic potential of adult human skeletal muscle cells

2.4.1 Potential origin of adult skeletal muscle osteoprogenitor cells

2.4.1.1 A plethora of possibilities

Cells isolated from adult human skeletal muscle can differentiate into multiple lineages in vitro (Alessandri et al. 2004; Williams et al. 1999). Importantly, these cells can also form new tissues, including bone, in animals in vivo (Mastrogiacomo et al. 2005; Sun et al. 2005; Musgrave et al. 2002). Many cell types within skeletal muscle that are shared with other tissues, including those within the interstitial connective tissue, adipose tissue, vascular system and blood are capable of contribution to HO. However, because of the preference of HO to form in skeletal muscle, attention has been centred on the osteogenic potential of muscle-specific cells, namely satellite cells and skeletal muscle stem cells (Tcacencu et al. 2005).
2.4.1.2 Satellite Cells and Skeletal Muscle Stem Cells

Several populations of skeletal muscle cells contain osteoprogenitor cells that have been labelled “stem cells”. Two core characteristics of stem cells are: (1) Self-renewal: the ability to persist in an undifferentiated state for the lifetime of the organism, and (2) Plasticity: the ability to differentiate into multiple highly specialised cell types given appropriate stimuli (Anderson et al. 2001; Deasy et al. 2001; Lemischka 2002; Tajbakhsh 2005; Ulloa-Montoya et al. 2005). However, true multipotency is not required for a cell that exhibits only unipotency within its resident tissue (Zammit and Beauchamp 2001). Satellite cells are ‘muscle stem cells’ because of their property of self-renewal and the ability to differentiate into the specialised myoblast. Recently, the lineage-restriction of satellite cells and their daughter myoblasts has been questioned by in vitro evidence. Wada et al. (2002) showed that myogenic precursor cells derived from human satellite cells spontaneously express MyoD and Cbfa1 and form Alizarin red staining nodules after exposure to BMP-2. This indicates the inherent in vitro ability of a population of sublaminar cells to differentiate into multiple mesodermal cell types. However, these experiments do not exclude the possibility of another cell type - morphologically similar to the satellite cell and sharing its compartment - being responsible for the observed multipotency. There is accumulating evidence that a heterogenous population of satellite cell progenitors that co-exists with and is distinct from the satellite cell population may generate this multipotency (Cao and Huard 2004). Firstly, satellite cells and one population of putative skeletal muscle stem cells (the so-called “side population” cells) are not co-purified during flow cytometric sorting, highlighting their distinct phenotypical properties (Asakura et al. 2002). Furthermore, Pax7/−/− mice have been shown to have normal levels of side population (sp) cells, albeit with a ten-fold increase in the haematopoietic capacity of the sp cells (Seale et al. 2000). This showed a requirement for Pax7 in driving stem cell differentiation toward the satellite cell phenotype and away from alternative development pathways (Asakura et al. 2002).

2.4.1.3 Other origins of potential osteoprogenitor cells

Many cell types exist in proximity to skeletal muscle fibres and satellite cells, such as endothelium and pericytes. Several groups have concluded that the vascular wall contains cells with stem cell characteristics (Diaz-Flores et al.
1992; Decker et al. 1995; Doherty et al. 1998; Collett and Canfield 2005; Tavian et al. 2005). Levy et al. (2001) and Mastrogiacomo et al. (2005) concluded that the multipotent cells they derived from human skeletal muscle were consistent with pericytes. It has been proposed that multipotent cells intimately associated with the vascular system during embryonic development exist in the post-natal organism as mesodermal stem cells (De Angelis et al. 1999; Minasi et al. 2002; Asakura 2003). The vast majority of skeletal muscle sp cells reside within the skeletal muscle and are not replaced by bone marrow derived progenitors, which suggests a non-haematopoietic source for these cells (Rivier et al. 2004). Despite this, there does appear to be a subset of the skeletal muscle stem cell population, expressing CD45, which is capable of limited myogenesis but significant haematopoietic ability. These cells are likely to either arise from or have a close relationship to the haematopoietic system (McKinney-Freeman et al. 2002). Conversely, CD45- cells are resident in skeletal muscle (Uezumi et al. 2006). However, the distinction between CD45+ and CD45- cells is not absolute. CD45+ sp cells, under the influence of Wnts, become CD45- (Polesskaya et al. 2003).

The essential role of mesenchymal progenitors in osteoblast development makes the population of mesenchymal cells present in the connective tissue of skeletal muscle an important potential origin of cells in HO. Recent unpublished cell-labelling experiments in transgenic mice have shown that the contribution to BMP-induced HO of cells from the skeletal muscle connective tissue, namely either endothelial cells or non-bone marrow derived skeletal muscle-resident stem cells, is more significant than the contribution by MyoD expressing cells (Goldhamer et al. 2003). In addition, the central role of skeletal muscle connective tissue cells is underlined by histological evidence (Urist 1965; Damanski 1961). Because the exact identification of mesenchymal stem cells is presently lacking, proof of multipotency is commonly given as evidence for the stem cell nature of rapidly dividing, colony-forming, morphologically undifferentiated cells (Pountos and Giannoudis 2005). For example, Williams et al. (1999) considered the fibroblastic cells within a skeletal muscle derived cell population to be mesenchymal stem cells based on bright field morphological characteristics and the ability to sustain multiligneage differentiation. Recently, thorough investigation into the specific markers of mesenchymal stem cells and
the use of new techniques are beginning to shed more light on their true nature and location (Young and Black 2004; Reyes et al. 2006).

2.4.2 Experimental osteoinduction of human skeletal muscle cells

The *in vivo* stimuli required for the differentiation of human skeletal muscle cells into the osteoblastic lineage, such as the putative factors present following CNS trauma, are largely unknown. The generic differentiation factor dexamethasone is commonly used to assess the osteogenic ability of skeletal muscle cells *in vitro*. However, the clinical applicability of dexamethasone-induced osteogenic differentiation is questionable because of the observed osteoporotic effects of glucocorticoids in patients. An increasing popularly tool for stimulating osteoinduction is the transduction or injection of BMPs into skeletal muscle cells. Since the initial experiments by Urist with demineralised matrix in animal muscle (Urist 1965; Urist et al. 1967; Urist et al. 1968), several studies have analysed the effect of matrix-derived osteogenic factors on animal muscle cells *in vivo and in vitro*. However, only a small subset has investigated the osteoinduction and implantation of human skeletal muscle cells (Table 4). Ethical barriers have prevented the reimplantation of genetically engineered human cells or the direct intramuscular injection of BMPs into human skeletal muscle. This raises questions as to the reliability of extrapolation into humans of results involving other species.
<table>
<thead>
<tr>
<th>Differentiation factor</th>
<th>Delivery Method (Carrier/Dose)</th>
<th>Study</th>
<th>Cell source</th>
<th>Cell isolation method</th>
<th>Implant Destination</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In vitro</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>n/a</td>
<td>(Sakaguchi et al. 2005)</td>
<td>3F, 5M, mean age 23Y</td>
<td>Primary cell culture: micro-filtration</td>
<td>n/a</td>
<td>None of three tested samples contained &gt;30% Alizarin-red staining colonies</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>n/a</td>
<td>(Sinanan et al. 2004)</td>
<td>Dental surgery patients</td>
<td>Selection of CD56+ cells using a microbead immuno-magnetic technique</td>
<td>n/a</td>
<td>Confluent cultures generated cells with and an osteoblastic phenotype</td>
</tr>
<tr>
<td>BMP-2</td>
<td>n/a</td>
<td>(Wada et al. 2002)</td>
<td>1F, 44Y</td>
<td>Single fibre extraction and low cell density culture</td>
<td>n/a</td>
<td>Intrinsic expression of Cbfa1 by putative satellite cells. Formation of Alizarin red staining nodules after exposure to BMP-2.</td>
</tr>
<tr>
<td>Dexamethasone, Insulin</td>
<td>n/a</td>
<td>(Young et al. 2001)</td>
<td>2 fetal, 2 mature and 2 elderly</td>
<td>Primary cell culture: micro-filtration, freeze-thaw</td>
<td>n/a</td>
<td>Progenitors and pluripotent cells present in culture. 1-5% of progenitors and 6-10% of pluripotent cells showed osteogenic differentiation through expression of bone sialoprotein and osteopontin, plus positive von Kossa staining.</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>n/a</td>
<td>(Levy et al. 2001)</td>
<td>Mean age 32Y, range 27-36Y</td>
<td>Primary cell culture: one preplate</td>
<td>n/a</td>
<td>Intrinsic ALP and osteocalcin expression. Increased ALP activity 3, 6 and 9 days after exposure to dexamethasone.</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>n/a</td>
<td>(Williams et al. 1999)</td>
<td>1F 75Y, 1M 27M</td>
<td>Primary cell culture: micro-filtration, freeze-thaw</td>
<td>n/a</td>
<td>Differentiation into five phenotypes, including bone (von Kossa).</td>
</tr>
</tbody>
</table>

Table 4 (continued on next page): Overview of studies into the ability of bone morphogenetic proteins (BMPs) and dexamethasone to cause osteoinduction in adult human skeletal muscle cells. So far, the reimplantation into humans of human muscle cells that have been modified *in vitro* has not been performed, nor has direct intramuscular injection of BMPs. Thus, it is difficult to make firm conclusions regarding the viability of transfer of osteogenic stimuli into patients using human skeletal muscle cells or the relevance of findings from animal models of induced HO in skeletal muscle.

(n/a = not applicable; F = female; M = male; Y = years of age; BMP = bone morphogenetic protein; hBMP = human bone morphogenetic protein; rhBMP = recombinant human bone morphogenetic protein; SCID = severe combined immune deficiency; Cbfa = core binding factor alpha; ALP = alkaline phosphatase)
<table>
<thead>
<tr>
<th>Differentiation factor</th>
<th>Delivery Method (Carrier/Dose)</th>
<th>Study</th>
<th>Cell source</th>
<th>Cell isolation method</th>
<th>Implant Destination</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In vivo</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>Hydroxyapatite scaffold</td>
<td>(Mastrogiacomo et al. 2005)</td>
<td>6F, 1M, age range 45-75Y</td>
<td>Primary cell culture: microfiltration</td>
<td>Immune deficient mice</td>
<td>Intrinsic Cbfa1, ALP and osteocalcin expression. New bone formed by human cells after 8 weeks.</td>
</tr>
<tr>
<td>BMP-2</td>
<td>hBMP, adenoviral and retroviral</td>
<td>(Lee et al. 2002)</td>
<td>Human</td>
<td>Primary cell culture</td>
<td>SCID mice</td>
<td>Enhanced bone healing with a small fraction of cells incorporated into the bony matrix, indicating healing due mainly to transport of BMP-2 to the defect</td>
</tr>
<tr>
<td>BMP-2</td>
<td>rhBMP-2, diffusion chamber</td>
<td>(Kawasaki et al. 1998)</td>
<td>5M, age range 11-36Y</td>
<td>Primary cell culture: one preplate</td>
<td>Athymic mice</td>
<td>Intrinsic ALP expression. Recombinant human BMP-2 stimulated proliferation of 3 samples, elevated ALP activity in all samples, inhibited vitamin D-dependent osteocalcin production and inhibited myotube formation. Diffusion chamber: induction of ALP-positive cells within the chamber, but no in vivo bone formation.</td>
</tr>
</tbody>
</table>
2.5 Central nervous system trauma: anatomy, epidemiology and associated factors

2.5.1 Central nervous system, blood-brain barrier and cerebrospinal fluid

Anatomically, the central nervous system (CNS) is comprised of the brain and spinal cord, which are bathed in cerebrospinal fluid (CSF). In humans, about 500ml per day of CSF are produced, mainly by the choroid plexus in the lateral, third and fourth cerebral ventricles. CSF gives the tissues of the CNS buoyancy and protects them against rises in intracranial pressure. It also serves as the lymphatic supply of the CNS and helps to regulate intracranial electrolyte balance (Han and Backous 2005). CNS tissue is separated from the systemic circulation by a network of intertwining cells known as the blood-brain barrier (BBB). Within the cerebral circulation, capillaries are lined by specialised endothelial cells that have reduced amounts of endocytotic vesicles (which restrict the flow of molecules through the cell) and that are joined together by tight junctions (which limit the passage of molecules between the cells) (Rubin and Staddon 1999) (Figure 11). The cerebral endothelial cells also possess surface carriers that modify and transport molecules between the blood and the CNS (Ribatti et al. 2006). In normal circumstances, the relatively impermeable barriers between the CNS and systemic circulation impede the passage of many molecules, especially large proteins. Thus, the BBB protects the CNS from potentially toxic compounds within the blood and prevents the flow of CNS contents into the systemic circulation. However, the BBB can be damaged by trauma and molecules released from injured CNS tissues can diffuse into the systemic circulation if they have suitable biophysical parameters (Allen and Geldenhuys 2006). These molecules may also leak into the CSF where they can be detected by lumbar puncture or catheterisation of the cerebral ventricles.
Figure 11: The perivascular environment in cerebral capillaries (left) and within the systemic vasculature (right). In the cerebral circulation, tight junctions between the endothelial cells and the close relationship to neighbouring pericytes and neural cells serve as a protective and selective barrier to compounds in the circulation. Trauma to the brain often results in breakdown of this barrier, allowing molecules that are normally secluded within the central nervous system to enter the systemic circulation (Misra et al. 2003).

2.5.2 CNS trauma

Trauma to the CNS can be classified into primary or secondary forms. Primary CNS trauma is the direct result of structural deformation imparted by an external physical force. It rapidly leads to cell death by means of local oncotic injury and necrosis, as well as grey matter and axonal contusions at distant sites (Ottens et al. 2006). Prevention of accidents and wearing of safety gear are the only ways to manage this form of CNS trauma as its direct effects occur too quickly for other interventions. Multiple cellular and molecular cascades are involved in the development of secondary CNS trauma. These include glutamate release from damaged neurons, an influx of intracellular calcium, microglia-mediated inflammation and, ultimately, protease activation with apoptosis (Zipfel et al. 2000). If the patient survives weeks to months after injury, then remodelling of the CNS tissue can occur and it has been proposed that stem cells are activated that aid in neuronal repair (Ottens et al. 2006). The majority of medical management of CNS trauma is targeted at prevention of the secondary form of CNS trauma, with the prime objective being to reduce intracranial pressure. Common treatment methods include hyperventilation, osmotic fluid therapy and craniectomy (Adamides et al. 2006).
2.5.3 Assessment of CNS trauma

The severity of brain trauma can be clinically assessed by using the Glasgow Coma Scale (GCS). This scale is calculated by using the best scores from a combination of eye, verbal and movement responses (Table 5). The highest possible score is 15 and the lowest 3. A GCS of 13 or more correlates with a mild brain injury, 9 to 12 is a moderate injury and 8 or less a severe brain injury (Teasdale and Jennett 1974).

<table>
<thead>
<tr>
<th>Motor response (M)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Follows commands</td>
<td>6</td>
</tr>
<tr>
<td>Localizes pain</td>
<td>5</td>
</tr>
<tr>
<td>Withdraws to pain</td>
<td>4</td>
</tr>
<tr>
<td>Flexion</td>
<td>3</td>
</tr>
<tr>
<td>Extension</td>
<td>2</td>
</tr>
<tr>
<td>None</td>
<td>1</td>
</tr>
<tr>
<td>Verbal response (V)</td>
<td></td>
</tr>
<tr>
<td>Oriented</td>
<td>5</td>
</tr>
<tr>
<td>Confused speech</td>
<td>4</td>
</tr>
<tr>
<td>Inappropriate words</td>
<td>3</td>
</tr>
<tr>
<td>Incomprehensible</td>
<td>2</td>
</tr>
<tr>
<td>None</td>
<td>1</td>
</tr>
<tr>
<td>Eye opening (E)</td>
<td></td>
</tr>
<tr>
<td>Spontaneous</td>
<td>4</td>
</tr>
<tr>
<td>To command</td>
<td>3</td>
</tr>
<tr>
<td>To pain</td>
<td>2</td>
</tr>
<tr>
<td>None</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 5: The Glasgow Coma Scale (Stevens and Bhardwaj 2006)

Spinal cord injury can be described by the level and completeness of the lesion. Quadriplegia, paraplegia and hemiplegia refer to paralysis of all four limbs, a pair of limbs, or an arm and a leg on one side of the body, respectively. A complete lesion results in loss of all neurological function in the affected region.

2.5.4 CNS trauma: a global epidemic

Trauma is the most common cause of acute CNS injury and affects more people every year in the United States than the neurodegenerative diseases Alzheimer’s and Parkinson’s disease in combination (Ottens et al. 2006; Urban
Brain and spinal cord trauma causes significant morbidity, mortality and financial burden. It occurs predominantly as a result of car and motorcycle accidents, falls, violent crimes and recreational activities (Lee et al. 2006; Ottens et al. 2006). CNS trauma typically affects young males, who are more often involved in activities that present a higher risk of serious injury (Day et al. 2006). Due to cognitive, emotional and physical morbidity, as well as high rates of mortality, in this young patient population, CNS trauma is responsible for the loss of a significant amount of quality adjusted life years. In fact, TBI is the leading cause of death and disability in young adults (Finfer and Cohen 2001). The hospitalisation rates for TBI vary widely throughout the world, from 91 in 100,000 patients in Spain to 300 in 100,000 in Johannesburg. In the United States, approximately a quarter of a million admissions per year are due to TBI. This translates to nearly two million presentations, 80,000 significant functional impairments and 50,000 deaths annually. Cumulatively, up to 6.5 million people may be living with TBI and its associated disabilities in the U.S. alone (Aimaretti and Ghigo 2005). Furthermore, society sustains a large financial burden due to lengthy periods of nursing, rehabilitation and convalescence. The cost of dealing with these patients is estimated to be between 10 and 56 billion dollars per year (Ottens et al. 2006; Urban 2006). SCI also represents a significant problem worldwide. Numbers of affected patients are reported to be as high as 28 per 100,000 population in Helsinki (Dahlberg et al. 2005). Again, motor vehicle accidents are a leading cause, together with falls in the elderly (Pickett et al. 2006). In Canada, the cost directly attributable to managing patients with a complete SCI is approximately $121,600 in the first year following injury (Dryden et al. 2005).

2.5.5 CNS trauma at Royal Perth Hospital

Royal Perth Hospital, the Level 1 Trauma Centre of Western Australia, is a forerunner in the collection and analysis of CNS trauma information. For the last decade, demographic, incidence and outcome information has been recorded for every trauma admission. This information is summarised annually in the “Royal Perth Hospital Trauma Report”. During the years 1995 to 2004, R.P.H. received approximately 300 admissions for SCI. Until 2004, the majority of these were admissions for relatively minor injuries (Injury Severity Score < 16). However, in 2004 two thirds of the SCI admissions were for major episodes of
injury (Injury Severity Score > 15). Fortunately, only an average of twenty SCI patients annually show evidence of neurological deficit. The number of TBI patients with significant injuries also rose during 2004, with 234 of the 268 total admissions falling into the major injury category (R.P.H. 2004). These figures compare favourably to nationally published data. For instance, 322 per 100 000 head of population in South Australia suffer TBI annually (Hillier et al. 1997) and the number of cases of SCI is approximately 10,000 across the entire Australian population. Of concern, O’Connor (2005) recently forecast that the number of spinal cord injuries in Australia would rise to 12,000 within fifteen years (O’Connor 2005). Taken together, these figures emphasise the tragedy of CNS trauma in Perth, Australia and around the world and the massive impact that this condition has on patients, their families and their societies. However, much effort is ongoing towards better management of neurotrauma and its complications, with significant improvements seen in many areas (Atkinson and Merry 2001). Heterotopic ossification is one complication of CNS trauma that is often neglected but is significant because it leads to considerable morbidity. An understanding of its pathogenesis could improve the quality of life of many patients.

2.5.6 Factors in the serum and CSF associated with CNS trauma and HO

Blood represents a potential physical link between damaged CNS tissue and peripheral sites of HO. A humoral mediated mechanism for HO has been suggested by several studies that have demonstrated the mitogenic and osteogenic effect of blood from CNS trauma subjects in mesenchymal and osteoblastic cells (Boes et al. 2006; Klein et al. 1999; Kurer et al. 1992; Bidner et al. 1990). Compared to serum, CSF represents a little-discussed bodily fluid that could be explored for the presence of osteoinductive substances released from damaged CNS tissue. Although often a disparity in the serum or CSF profile between CNS trauma and control patients has been discovered, results are inconsistent and often reflect an incidental consequence of the HO or the CNS damage (Table 6). As yet, the identification of serum or CSF-borne factors definitively involved in the pathogenesis of HO has not been achieved.
Table 6 (continued on next page): Changes in the concentration of factors in the serum and CSF of patients following CNS trauma. Several factors have potential connections to the development of HO because of their influence on osteogenesis.

(↑/↓ = increase/decrease in factor; TBI = traumatic brain injury; HO = heterotopic ossification; bFGF = basic fibroblast growth factor; PCIP = propeptide of type I procollagen; LDL = low density lipoprotein; MMP = matrix metalloproteinase; CRP = C reactive protein; ESR = erythrocyte sedimentation rate; VEGF = vascular endothelial growth factor; IGF = insulin-like growth factor; GH = growth hormone; mRNA = messenger ribonucleic acid; TSH = thyroid stimulating hormone; SCI = spinal cord injury; GFAP = glial fibrillary acidic protein; NFL = neurofilament protein; TNF = tumour necrosis factor; FDPs = fibrinogen degradation products; RANKL = receptor activator of nuclear factor kappa beta ligand; PTH = parathyroid hormone)

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Study</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TBI Serum</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>(Wildburger, Zarkovic, Dobnig et al. 1994)</td>
<td>↑ 2nd week post-TBI</td>
</tr>
<tr>
<td></td>
<td>(Singh et al. 2003)</td>
<td>No correlation with HO</td>
</tr>
<tr>
<td></td>
<td>(Rigaux et al. 2005)</td>
<td>↑TBI+HO 90 days post-injury</td>
</tr>
<tr>
<td>bFGF</td>
<td>(Wildburger, Zarkovic, Egger et al. 1994), (Wildburger et al. 1995), (Wildburger et al. 1996)</td>
<td>Cyclic ↑ in TBI + fracture group, 1st week post-injury, unrelated to fibroblast growth</td>
</tr>
<tr>
<td>Carboxy terminal PCIP</td>
<td>(Wildburger, Zarkovic, Dobnig et al. 1994)</td>
<td>↑ 1st week post-TBI</td>
</tr>
<tr>
<td>Collagen breakdown products</td>
<td>(Andermahr et al. 2006)</td>
<td>↓ TBI</td>
</tr>
<tr>
<td></td>
<td>(Trentz et al. 2005)</td>
<td></td>
</tr>
<tr>
<td>Prolactin</td>
<td>(Wildburger et al. 1998)</td>
<td>↑ TBI + fracture, 5th week</td>
</tr>
<tr>
<td></td>
<td>(Chiolero et al. 1988)</td>
<td>↓ TBI</td>
</tr>
<tr>
<td>S100B</td>
<td>(Hayakata et al. 2004; Savola et al. 2004; Raabe et al. 2003)</td>
<td>↑ TBI</td>
</tr>
<tr>
<td>Oxidized LDL</td>
<td>(Wildburger et al. 2000)</td>
<td>↑ TBI &gt; fracture alone</td>
</tr>
<tr>
<td>Osteocalcin</td>
<td>(Trentz et al. 2005)</td>
<td>↓ 1st week post-TBI</td>
</tr>
<tr>
<td>PTH</td>
<td>(Trentz et al. 2005)</td>
<td>↑ in those with TBI + fracture</td>
</tr>
<tr>
<td>IGF-1, GH</td>
<td>(Beeton et al. 2002)</td>
<td>Variable levels</td>
</tr>
<tr>
<td></td>
<td>(Wildburger et al. 2001)</td>
<td>↑TBI alone for 14 weeks, but gradual ↓ if associated fracture</td>
</tr>
<tr>
<td>Triiodothyronine</td>
<td>(Tenedieva et al. 2000)</td>
<td>↓ comatose TBI patients</td>
</tr>
<tr>
<td>TSH</td>
<td>(Chiolero et al. 1988)</td>
<td>↓ TBI</td>
</tr>
<tr>
<td>Interleukins</td>
<td>(Maier et al. 2001; Beeton et al. 2004; Dziurdzik et al. 2004; Suehiro et al. 2004; Shiozaki et al. 2005)</td>
<td>↑IL-1β/6/-8/-10; ↑IL-1β and IL-10 mainly due to other associated trauma not TBI</td>
</tr>
<tr>
<td>MMP-9</td>
<td>(Suehiro et al. 2004)</td>
<td>↑ TBI</td>
</tr>
<tr>
<td>FDPs</td>
<td>(Ueda et al. 1985)</td>
<td>↑ proportional to brain damage</td>
</tr>
<tr>
<td></td>
<td>(Kushimoto et al. 2003)</td>
<td></td>
</tr>
<tr>
<td>Thrombomodulin, von Willebrand factor</td>
<td>(Yokota et al. 2002)</td>
<td>Indicate cerebral endothelial injury and activation in TBI</td>
</tr>
<tr>
<td>Leptin</td>
<td>(Rigaux et al. 2005)</td>
<td>↓ TBI+HO 90 days post-injury</td>
</tr>
<tr>
<td>Constituents</td>
<td>Study</td>
<td>Findings</td>
</tr>
<tr>
<td>----------------------------------</td>
<td>-----------------------------------------------------------------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>CSF</td>
<td>Alpha II-spectrin breakdown products</td>
<td>↑TBI</td>
</tr>
<tr>
<td></td>
<td>(Ringger et al. 2004)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Pike et al. 2002)</td>
<td></td>
</tr>
<tr>
<td>VEGF</td>
<td>(Shore et al. 2004)</td>
<td>↑TBI</td>
</tr>
<tr>
<td>Interleukins</td>
<td>(Stahel et al. 1998; Whalen et al. 2000; Maier et al. 2001; Yatsiv et al. 2002; Kushi et al. 2003; Hayakata et al. 2004; Shiozaki et al. 2005)</td>
<td>↑IL-1β/-6/-8/-10/-12/-18 acutely</td>
</tr>
<tr>
<td>FDPs</td>
<td>(Conti et al. 2004)</td>
<td>Found in TBI alone</td>
</tr>
<tr>
<td>SCI Serum</td>
<td>CRP, ESR</td>
<td>Specificity of CRP &gt; ESR for inflammatory phase of HO</td>
</tr>
<tr>
<td></td>
<td>(Estrores et al. 2004)</td>
<td></td>
</tr>
<tr>
<td>Haemoglobin</td>
<td>(Huang et al. 1990)</td>
<td>↓SCI</td>
</tr>
<tr>
<td></td>
<td>(Lipetz et al. 1997)</td>
<td>↓ acute SCI, tetra-, complete</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>(Kim et al. 1990; Singh et al. 2003)</td>
<td>No correlation with HO</td>
</tr>
<tr>
<td>Osteocalcin</td>
<td>(Pietschmann et al. 1992)</td>
<td>↑ SCI</td>
</tr>
<tr>
<td>PTH</td>
<td>(Mechanick et al. 1997)</td>
<td>↓ SCI</td>
</tr>
<tr>
<td>RANKL</td>
<td>(Maimoun et al. 2005)</td>
<td>↓ SCI</td>
</tr>
<tr>
<td>Osteoprotegerin</td>
<td>(Maimoun et al. 2005)</td>
<td>↑ SCI</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>(Mechanick et al. 1997)</td>
<td>↓ SCI</td>
</tr>
<tr>
<td>Prolactin</td>
<td>(Mechanick et al. 1997)</td>
<td>SCI &gt; TBI</td>
</tr>
<tr>
<td>Leptin</td>
<td>(Wang, Huang et al. 2005; Bauman et al. 1996)</td>
<td>↑ SCI</td>
</tr>
<tr>
<td>Erythropoietin</td>
<td>(Claus-Walker and Dunn 1984)</td>
<td>↑ all patients, normalised after 8 weeks in those with thoracic cord lesions only</td>
</tr>
<tr>
<td>Dehydroepiandrosterone, dehydroepiandrosterone</td>
<td>(Campagnolo et al. 1999)</td>
<td>Tetra &gt; paraplegic</td>
</tr>
<tr>
<td>Melatonin</td>
<td>(Zeitzer et al. 2000)</td>
<td>Absent in complete cervical spinal cord lesions</td>
</tr>
<tr>
<td>Triiodothyronine</td>
<td>(Prakash 1983)</td>
<td>↓ SCI</td>
</tr>
<tr>
<td></td>
<td>(Bugaresti et al. 1992)</td>
<td>↓ SCI 1st week post-injury</td>
</tr>
<tr>
<td>Creatine kinase</td>
<td>(Singh et al. 2003)</td>
<td>↑ in those with SCI + HO</td>
</tr>
<tr>
<td></td>
<td>(Sherman et al. 2003)</td>
<td>↑ aggressive, drug-resistant HO</td>
</tr>
<tr>
<td>CSF</td>
<td>GFAP, NFL</td>
<td>↓ tetraplegic, complete</td>
</tr>
<tr>
<td></td>
<td>(Guez et al. 2003)</td>
<td></td>
</tr>
<tr>
<td>Eicosanoids</td>
<td>(Nishisho et al. 1996)</td>
<td>↑ acute SCI</td>
</tr>
<tr>
<td>White blood cells</td>
<td>(Travlos et al. 1994)</td>
<td>↑ SCI, &lt;3 weeks post-injury</td>
</tr>
</tbody>
</table>
2.5.6.1 Markers of CNS trauma and inflammation

Factors that have been investigated as markers of the severity of TBI may be responsible for the osteogenesis seen in patients with TBI. The most commonly described of these putative biomarkers are S100B, neuron specific enolase, alpha II spectrin and the ubiquitins (Pike et al. 2001; Raabe et al. 2003; Hayakata et al. 2004; Ringger et al. 2004; Savola et al. 2004). Their exact relevance to HO is largely unknown. Interleukins are key mediators of the inflammatory response and are also found in elevated levels within the brain and systemically following TBI. The influence of several interleukins on osteogenesis is better described.

- Interleukins

The activation of cytokine and neuroendocrine cascades by trauma closely replicates sepsis (Matsuda and Hattori 2006). Vascular alterations, oedema and parenchymal swelling can occur, potentially leading to permanent tissue damage and multi-organ failure (Lucas et al. 2006). The interleukin (IL) family of cytokines are intensely involved in the production of this immunological and inflammatory response (Maier et al. 2005). After severe TBI, IL-6 concentrations within the CSF reach maximal levels within six hours (Kushi et al. 2003). TBI patients also show increased serum levels of IL-6 less than twelve hours after injury, in tandem with a decrease in the circulating concentrations of the IL-6 inhibitor soluble gp130 (Beeton et al. 2004). SCI patients also possess elevated serum levels of IL-6 compared to controls (Segal and Brunnemann 1993; Segal et al. 1997). IL-6 has been suspected of early involvement in the accelerated fracture healing seen in TBI patients. Osteoblasts secrete IL-6 in response to systemic factors such as PTH and IL—8 (Beeton et al. 2004). Osteoblasts also possess receptors for this cytokine (Bellido et al. 1996). IL-6 has been shown to dose-dependently induce ALP and osteocalcin expression and the development of mineralisation in a rat embryonic fibroblast line, acting through the glycoprotein 130 (gp130) receptor (Taguchi et al. 1998). IL-6 also has indirect effects on osteoblastogenesis by controlling production of other factors, such as BMP-6 and IGF-1 (Franchimont et al. 2005). Thus, it is feasible that IL-6 represents a pro-osteogenic factor early in the post-CNS trauma. Recently, Rifas et al (2006) suggested that interleukins are involved in the formation of heterotopic bone by induction of BMP-2 expression in human mesenchymal
stromal cells (hMSCs) (Rifas 2006). IL-17, working synergistically with other T lymphocyte inflammatory cytokines, caused hMSCs to upregulate BMP-2 expression by a factor of ten-fold above control values. Moreover, ALP production was increased and a bony matrix developed in vitro. These processes could be blocked by inhibition of the p38 MAPK pathway. Hence, it appears that inflammation, such as exists following CNS trauma, promotes the development of the osteoblast phenotype through a mechanism that involves interleukins, BMP-2 and the p38 MAPK pathway.

2.5.6.2 Neuroendocrine

Neuroendocrine causes for HO after CNS trauma have gained more exposure in recent times after the discovery of neural synapses within close proximity to bone (Spencer et al. 2004) and the realisation that bone remodelling may be controlled centrally by mediators such as leptin, pituitary hormones, melatonin and prostaglandins. CNS trauma can cause direct damage to the intracranial endocrine glands or indirectly influence bone formation by modification of synapse behaviour, thus creating a hormonal milieu that predisposes to ossification (Trentz et al. 2005).

- Leptin and the hypothalamus

The satiety hormone leptin, a 16-kDa peptide, has been implicated in the neuroendocrine control of bone mass (Elefteriou et al. 2004; Thomas 2004). Ducy et al. (2000) showed that bone formation in mice was inhibited after injection of leptin into the cerebral ventricles (Ducy et al. 2000). Leptin signals through receptors in the ventral hypothalamus to cause noradrenaline release from peripheral sympathetic nerves (Harada and Rodan 2003). Noradrenaline binds to β2 adrenoreceptors on osteoblasts to mediate the osteoinhibitory effect of leptin (Takeda et al. 2002). Administration of antagonists to β2 receptors results in increased bone mineral density in elderly human populations (Bonnet et al. 2005; Turker et al. 2006). Leptin also appears to have direct cellular functions, such as the ability to enhance osteogenesis in both bone marrow and cord blood-derived mesenchymal stem cells (Chang et al. 2006) and the differentiation of human osteoblasts in vitro (Gordeladze et al. 2002). SCI patients have increased serum leptin levels. Wang, Huang et al. (2005) showed a trend for tetraplegic patients to have a higher serum concentration of leptin.
than those with paraplegia, although this difference did not reach statistical significance (Wang, Huang et al. 2005). A recent human study has also documented alterations in the leptin-hypothalamic-sympathetic signalling pathways following severe TBI (Rigaux et al. 2005). Serum was obtained from 31 men three months after severe TBI. A significant decrease in leptin level was found in the patients diagnosed with HO. However, the recorded change in leptin concentration may be ascribable to a variety of other causes aside from hypothalamic damage, such as diet and body fat composition (Bauman et al. 1996). Hence, whether leptin is capable of inducing HO after CNS trauma is yet to be clarified.

- Pituitary gland and hypopituitarism

The pituitary gland is a small endocrine structure sitting below the hypothalamus and connected to it by a dense collection of blood vessels and neurons that allow rapid transmission of neurohormonal signals. Under normal physiological conditions, the pituitary is responsible for the secretion of growth hormone, prolactin, thyroid stimulating hormone, adrenocorticotropic hormone, follicle stimulating hormone, and luteinising hormone (anterior pituitary hormones), as well as the release of antidiuretic hormone and oxytocin (posterior pituitary hormones) (Amar and Weiss 2003). TBI can result in damage to the pituitary through compression from surrounding oedema, which can resolve as the swelling improves. It can also cause direct damage to the pituitary stalk or hypothalamic neuron, pituitary hemorrhagic infarction or hypothalamic micro-haemorrhages, which may be irreversible (Agha et al. 2004; Aimaretti and Ghigo 2005). SCI can result in hypothalamus-pituitary-adrenal axis dysfunction by unclear means (Wang et al. 1992; Huang et al. 1995; Huang et al. 1998; Campagnolo et al. 1999; Safarinejad 2001; Naderi and Safarinejad 2003). Up to 80% of patients with TBI have pituitary dysfunction (Aimaretti and Ghigo 2005). The osteogenic effect of this dysfunction has been explored in a rat model of hypophysectomised rats. Rat bone marrow stroma cells were taken from both hypophysectomised rats and normal controls. Those osteoprogenitor cells taken from rats lacking pituitary function had increased levels of recruitment, proliferation, ALP and mineralisation potential when exposed to optimal osteogenic differentiation medium in vitro (Yeh et al. 1999). These results may be relevant to the
pathogenesis of HO because they suggest a differential upregulation of osteoprogenitor cell sensitivity to osteogenic stimuli following brain damage.

- **Growth factors: bFGF, GH and IGF-1**

Basic fibroblast growth factor (bFGF), growth hormone (GH) and its downstream partner insulin-like growth factor-1 (IGF-1) have been implicated as osteogenic factors after CNS trauma because of their presence in raised concentrations in the serum and their osteoblast growth-stimulating abilities.

bFGF, a 17kDa polypeptide, is present in both brain and bone tissue. It is stored in an active form within the bony matrix and promotes fracture healing when the matrix is disrupted (Solheim 1998). Humans possessing disrupted bFGF signalling exhibit signs of achondroplasia (Minina et al. 2002). bFGF has direct proliferative effects in a variety of osteoblastic cell *in vitro* and *in vivo*. In rat osteoprogenitor cells derived from bone marrow, bFGF demonstrates a stimulatory effect on proliferation and differentiation (Tanaka et al. 1999). It also increases the efficiency of HO caused by demineralised matrix and BMP-2 in rats (Aspenberg and Lohmander 1989; Nakamura et al. 2005). This effect occurs in a biphasic fashion, with inhibition of BMP-2 osteoinductivity at higher doses of bFGF (Fujimura et al. 2002; Nakamura et al. 2005). The Wildburger group’s extensive investigations have revealed the fluctuating behaviour of bFGF in the serum of TBI patients with and without fractures. Patients with TBI and fracture demonstrated raised bFGF levels compared to patients with isolated fracture at 1, 2, 4 and 7 weeks after injury. Wildburger et al related these findings to increased osteogenesis because of a temporal association between bFGF levels and stages of fracture healing. Despite these changes in serum concentrations, there was no relationship of the TBI patient serum bFGF level to the growth of a fibroblast cell line (Wildburger, Zarkovic, Egger et al. 1994; Wildburger et al. 1995; Wildburger et al. 1996).

Although hypopituitarism is relatively common following CNS trauma, disorders of increased function of pituitary hormones are also documented and these may have greater relevance to bone formation. GH induces the release of IGF-1 from cells such as osteoblasts. IGF-I has been shown to stimulate proliferation and differentiation of fetal rat calvarial cells and rat bone marrow stromal cells, and the proliferation of human osteoblasts (Solheim 1998; Nakayama et al.
It also has a role in the recruitment and proliferation of mesenchymal stem cells (Solheim 1998). The involvement of GH and IGF-1 in the development of HO following brain injury has long been suspected because of the observation of high levels of GH in patients with brain lesions who develop HO compared to those that do not (Sazbon et al. 1983). Wildburger et al. (2001) measured increased levels of GH in all patients following TBI and/or fracture but only in those patients with TBI plus fracture did the GH level rise a second time after an initial period of normalisation. The timing of this second GH peak correlated with the initiation of clinically observable ossification. Only patients with TBI alone had increased IGF-1 levels for the entire fourteen week duration of the study. Conversely, the most recent investigation found that the levels of IGF-1 were significantly lower in those patients with TBI than a healthy control group (Beeton et al. 2002). Huang et al. (1995) also found that one third of SCI patients have reduced IGF-1 levels. This may be attributable to systemically decreased responsiveness to GH. However, it was argued that the effect of GH to stimulate IGF-1 upregulation locally at fracture sites might persist, even without systemic release of IGF-1 (Beeton et al. 2002).

2.5.6.3 Prostaglandins
Cyclo-oxygenase (COX) converts arachidonic acid released from membrane phospholipids into prostaglandins (Bondesen et al. 2004). The most important family of prostaglandin receptors in the development of HO is the EP family, which consists of EP1 to EP4 (Bartlett et al. 2006). Addition of an agonist to the EP4 receptor improves the osteoinductive effects of BMP-2 implanted into rats and increases the expression of BMP-2 in human mesenchymal stem cells (Arikawa et al. 2004; Toyoda et al. 2005). Prostaglandin E2 is the primary prostaglandin isoform synthesised by osteoblastic cells and has been shown to have an anabolic effect on bone in rats in vivo (Keila et al. 2001; Bartlett et al. 2006). A distinctly orchestrated cascade of prostaglandin expression has recently been discovered within the skeletal muscles of rabbits in an experimental model of HO (Bartlett et al. 2006). This cascade was inhibited by administration of a prostaglandin E2 (EP4) receptor (Bartlett et al. 2006). In humans, a 5-year prospective study of 44 patients with SCI found that the 8 patients who developed HO had significantly increased urinary excretion of prostaglandin E2 until the heterotopic bone was fully mature (Schurch et al. 2006).
The use of a non-steroidal anti-inflammatory drug in these patients,
thereby inhibiting COX activity and prostaglandin production, slowed the
development of HO, further supporting the theory that prostaglandins are
involved in its pathogenesis (Schurch et al. 1997).

2.5.7 Evidence of increased osteogenesis after CNS trauma

2.5.7.1 Clinical evidence

- Against increased heterotopic ossification following CNS trauma
The high rates of HO in patients with CNS trauma and various non-traumatic
CNS disorders suggest a common, central malfunction that predisposes to HO.
Few authors have questioned the veracity of the increased incidence of HO in
CNS trauma patients. However, a recent retrospective study has found that the
incidence of HO in patients with polytrauma and TBI was similar to that in a
matched polytrauma group without TBI (Pape et al. 2001). Although the age
and injury severity of the groups were statistically similar, the group without TBI
spent significantly more time under artificial ventilation. Based on this finding,
the authors hypothesised that artificial ventilation is a major risk factor for HO,
although they were not able to exclude the influence of other unidentified
factors. Consequently, it is difficult to conclude that both groups truly had similar
rates of HO.

- Against accelerated fracture healing following CNS trauma
Osteogenesis is a term that encompasses both physiological fracture healing
and pathological HO. Some authors feel that the distinction between heterotopic
and healing ossification should be emphasised as they may represent different
processes, albeit within the context of traumatic CNS injury (Klein et al. 1999;
Cope 1990). There is persisting debate around the quality of the callus formed
and the clinical scenarios in which fracture healing is accelerated (Morley et al.
2005). In CNS trauma patients, a distinction from acceleration of typical callus
formation can be made by the predominance of bone at sites peripheral to the
fracture site (Spencer 1987). The Garland group have concluded that the
excess bone seen in soft tissues around the fracture site is not consistent with
normal fracture healing, but is rather a misinterpreted version of HO (Garland et
al. 1980; Garland et al. 1982; Garland and Dowling 1983; Garland et al. 1989;
Kushwaha and Garland 1998). In addition, they claim that, with the data
available at the time, it could only be stated that a surgically fixated femoral fracture in a patient with TBI exhibits early union (Garland 1988; Garland 1992; Kushwaha and Garland 1998). Criticisms of these findings have been the reliance on clinical observations rather than statistical substantiation with the use of a control group (Bidner et al. 1992). Moreover, the relevance of older studies may be limited due to differences in the current methods of fracture management, especially the increased frequency of conservative management (Giannoudis et al. 2006).

A recent review (Morley et al. 2005) has addressed the specific question of whether or not there is conclusive evidence of accelerated fracture healing following CNS trauma. Six studies on the relationship of TBI to accelerated fracture healing from 1980 to 1987 were analysed. The conclusions drawn from the available data were that the new bone observed surrounding fracture sites in TBI patients could be a variant of HO and that there was a lack of evidence to support the assertion that this bone leads to accelerated fracture healing. However, the only two reviewed studies with control groups (Perkins and Skirving 1987; Spencer 1987) were in favour of accelerated fracture healing. Furthermore, later research by the authors of this review may have successfully answered their own question (Giannoudis et al. 2006; see below).

- In favour of enhanced osteogenesis in the form of accelerated fracture healing

In the same edition of the British Journal of Bone and Joint Surgery (Vol 69B, 1987), two articles appeared that would become crucial to reinvigorating the topic of head injury and accelerated fracture healing. Perkins and Skirving (1987), from Royal Perth Hospital, enrolled 22 patients with TBI who underwent intramedullary nailing (IMN) of a femoral fracture, and a control group of 22 patients with only femoral fractures also treated with IMN. The head injury was graded into mild, moderate or severe types, depending on the length of coma. There was a statistically significant increase in callus volume and decrease in fracture healing time in the TBI group, although there was no correlation with coma duration. Potential biases, such as differences in the timing of surgical management, type of fracture and method of surgical repair, were not statistically significant between groups. Spencer (1987) compared 30 age-,
gender- and fracture-matched control patients with 53 patients with severe TBI and fractures of the limbs, pelvis or spine. The criterion for severe TBI was a GCS score of ten or less within the first 48 hours of admission. As opposed to the study by Perkins & Skirving (1987), this study was focussed primarily on the effect of TBI on conservatively managed fractures. Of 82 fracture sustained by the patients with TBI, only 8 fractures were treated with internal fixation. Again, there was a statistically significant improvement in time to union in those with TBI, but no correlation with GCS on admission or limb spasticity. The acceleration of union occurred regardless of the treatment method, refuting the claim that only internally fixed femurs heal more quickly (Kushwaha and Garland 1998; Garland 1992; Garland 1988).

The most recent published contribution reinforced that TBI patients show accelerated fracture healing (Giannoudis et al. 2006). In this study, time to union was defined as circumferential callus in two planes and full painless weight bearing. There was a dramatic difference between TBI and non-TBI groups with regards to time to union of a diaphyseal femur fracture treated by an intramedullary nail. Patients in the TBI group had evidence of fracture healing in an average of 10.5 weeks, whereas the non-TBI group had a time to union of 22.9 weeks. TBI patients also had more exuberant callus formation, as measured using the same technique employed by Spencer (1987). There were no demographic differences described between the groups and consideration of the use of a reamed or unreamed nail technique did not affect the conclusion. The severity of TBI and the duration of artificial ventilation did not significantly affect the rate of union in the injured group.

With regards to the effect of SCI on the rate of fracture healing, there are only limited and dated animal models (Aro et al. 1981; Aro 1985; Aro et al. 1985; Miyamoto 1987). There is a distinct lack of clinical evidence regarding the rate of fracture healing in SCI patients in comparison to control groups (Table 7).
<table>
<thead>
<tr>
<th>Study</th>
<th>Patients</th>
<th>Accelerated union?</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Giannoudis et al. 2006)</td>
<td>67 patients: 17 TBI, 50 without TBI, all with femoral fracture</td>
<td>Yes</td>
<td>No difference with reamed or unreamed nails; larger callus in TBI group</td>
</tr>
<tr>
<td>(Perkins and Skirving 1987)</td>
<td>44 patients: 22 TBI plus femur fracture, 22 femur fracture alone, treated with IMN.</td>
<td>Yes</td>
<td>Larger callus in TBI group</td>
</tr>
<tr>
<td>(Spencer 1987)</td>
<td>83 patients: 53 TBI plus fracture, 30 fracture alone, treated conservatively.</td>
<td>Yes</td>
<td>Peripheral ossification in TBI group</td>
</tr>
<tr>
<td>(Garland and Dowling 1983)</td>
<td>47 patients: TBI plus forearm fracture, mostly ORIF</td>
<td>No</td>
<td>No control group</td>
</tr>
<tr>
<td>(Garland et al. 1982)</td>
<td>65 patients: TBI plus femoral fracture, mostly conservative treatment</td>
<td>No</td>
<td>No control group</td>
</tr>
<tr>
<td>(Garland et al. 1980)</td>
<td>41 patients: TBI plus tibia fracture, mostly conservative treatment</td>
<td>No</td>
<td>No control group</td>
</tr>
<tr>
<td>(Calandriello 1964)</td>
<td>13 patients: severe TBI plus fracture, treated conservatively</td>
<td>Yes</td>
<td>No control group</td>
</tr>
</tbody>
</table>

Table 7: Clinical studies investigating the rate of fracture healing following central nervous system trauma
(TBI = traumatic brain injury; SCI = spinal cord injury; HO = heterotopic ossification; GCS = Glasgow Coma Scale; IMN = intramedullary nail; ORIF = open reduction internal fixation; ↑ = increased)
2.5.7.2 Laboratory evidence

Five studies have analysed the effect of sera from patients with CNS injuries on cell proliferation and differentiation (Table 8). Only one of these tested human cells (Kurer et al. 1992) and only one other study examined the osteogenic effect of CSF (Klein et al. 1999).

- In favour of a serum-mediated or CSF-mediated osteogenic effect

Kurer et al. (1992) supported the existence of a serum-mediated osteogenic factor following SCI. Sera from 4 patients with SCI and HO and 4 patients with SCI but no HO were obtained between 4 and 7 months after injury. The groups were matched for age and gender to healthy controls, and the SCI groups had comparable lesion levels. Compared to control sera, serum from SCI patients induced a statistically significant increase in proliferation and ALP levels of cells extracted from human femurs. Furthermore, serum from SCI patients with HO had a significantly increased stimulatory effect compared to those SCI patients without evidence of HO, at all serum concentrations (1 to 20%).

Serum from rats with TBI has also been shown to induce a novel relationship to cell number, ALP and mineralisation capacity of rat bone marrow stromal cells (BMSCs) (Klein et al. 1999). Exposure to 2% serum taken from rats 48 hours after TBI resulted in a reduced number of BMSCs compared to normal rat serum. At the same time point, the ALP activity was at its maximal value, significantly higher than the normal values. The mineralisation potential of serum from TBI rats was 48% of a dexamethasone control, at best, and there was a direct correlation between mineralisation capacity before TBI and after TBI. Aside from describing the effects of serum from TBI rats on rat BMSCs, the only data regarding the osteogenic effects of CSF following TBI were presented in this study. CSF was collected from four rats seven days after TBI and pooled. Normal rat CSF was also collected and pooled for comparison. Rat BMSCs were exposed to pooled CSF from each group at a concentration of 2%. This resulted in a statistically similar decrease in cell number and higher ALP activity in both TBI and normal groups. The amount of mineralisation induced by these two groups was higher than that of a dexamethasone control, suggesting that CSF is intrinsically able to support osteogenesis.
Earlier, Bidner et al. (1990) proposed that serum from humans with TBI has a proliferative effect on fetal rat calvarial cells (FRCs). Thirty-two patients were divided into four equal groups consisting of patients with TBI alone, TBI plus fracture, fracture alone and healthy controls. The TBI patients all had coma duration of at least 3 days and the mean age and gender distribution of all groups were equivalent. Fetal rat skin fibroblasts were used as control cells for the FRCs. After five days incubation in 10% serum, sera from the TBI groups dose-dependently increased FRC proliferation significantly compared to the non-TBI groups. The same sera had no effect on the proliferation of the skin fibroblasts, indicating that the TBI serum might contain a proliferative factor specific to osteoblastic cells. However, FRC cells are heterogenous and it is not clear that the observed proliferation involved mature osteoblasts, as opposed to earlier osteoprogenitor cells or mesenchymal cells.

Recently, Boes et al. (2006) found that serum after TBI had a proliferative effect on mesenchymal stem cells, but not fibroblasts or committed osteoblastic cells. The effect of pooled serum from 23 rats with TBI and femur fracture was compared to pooled serum from 20 rats with femur fracture alone on the proliferation of three cell lines representative of mesenchymal stem cells, fibroblasts and committed osteoblastic cells. The serum from the TBI group (5% concentration) had a proliferative effect on the mesenchymal stem cell line only. An in vivo rat model of TBI and fracture established an association between this mesenchymal cell proliferation and the production of a stiffer callus. This suggests that humoral factors after TBI play a developmentally early role in HO and fracture healing through the expansion of mesenchymal progenitors.

- Against a serum-mediated osteogenic effect

One contradictory in vitro study showed that sera from humans with SCI and TBI did not increase the proliferation rate of one-day old rat calvarial cells (Renfree et al. 1994). Sera were obtained 48 hours and 3, 6 and 12 weeks after injury in 32 humans: 16 with SCI and 16 with TBI. No increase in the proliferation in osteoblastic cells treated with sera from the patients with CNS trauma was measured. In fact, the only significant difference between the CNS trauma and control groups was a decrease in osteoblast number at the time of the initial blood sample. Otherwise, the osteoblast numbers were stable in all
groups at all time points, with no significant differences. This osteoblast response was specific as fibroblasts exposed to the same sera showed no statistically significant difference between groups at any time point. A sub-analysis of the patients with HO also showed no difference in osteoblast number compared to those patients without HO. However, there was a statistically significant increase in thymidine incorporation in the HO group, indicating an increase in DNA synthesis.

<table>
<thead>
<tr>
<th>Study</th>
<th>Cells</th>
<th>Source of sera</th>
<th>Classification of trauma</th>
<th>Blood sample post-injury</th>
<th>Serum or CSF-mediated osteogenic effect of CNS trauma?</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Boes et al. 2006)</td>
<td>C3H10T1/2 MSCs; MC3T3-14 committed osteoblastic cells; NIH3T3 fibroblasts</td>
<td>43 rats: 23 TBI plus fracture, 20 fracture alone</td>
<td>Histological analysis of brain tissue; behaviour assessment scale</td>
<td>2 days and 3 weeks</td>
<td>Yes: increased proliferation of mesenchymal stem cells but not mature osteoblasts or fibroblasts</td>
</tr>
<tr>
<td>(Klein et al. 1999)</td>
<td>Rat BMSC</td>
<td>16 rats: 8 TBI model; 8 sham-operated controls</td>
<td>Neurological Severity Score 16-19/24 (severe TBI) one hour post-injury</td>
<td>1, 2, 7 and 14 days</td>
<td>Yes: intrinsic CSF osteogenic effect; reduced cell number but increased ALP activity due to serum 48 hours after TBI</td>
</tr>
<tr>
<td>(Renfree et al. 1994)</td>
<td>One-day old rat calvarial cells</td>
<td>43 humans: 16 SCI (13 with HO), 16 TBI (5 with HO), 11 controls</td>
<td>Complete SCI. TBI with GCS &lt;9.</td>
<td>48 hrs and 3, 6, 12 weeks</td>
<td>No: Decreased osteoblast number at time of injury in TBI and SCI groups. Increased thymidine incorporation with HO but same osteoblast number.</td>
</tr>
<tr>
<td>(Kurer et al. 1992)</td>
<td>hOB from THR</td>
<td>12 humans: 4 SCI, with and without HO; 4 controls</td>
<td>Complete lesions, matched levels</td>
<td>4-7 months</td>
<td>Yes: increased osteoblast proliferation and ALP levels at 1-20% serum concentrations (p&lt;0.01)</td>
</tr>
<tr>
<td>(Bidner et al. 1990)</td>
<td>FRC</td>
<td>32 humans: 8 TBI alone, TBI and fracture, fracture alone, controls</td>
<td>Coma of at least 3 days' duration</td>
<td>1-65 days</td>
<td>Yes: increased osteoblastic proliferation of TBI and TBI plus fracture groups at 10% serum concentrations (p&lt;0.001). No increased skin fibroblast proliferation.</td>
</tr>
</tbody>
</table>

Table 8: Studies investigating serum-mediated osteogenic effects following central nervous system trauma
(MSC = mesenchymal stem cells; BMSC = bone marrow stromal cells; FRC = fetal rat calvarial cells; SCI = spinal cord injury; HO = heterotopic ossification; ALP = alkaline phosphatase; TBI = traumatic brain injury; CSF = cerebrospinal fluid; GCS = Glasgow coma scale; hOB = human osteoblasts; THR = total hip replacement)
Overall, the available laboratory evidence favours the assertion that serum from CNS trauma patients increases the proliferation and ALP expression of mesenchymal and early osteoblastic cells. A link between these serum-mediated effects and improved fracture callus formation has also been demonstrated. The one study that looked into the osteogenic effect of CSF from rats with TBI concluded in favour of an osteogenic effect of this body fluid. However, none of the published studies analysed the effect of serum or CSF from patients with CNS trauma on human cells from skeletal muscle.

2.5.8 Summary
Within the orthopaedic community there is a widespread assumption that traumatic injury to the brain or spinal cord is associated with increased osteogenesis in the form of HO and accelerated fracture healing. It is accepted that CNS trauma results in high rates of HO, even compared to other CNS conditions. Although histologically the callus formed after CNS trauma is not consistent with typical callus, the latest clinical studies involving matched control groups confirm that patients with TBI have accelerated fracture healing. Similar clinical data for SCI is lacking. This phenomenon of excess bone formation, whether within soft tissue such as skeletal muscle, or at a fracture site, is reliant on activation of osteogenic cells and is yet to be fully explained. The majority of in vitro evidence supports the notion that serum from patients with CNS trauma has mitogenic and osteogenic properties. There is currently only one published article on the osteogenic effect of CSF after traumatic CNS injury and this, too, supports a CSF-mediated osteogenic effect on rat bone marrow stromal cells. As yet, no investigation has been performed into the effect of serum and CSF from CNS trauma patients on the usual location of heterotopic ossification: skeletal muscle.
Chapter 3: Hypotheses and Experimental Aims

This project differs from those that have preceded it because it investigated the role of adult human skeletal muscle in the phenomenon of HO following CNS trauma. This study proposed, for the first time, to expose adult human skeletal muscle cells to serum and CSF from patients following CNS trauma, and analyse subsequent proliferation and osteoblastic differentiation of the muscle cells.

The key question to be answered was:

**Can cells derived from adult human skeletal muscle form bone when exposed to blood and cerebrospinal fluid from patients with central nervous system trauma?**

Based on current literature and observation of pathophysiology, the hypothesis for this study was that skeletal muscle cells exposed to serum and CSF from patients with CNS trauma would proliferate and undergo osteoblastic differentiation. This theory was tested by the collection of skeletal muscle, blood and CSF from patients at Royal Perth Hospital, Western Australia, and subsequent laboratory investigation at the School of Anatomy and Human Biology of the University of Western Australia.

During the course of this study, the specifics aims to be satisfied were to:

1. Isolate, culture and characterise cells obtained from adult human skeletal muscle
2. Determine the proliferation rates of these skeletal muscle cells in response to serum and CSF from patients following trauma to the brain or spinal cord
3. Investigate the potential of the skeletal muscle cells to differentiate into the osteoblastic lineage and, ultimately, to form a mineralised matrix after exposure to serum and CSF from patients with CNS trauma
The fulfilment of these aims and confirmation of the central hypothesis would help to explain the phenomenon of HO following CNS trauma. Clinically, a better understanding of this condition may result in a reduction in morbidity and societal financial burden. In addition, the isolation of adult skeletal muscle cells that are capable of participating in bone formation may open novel frontiers of investigation in the field of tissue engineering. Finally, if this study serves as a stimulus for the isolation of an osteoinductive factor derived from patients with CNS trauma, a novel therapeutic agent that could be used in the treatment of a variety of skeletal conditions, such as recalcitrant fractures and osteoporosis, may be revealed in the future.
Chapter 4: Materials and Methods

To achieve the experimental objectives, numerous materials and laboratory techniques were employed in experiments involving cells isolated from adult human skeletal muscle. 

Firstly, specimens of skeletal muscle, blood and cerebrospinal fluid were collected from patients and processed to prepare them for experimentation. 

Secondly, cells were isolated from adult human skeletal muscle and characterised using light microscopic, immunohistochemical and reverse transcriptase polymerase chain reaction techniques.

Thirdly, the effect of serum and cerebrospinal fluid from patients with traumatic brain or spinal cord injury on the proliferation of the muscle cells was analysed by comparison to normal controls using a cell assay and a 5-bromo-2-deoxyuridine assay.

Finally, the effect of serum and cerebrospinal fluid from patients with traumatic brain or spinal cord injury on the osteogenic differentiation of the skeletal muscle cells was analysed by comparison to normal controls through Western blotting, alkaline phosphatase activity assay and Villanueva bone stain.
4.1 Patient selection

4.1.1 Skeletal muscle
Human skeletal muscle was obtained from patients undergoing orthopaedic trauma surgery at Royal Perth Hospital (R.P.H.).

4.1.1.1 Inclusion criteria
- Surgical procedure involving excision of skeletal muscle
- Age 18 to 80 years

4.1.1.2 Exclusion criteria
- Myopathic or connective tissue disorder

4.1.2 Sera and cerebrospinal fluid
Blood and cerebrospinal fluid (CSF) was obtained from patients with traumatic central nervous system (CNS) injury and control subjects.

4.1.2.1 Inclusion criteria
- Traumatic brain injury (admission score ≤ 9 on GCS); or
- Spinal cord injury (complete paraplegia or quadriplegia)
- Age 18 to 80 years

4.1.2.2 Exclusion criteria
- Open/penetrating head injury
- Significant fractures (involving long bones or pelvis)
- Previous head injury or metabolic bone disease

For the serum experiments, healthy control subjects were randomly selected healthy colleagues. For the CSF experiments, patients without traumatic brain or spinal cord injury formed the control group. Only traumatic brain injured (TBI) patients were included in the CSF experiments as CSF is not routinely obtained from patients with spinal cord injury (SCI). Diagnostic tests, such as computed tomography scans, were accessible for each patient.
4.1.2.3 Sample collection

- Serum
The CNS trauma patients were venesected at approximately 6 hours, 24 hours and 72 hours after the time of injury. Control subjects were venesected on one occasion. The potential influence of circadian hormonal changes was negated by obtaining specimens in the early morning when practical. After collection, whole blood was processed by the laboratory at R.P.H. to isolate the serum component, which was stored at -20°C until used in experiments.

- CSF
CSF was collected from patients who had undergone a lumbar puncture or who had an intracranial CSF drainage device in situ. After collection, the R.P.H. laboratory first processed the samples for diagnostic purposes, involving centrifugation to obtain a cell-free supernatant. These supernatants were stored at +4°C until used in experiments.

4.1.3 Ethics Approval
Ethics approval for the collection of human skeletal muscle, serum and CSF was obtained from the R.P.H. Ethics Committee (Appendix A).

4.2 Skeletal muscle cell culture

4.2.1 Cell isolation
Human skeletal muscle samples (approximately 1cm³ per patient) were excised intra-operatively under sterile conditions with scissors or a scalpel and placed into a sterile container with 0.9% saline water. At no time were muscle specimens from different patients pooled. Each patient was given a unique study number and each patient’s cell line was individually maintained. After transport to the laboratory, the saline was discarded and the muscle samples were finely minced with scissors then agitated in the presence of 0.05% trypsin–ethylenediamine-tetraacetic acid (trypsin-EDTA; from Gibco Invitrogen, NZ) for one hour. Approximately 10ml of trypsin-EDTA was used for every 1cm³ of tissue. The supernatant was then retrieved and centrifuged at 1200rpm for eight minutes using an Eppendorf 5810R centrifuge (Eppendorf, Germany). After centrifugation, the supernatant was discarded and the resultant cell pellet
was broken. The cells were then distributed into 25cm² culture flasks (Sarstedt, Germany) containing 5ml of Dulbecco’s modified essential medium/Ham’s F12 (DMEM/F-12; Gibco Invitrogen) supplemented with 15% fetal calf serum (FCS; from JRH Biosciences, USA) and 1% antibiotics (10,000 units/ml penicillin G sodium, 10,000µg/ml streptomycin sulfate, 25µg/ml amphotericin in 0.85% saline; from Gibco Invitrogen).

4.2.2 Maintenance of cell cultures

The cell cultures were incubated at 37°C in a humidified atmosphere containing 5% CO₂ in air. The medium was changed weekly. Cells were passaged by trypsinisation of the adherent cells and redistribution into new flasks in a 1:2 ratio when they approached confluence. At the time of passage, the flask supernatant was discarded and the cells washed with 5ml of phosphate buffered saline (PBS; Invitrogen). 3 ml of trypsin-EDTA was used to detach the cells from the culture flasks. Usually, an incubation period of 5-10 minutes at room temperature was sufficient for cell detachment, which was confirmed by observation under a light microscope. The suspended cells were pipetted into sterile 15ml tubes (Sarstedt) and centrifuged at 1200 rpm for 8 minutes. The supernatant was discarded, the cell pellet was broken and the cells resuspended in DMEM/F-12 supplemented with 15% FCS and 1% antibiotics. 5ml of cells plus medium were redistributed into new flasks. Characterisation of cell lines was performed within the first three passages and further experimentation occurred within three passages of the characterisation step to minimise the effects of passaging on cell phenotype and behaviour. Muscle cells from different patients were tested with sera and CSF from a number of different patients in multiple experiments.
4.3 Skeletal muscle cell characterisation

Visualisation of cells using light microscopy and immunohistochemical techniques, plus determination of cell mRNA content by way of reverse transcriptase polymerase chain reaction, were performed to characterise the skeletal muscle cell populations. In addition, Western blot analysis of protein content was indirectly used as a characterisation technique on the control cells during later differentiation experiments (Table 9).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Marker</th>
<th>Technique</th>
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<tbody>
<tr>
<td>Skeletal muscle</td>
<td>MyoD</td>
<td>RT-PCR</td>
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<tr>
<td></td>
<td>Desmin</td>
<td>IHC</td>
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<tr>
<td></td>
<td>Myosin heavy chain IIa</td>
<td>RT-PCR</td>
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<td></td>
<td>Myotube formation</td>
<td>Bright field, IHC</td>
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<tr>
<td>Osteoblastic</td>
<td>Alkaline phosphatase</td>
<td>IHC, RT-PCR, activity assay</td>
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<td></td>
<td>Cbfa1</td>
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<td>Osterix</td>
<td>RT-PCR, WB</td>
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<tr>
<td>Adipocytic</td>
<td>PPARγ</td>
<td>RT-PCR</td>
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<td>Stromal</td>
<td>Stro-1</td>
<td>IHC</td>
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<td></td>
<td>Vimentin</td>
<td>IHC</td>
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<td>Cytoskeletal</td>
<td>β-Actin</td>
<td>IHC, RT-PCR, WB</td>
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<tr>
<td>Nuclear</td>
<td>DAPI nuclear stain</td>
<td>IHC</td>
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<td></td>
<td>SYTO Green No. 25</td>
<td>IHC</td>
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<tr>
<td>Non-specific</td>
<td>BMP-R1A/1B/2</td>
<td>RT-PCR</td>
</tr>
</tbody>
</table>

Table 9: Techniques used for the characterisation of the skeletal muscle cell populations
(MyoD = myogenic determination factor; Cbfa = core binding factor alpha; ALP = alkaline phosphatase; DAPI = 4', 6-diamidino-2-phenylindol-dihydrochloride; PPAR = peroxisome proliferative activated receptor; BMPR = bone morphogenetic protein receptor; IHC = immunohistochemistry; RT-PCR = reverse transcriptase-polymerase chain reaction; WB = Western blot)

4.3.1 Light microscopy

Cells in flasks and plates were viewed under bright light conditions with a Nikon Eclipse TE300 inverted microscope. Images were captured on a Hitachi-HV-C20M camera using Metamorph v6.2r6 and Q Capture v2.81.0 software, and then saved digitally.
4.3.2 Immunohistochemistry

Characterisation of specific markers within the nucleus, cytoplasm and membrane of the skeletal muscle cells was conducted using immunohistochemical techniques.

4.3.2.1 Coverslip and cytospin preparation

Two techniques were used to prepare cells for staining and mounting on slides, namely coverslips and cytopsin. For the coverslips procedure, cells were trypsinised and centrifuged, and then the cell pellet was resuspended in DMEM with 12.5% FCS and 1% antibiotics. The suspended cells were then redistributed into 24 well plates (Sarstedt) and allowed to grow onto glass coverslips at the bottom of each well. One confluent 25cm² flask of cells was used for each 24 well plate. For the cytopsin technique, cells were maintained in standard medium in 25cm² culture flasks (Sarstedt).

4.3.2.2 Cell fixation and permeabilisation

Initially, cells were fixed in 1% paraformaldehyde in PBS (Invitrogen) for 10 minutes at room temperature. 3 ml of fixative was used for every flask of cells and 1ml for each well on a 24 well plate (Sarstedt). After fixation, the cells within flasks were detached using a cell scraper and resuspended in 1ml PBS. This was then centrifuged and the resulting cell pellet broken and suspended again in 1ml PBS for transfer to the cytopsin machine. The cytopsin was run at 600rpm for 4 minutes and the slides allowed to dry for thirty minutes. 0.1% Triton X-100 in PBS was then used as a cell membrane permeabilising agent. The cells were then exposed to 100µl of Triton per well or slide for one minute. The cells were then washed in PBS twice for five minutes each time.

4.3.2.3 Staining protocols

- Primary antibodies

Primary monoclonal antibodies for alkaline phosphatase (ALP), desmin, Stro-1 and vimentin were used. Table 10 lists the dilution and manufacturer of each antibody. 100µl volume of each was used for cells prepared by cytopsin and 200µl volume was used in each well in the coverslip method. Following incubation with the primary antibody for 4 hours, cells were washed three times in PBS for five minutes each time.
Table 10: Primary antibodies used during immunohistochemical characterisation of the skeletal muscle cells. Dilutions were performed in phosphate buffered saline as indicated. All incubations were performed at room temperature for 4 hours.

- **Secondary antibodies**
  A donkey anti-mouse Alexa Fluor 488 secondary antibody (Molecular Probes, USA) was added at a concentration of 1:50 in PBS after incubation with the primary antibody. Incubation was for one hour at room temperature. Following this, cells were washed three times in PBS for five minutes each time.

- **DAPI and Phalloidin**
  After staining with the secondary antibody, counterstaining with DAPI (4’, 6-diamidino-2-phenylindol-dihydrochloride) (Roche, Switzerland) and Alexa Fluor 546 Phalloidin (Molecular Probes) was performed to visualise the cell nucleus and β-actin filaments, respectively. DAPI was used in a dilution of 1:1000 and Phalloidin at 1:100 in PBS. Cells were incubated with these antibodies for thirty minutes at room temperature. 100µl of each was used for cells prepared by cytospin and 200µl in each well in the coverslip method. Following this, cells were washed three times in PBS for five minutes each time.

- **SYTO Green**
  SYTO Green number 25 (Molecular Probes) was used as a nuclear stain in living cells. A cell permeant was not used, as the cell membranes of living cells were already permissive to the passage of the dye. Cells were exposed to SYTO Green in PBS at a concentration of 1:1000 for one hour at room temperature. Following this, cells were washed three times in PBS for five minutes each time.
4.3.2.4 Mounting and sealing
Mounting was performed with DAKO fluorescent mounting medium (DAKO, USA). Nail polish was used to seal the coverslips and slide covers.

4.3.2.5 Microscopes
A Nikon Eklipse 90i microscope was used to visualise fluorescent stained cells. Images were captured by Nikon DXM1200F and Photometric CoolSnap ES cameras using Nikon ACT-1 v3.63 software. Digital Optics V++ v4.0 software was used for colour overlay and to save images digitally.

4.3.3 Reverse transcriptase polymerase chain reaction (RT-PCR)
4.3.3.1 RNA isolation
One flask of nearly confluent cultured skeletal muscle cells was used for each RT-PCR reaction to obtain adequate amounts of RNA. Under strict sterile conditions, the cells were scraped from the bottom of the culture flasks within the existing culture medium. The subsequent cell suspension was centrifuged at 1200rpm for 12 minutes using an Eppendorf 5815R centrifuge (Eppendorf) to obtain a cell pellet. This pellet was mixed with 1ml of PBS, transferred to a 1.5ml tube (Eppendorf, Germany) and centrifuged at maximal revolutions for 15 seconds using an Eppendorf 5815R centrifuge (Eppendorf). 800µl of Ultraspec-II RNA Isolation System (Biotecx Laboratories, Houston, U.S.A.) was vigorously shaken and then added to the resultant cell pellet in each Eppendorf tube. After vortexing, 200µl of chloroform was added and the mixture vortexed again and then centrifuged at maximal rpm for 15 minutes at +4°C. RNA could then be found within the aqueous phase of the resultant suspension. 450µl of this aqueous phase was removed and mixed with an equal amount of isopropanol. After another 15 minutes of maximal centrifugation at +4°C, the RNA pellet was subjected to two cycles of washing by ethanol. It was then left to dry overnight. The next morning, 40µl of nuclease-free water was added to the dried RNA pellet and 100µl of a 1:50 dilution of this solution was made using further nuclease-free water. The RNA concentration was estimated by the absorbance of the 1:50 solution at 260nm on a Beckman 640 spectrophotometer.
4.3.3.2 Reverse transcriptase reaction

Complimentary DNA (cDNA) was generated from the isolated RNA via the reverse transcriptase reaction. A 20µl reaction volume was constructed by combination of 4 µl MgCl₂, 2µl of reverse transcriptase buffer, 2µl deoxyribonucleotide triphosphate, 1 µl oligonucleotide and 0.3µl of avian myeloblastosis virus reverse transcriptase (all from Promega, USA) with variable amounts of RNA and nuclease-free water, depending on the RNA yield. This mixture was incubated at +42°C for 15 minutes to form cDNA.

4.3.3.3 Real time RT-PCR

Real time analysis of the RT-PCR was performed using the SYBR Green PCR master mix (Bio-Rad, USA) on a Corbett Rotorgene 3000 real time thermal cycler. PCR was performed over 40 amplification cycles, involving denaturation at +95°C for 90 seconds, annealing for +54°C for 40 seconds and extension for +72°C for 40 seconds. Rotorgene software v5.0 (Corbett Life Sciences, USA) was used to analyse the resultant fluorescence and melting curve peaks. The specific primers listed in Table 11 were used for the characterisation of the cultured cells. A positive control containing β-actin and a negative control lacking cDNA were also tested on all runs. Only those primer products with distinct melting curve peaks were subjected to post-PCR gel electrophoresis.

4.3.4 Post-PCR gel electrophoresis

Following the PCR reaction, the cDNA was loaded onto a 1.5% agarose gel containing 3 drops of ethidium bromide per 100ml of gel. 3µl of loading dye and 10µl of cDNA were mixed and inserted into each well. 3µl of a 100 base pair standard (Blue/Orange 6x Loading Dye, Promega) was included on all gels for identification of band size. PCR products were separated on the gel under 100V until the standard bands were distinct when observed under an ultraviolet light. Digital photographs of the gels were taken with a Ricoh RDC-7 3.3 megapixel digital camera and stored digitally.
### Table 11: Primers used for characterisation of the skeletal muscle cells using reverse transcriptase polymerase chain reaction. All primers were supplied by Geneworks, Australia. Expected primer product lengths were determined by analysis of the genetic sequence at the nucleotide resource of Pubmed.com.

(bp = base pairs; MyoD = myogenic determination factor; MyHC = myosin heavy chain; ALP = alkaline phosphatase; Cbfa = core binding factor alpha; Osx-L = Osterix-Long splice variant; Osx-S = Osterix-Short splice variant; BMPR = bone morphogenetic protein receptor; PPAR = peroxisome proliferative activated receptor)
4.4 Assessment of proliferation

4.4.1 Plate protocols

4.4.1.1 Serum

For serum proliferation experiments, one flask of cultured muscle cells was agitated in 2.5ml of trypsin-EDTA until the cells were no longer adherent. The supernatant was then centrifuged at 1200rpm for 8 minutes and the resultant cell pellet resuspended in DMEM/F-12 and 1% antibiotics at a concentration of 5-10,000 cells per ml (as determined using a manual cytometer). Muscle cells were cultured in 200µl of DMEM/F-12 and 1% antibiotics in each well of a 96-well plate. A 2:1 serum dilution series was constructed so that the serum concentrations sequentially decreased from 6.25% of the total well volume (Figure 12). Experiments were conducted in triplicate with three to six dilutions. On all plates, the proliferation rate of the serum-treated skeletal muscle cells was standardised against a quadruplicated negative control, comprising muscle cells in 200µl of medium alone. A quadruplicated positive control, comprising muscle cells in 200µl of medium supplemented with 12.5% FCS, was also included for comparison.

4.4.1.2 CSF

The CSF proliferation assay was constructed in a similar fashion to the serum proliferation assay except that CSF was substituted for serum in the 96-well plate (Figure 12). Like the serum proliferation plates, cells were exposed to CSF in a 1:2 dilutional series that ranged from a CSF concentration of 6.25% to 0.20%. Again, experiments were conducted in triplicate and controls were quadruplicated.
Figure 12: Organisation of a typical 96-well plate with six dilutions per sample for serum and CSF proliferation experiments. Each well contained skeletal muscle cells in 200µl DMEM/F-12 plus 1% antibiotics and varying concentrations of serum/CSF. Darker wells represent greater concentrations of serum/CSF (6.25% → 3.13% → 1.56% → 0.78% → 0.39% → 0.20% to 2 decimal places). On many plates, samples were tested in only three or four dilutions. Cells were derived from the same patient. Serum/CSF were from different patients. Experiments were performed in triplicate on each serum/CSF sample. Positive and negative controls were performed on each plate in quadruplicate.

4.4.2 CellTiter96 Aqueous One Solution Cell Proliferation Assay

To assess proliferation of the skeletal muscle cells in response to the different factors, the CellTiter96 Aqueous One Solution Cell Proliferation Assay (Promega Corporation, USA) was used. This assay employs a colorimetric [3-(4,5-dimethylthiazol-s-yl)-5-(3-carboxymethoxyphenol)-2-(4-sulfonphenyl)-2H-tetrazolium (MTS) test to measure mitochondrial activity, an indicator of cell number, through measurement of absorbance. Cells in the 96-well plate were exposed to the experimental conditions for 5 days, and then 20µl of the MTS reagent was added to each well and the plates placed back into the +37°C incubator. Twenty hours after addition of the reagent, the absorbance of each well on the 96-well plate was measured through the 492nm filter on the Multiskan plate reader (Labsystems, Finland). Proliferation rates were standardised to the negative plate control to account for variations between plates brought about by slight differences in aspects such as incubation period and initial cell number.
4.4.2.1 Determination of maximal proliferative effect
The FCS concentration used for the positive control (12.5%) was determined by the maximal proliferative effect of FCS in a 2:1 dilution series (involving 12 wells, starting at a concentration of 50%) in one muscle sample. Cells were added to each concentration of FCS in a suspension of 10,000 cells/ml of standard medium. The resultant proliferation was measured using the CellTiter96 Aqueous One Solution Cell Proliferation MTS Assay at 492nm on the Multiskan reader after 5 days.

4.4.3 5-bromo-2-deoxyuridine (BrdU) assay and stain
The accuracy of the proliferation measured by the MTS Assay was confirmed by testing with 5-bromo-2-deoxyuridine (BrdU), a thymidine analogue that can be bound by a secondary antibody to reveal cells in which DNA synthesis has occurred.

4.4.3.1 BrdU assay
The proliferation of the skeletal muscle cells was measured using a BrdU proliferation assay (Roche Diagnostics, Germany). The manufacturer’s protocol was used as a guideline. The initial steps of the BrdU assay replicated those of the MTS assay. Cells were suspended in DMEM/F12 supplemented with 1% antibiotics and patient serum, and allowed to attach to a 96 well plate for two days (as described previously). Quadruplicated positive and negative controls were also included on the plate. 20\(\mu\)l of 1:1000 BrdU in PBS was then added to each well and the cells were allowed to proliferate for another 3 days. At this time, the wells were tapped dry and left overnight. 100\(\mu\)l of the FixDenat solution from the Roche kit was then placed into each well and the plate incubated at room temperature for 30 minutes. After removal of the FixDenat solution by tapping, 100\(\mu\)l/well of the anti-BrdU peroxidase (POD) working solution was added. This was removed after 90 minutes and the wells washed with 200\(\mu\)l/well of the kit Washing solution. Finally, the cells were incubated with 100\(\mu\)l/well of the kit Substrate solution for two hours. The plate was viewed under the 405nm filter of the Multiskan reader.
4.4.3.2 BrdU stain

For this method of identifying cells that have undergone proliferation, cells were cultured in flasks and allowed to grow in DMEM/F12 plus 1% antibiotics and 15% FCS until they were approximately 70% confluent. At this point, the medium was changed to a serum-free medium. After a further 24 hours, 5% patient serum and 10mM BrdU were added to the wells and cells were allowed to proliferate for 5 days at +37°C and 5% CO₂ in a humidified environment. Several techniques were employed in an attempt to gain a positive intra-nuclear BrdU stain. Initially, cells were fixed with 3ml of 1% PFA in PBS, then the cytospin procedure was used to distribute the cells onto slides for the staining steps. 100µl of Fixation buffer (Roche Diagnostics) was placed on the cells for 10 minutes to fix-permeabilise them before incubation with a biotin labelled mouse anti-BrdU-antibody (Molecular Probes) (3µl in 60µl PBS per slide) for 4 hours. The slides were then washed twice in PBS and incubated with Streptavidine-Alexa Fluor 488 (Molecular Probes) (1µl in 50µl PBS per slide) for 1 hour, followed by further washing in PBS. Because of the lack of success at visualisation of a positive BrdU stain using this technique, other techniques were trialled including different incubation periods and concentration of the Roche fixation buffer, use of the anti-POD secondary antibody from the Roche kit, assembling antibodies in the Roche antibody dilution solution and trypsinisation of cells without fixation to obtain suspended cells for cytospin.

4.4.4 Muscle validation

To assess the effect of different patient sources of skeletal muscle cells on the proliferation results, a validation study was conducted. This involved an assessment of the variance of the proliferation rates of three muscle samples after exposure to the same ten sera samples as measured by the MTS assay. The three muscle specimens were selected to represent a cross-section of the patients who contributed skeletal muscle. The serum samples were selected based on the injury to the patient, such that the TBI and SCI patients and the normal control subjects were represented.
4.5 Assessment of differentiation

4.5.1 Culture flask protocols

4.5.1.1 Serum

Four flasks of cultured skeletal muscle cells were required for each in vitro serum differentiation experiment (Figure 13). At approximately 70% confluence, the standard culture medium was replaced with 3ml of serum-free DMEM/F12 supplemented with 1% antibiotics. After 24 hours, the cultured cells were exposed to 5% serum from TBI patients and incubated at +37°C and 5% CO₂. Sera from TBI patients with high and low proliferation rates as shown by the proliferation assays were compared to assess the relationship between proliferation and differentiation. The cells were cultured under these conditions for one week before assessment of protein content by Western blotting. Differentiation was compared to that of the same cells cultured in sera from control patients and serum-free medium alone. The experiments were repeated in triplicate.

![Figure 13: Culture flask protocol for serum differentiation experiments. Four flasks were used to allow comparison of the differentiation effect of sera from TBI patients (high and low proliferating serum) with sera from SCI patients, normal control subjects and a serum-free medium.](image)

4.5.1.2 CSF

Three flasks of cultured skeletal muscle cells were required for each CSF differentiation experiment (Figure 14). At approximately 70% confluence, the standard medium was replaced with 3 ml of serum-free DMEM/F12 supplemented with 1% antibiotics. After 24 hours, the cultured cells were
exposed to 5% CSF from TBI patients and incubated at +37°C and 5% CO₂ for one week before Western blotting. Differentiation was compared to that of the same cells cultured in CSF from control patients and CSF-free medium alone. The experiments were repeated in triplicate.

![Culture flask protocol for CSF differentiation experiments.](image)

**Figure 14**: Culture flask protocol for CSF differentiation experiments. Three flasks were used to allow comparison of the differentiation effect of CSF from TBI patients with CSF from uninjured patients and a CSF-free medium.

### 4.5.2 Western blot

The Western blot technique was used to assess the maturation of putative osteoprogenitor cells within skeletal muscle by their expression of the Osterix protein. β-actin was used as an internal positive control and human fetal osteoblasts (hFOB 1.19, from the American Type Culture Collection, USA) were used to ensure viability of the Osterix antibody.

#### 4.5.2.1 Extraction of whole cell lysate

After incubation of the skeletal muscle cells in the different experimental conditions for one week, the supernatant was removed and the cells were rinsed in 1ml of PBS. The cells were then transferred to a 1.5ml Eppendorf tube, spun at maximum speed in a +4°C centrifuge for three minutes and then placed on ice. The resultant supernatant was removed and 200µl of cold whole cell lysate buffer (from a solution of 12.5 ml 20% sucrose, 1.25 ml 1M Tris-HCl, 2.5 ml SDS, 1.25 ml 2-mercaptoethanol, 7.5ml deionized water) was added to each tube. A 23-gauge needle and syringe were then used to evenly disperse the cell lysate, which was stored at -80°C until used in experiments.
4.5.2.2 Gel electrophoresis

Gel electrophoresis was employed to separate the cell population proteins into bands, based on their molecular weight, which could be identified by specific antibodies. A routine Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) protocol was used for this process. The proteins of interest had expected molecular weights of 43kDa (β-actin) and 45kDa (Osterix), respectively, so a 12% agarose stacking gel was used for all separations. The components of two separating gels were 2.5ml 1.5M Tris HCL (pH 8.8), 100µl 10% SDS, 10µl Tetramethyl-ethylenediamine (TEMED), 100µl 10% ammonium persulphate (APS), 4ml 30% acrylamide and 3.35ml ddH₂O. The gel was poured into the blot apparatus and allowed to set. The stacking gel consisted of 6.1ml of ddH₂O, 2.5ml of 0.5M Tris-HCl (pH = 6.8), 100µl of 10% SDS, 1.3ml of 30% Acrylamide, 10µl TEMED and 50µl of 10% APS. This gel was poured onto the set separating gel and lane combs inserted. Once the stacking gel had set, the apparatus was placed into an electrophoresis tank and the inner chamber was filled with electrode buffer. Bromophenol blue was used as a loading dye to assess migration. For each lane, 25µl of protein was combined with 2µl of bromophenol blue and heated at +95°C for five minutes. Because of condensation in the lid of the Eppendorf tubes after heating, the tubes were centrifuged briefly. The samples were then loaded into the wells of the separating gel with a metal syringe. The SeeBlue Plus2 protein standard (Gibco Invitrogen) was loaded into one lane as a size marker and to assess progress of the run. Initially, the gel was run at 100V until the proteins had migrated through the stacking gel. At this point, the voltage was increased to 130V. The electrophoresis was stopped when the protein size of interest was likely to be present between two well-separated bands of the protein standard.

4.5.2.3 Transfer From Gel To Membrane

After electrophoresis, the proteins in the separating gel were transferred to a Hybond-C Super Nitrocellulose Membrane (Amersham, Uppsala, Sweden) for immunoblotting. A cassette was constructed consisting of the separating gel and a membrane, sandwiched between blotting paper and fibrous pads that facilitate protein transfer. The cassette was placed in a chamber filled with transfer buffer and the transfer induced by 100V for 75 minutes.
4.5.2.4 Immunoblot: fluorescence and chemiluminescence

After transfer of the proteins from the agarose gel to the nitrocellulose membrane, immune staining of the membranes was performed to determine the presence of Osterix and β-actin proteins. The membrane was released from the transfer cassette and washed for ten minutes three times in 0.5% Tris-Buffered Saline Tween-20 (TBS-T) on a rocker at room temperature. Ponceau S confirmed the success of the transfer of the proteins to the membrane. Ponceau S was washed off with 0.5% TBS-T. Next, excess antigens were blocked by incubation with 5% milk in 0.5% TBS-T for 30 minutes at +37°C. Then, the membrane was incubated with 1ml of 1:500 primary antibody within a plastic envelope overnight at +4°C on a rocker. The next morning, the membrane was freed from the plastic envelope and washed three times in 0.5% TBS-T for ten minutes each time.

- Fluorescent antibodies

For the fluorescent immunoblot method, 10ml of 1:1000 secondary antibody was used on the membrane for 1 hour at room temperature. For the Osterix biotinylated secondary antibody, a tertiary antibody was used (10ml 1:1000; Streptavidine-Alexa Fluor 488, Molecular Probes) for thirty minutes at room temperature and then the membrane was washed again (Table 12).

<table>
<thead>
<tr>
<th>Marker</th>
<th>1º antibody</th>
<th>2º antibody</th>
<th>3º antibody</th>
<th>Expected weight (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osterix</td>
<td>Goat polyclonal anti-osterix (Santa Cruz, USA)</td>
<td>Biotinylated donkey anti-goat</td>
<td>Streptavidine-Alexa Fluor 488</td>
<td>45</td>
</tr>
<tr>
<td>β-actin</td>
<td>Mouse monoclonal anti-actin (Sigma)</td>
<td>Donkey anti-mouse Alexa Fluor 488</td>
<td>-</td>
<td>43</td>
</tr>
</tbody>
</table>

Table 12: Antibodies used during Immunoblot of the skeletal muscle cells using the fluorescent technique. All primary antibodies were incubated overnight at +4C. Secondary antibodies (all from Molecular Probes) were incubated for one hour at room temperature. For Osterix, Streptavidine-Alexa Fluor 488 (Molecular Probes) was used for thirty minutes at room temperature. Expected molecular weights were obtained from the protein database at www.expasy.org/sprot/.
Enhanced Chemiluminescence

In addition to fluorescent labeling, membranes were tested using the Enhanced Chemiluminescent (ECL) technique. For this method, the membranes were incubated with a horseradish peroxidase conjugated goat anti-rabbit IgG (1:10,000; DAKO, Denmark) for one hour at room temperature after the primary antibody (β-actin) or secondary antibody (osterix). Luminol/Enhancer solution and Stable Peroxide solution from the SuperSignal West Pico ECL substrate kit (Pierce, USA) was added to the membrane in equal quantities for five minutes. For both techniques, the membranes were viewed using the Kodak 2000MM Imaging System (Kodak, Australasia) and the images were stored digitally.

4.5.3 Villanueva osteochrome bone stain

Assessment of the ability of the skeletal muscle cells to generate a mineralised matrix after three weeks in the different experimental conditions and a mineralisation medium was performed using the Villanueva Osteochrome Bone Stain (Polysciences Inc, Germany).

4.5.3.1 Mineralisation medium

A supplemented medium was used to promote mineralisation. This consisted of:

- DMEM/F12 supplemented with 1% antibiotics
- 2mM β-glycerophosphate
- 12.5 µg/ml ascorbic acid

Before finalising these conditions, varying concentrations of β-glycerophosphate and ascorbic acid were trialled by culture of cells in 6-well plates with a range of different supplement concentrations, followed by bright field microscopic assessment of cell viability.

4.5.3.2 Plate protocols

- Serum

Cultured cells were trypsinised and resuspended in DMEM/F12 supplemented with 15% FCS and 1% antibiotics. They were then redistributed into a 6-well plate and allowed to grow to confluence. The supernatant was then removed and replaced with FCS-free DMEM/F12. After 24 hours, 3ml of the mineralisation medium (as described above) and 5% (150 µl) patient serum was
added to each well. The serum was derived from TBI serum samples with high or low proliferative activity (based on the proliferation experiments), a SCI patient and a normal control subject. Positive and negative controls were used on each plate for comparison (Figure 15). The medium was changed every 5 days and the supernatant collected for measurement of ALP at the time of change of medium. After three weeks, the Villanueva osteochrome stain was performed on the cells in situ. The experiments were performed in triplicate.

Figure 15: Organisation of 6-well plate for Villanueva stain of muscle cells cultured in the presence of 5% serum and a mineralisation medium (n=3)

- CSF
Similar to the serum differentiation 6-well experiments, cultured cells were first trypsinised and resuspended in DMEM/F12 supplemented with 15% FCS and 1% antibiotics. They were then redistributed into a 6-well plate and allowed to grow to confluence. The supernatant was then removed and replaced with serum-free medium. After 24 hours, 3ml of the mineralisation medium and 5% (150 µl) patient CSF was added to each well. The CSF was derived from 2 TBI patients and 2 uninjured subjects. Positive and negative controls were used on each plate for comparison (Figure 16). The medium was changed every 5 days and the supernatant collected for measurement of ALP at the time of change of medium. After three weeks, the Villanueva osteochrome stain was performed on the cells in situ. The experiments were performed in triplicate.
Figure 16: Organisation of 6-well plate for Villanueva stain of muscle cells cultured in the presence of 5% CSF and a mineralisation medium (n=3)

4.5.3.3 Villanueva osteochrome bone stain protocol
After three weeks culture, the cells were washed once in 2ml PBS (per well). Then 2ml of 70% ethanol was added to each well for 15 minutes, followed by three washes with 2ml of distilled water. 1ml of Villanueva Bone Stain was then added per well and left for 90 minutes, followed by a further three washes with distilled water. The final washing step involved multiple washes with 2ml of 70% ethanol until the resultant wash solution was colourless. Finally, 1ml of 1% PFA was added to the flask to fix the stained cells. The cells were then viewed using a Nikon Eklipse TE300 microscope under bright field conditions and the positively stained nodules were counted.

4.5.4 Alkaline phosphatase assay
The supernatant from the 6-well plates involved in the differentiation experiments was collected at the time of medium change. The supernatants were stored at -20°C until they were processed. At the R.P.H. laboratory, the supernatants were analysed with the Roche/Hitachi 917 analyser system, which employs a spectrophotometer to determine ALP activity through the production of p-nitrophenol.
4.6 Statistical analysis

SPSS for Windows Version 14.0 (SPSS Inc, USA) and Microsoft Excel (Microsoft, USA) were used to perform statistical analyses and to produce graphics. The independent sample t-test was used to compare two group means and one-way analysis of variance (ANOVA) was used to compare multiple group means simultaneously. The Bonferroni post hoc test was used to identify specific differences between multiple values. The chi-square test was used to assess differences in between categorical variables. Initially, the data were examined to ensure the test assumptions were met, including multivariate normality, and transformations were performed on dependent variables accordingly. In both proliferation and differentiation experiments, 95% confidence intervals were calculated to compare the study group means to the negative control (value = 1). A significant difference between values was defined as a $p$ value of less than 0.05 or a confidence interval that did not include the control value.
Chapter 5: Results and Achievements

The results obtained from the experiments involving adult human skeletal muscle cells, serum and cerebrospinal fluid are described in this chapter. Achievements are outlined at the end of the chapter.
5.1 Patient selection

5.1.1 Skeletal muscle

One specimen of skeletal muscle (approximately 1cm$^3$) was obtained from each of 38 patients who underwent orthopaedic trauma operations at Royal Perth Hospital. The mean age of the patients was 37.6 years [range = 18 to 80, standard deviation (SD) = 19.3]. 78.9% were male. The average age of males was 35.8 years (SD = 18) and the average age of females was 45.8 years (SD = 24.6). There was no significant difference in the average age of males compared to the average age of females ($p = 0.211$) (Figure 17). Muscle was obtained from muscle groups in the lower limbs (22 samples) and the upper limbs (15 samples). A sample of rectus abdominis was excised from one patient. Table 13 summarises the age, gender and muscle group of each patient who contributed skeletal muscle.

![Figure 17: Age and gender distribution of patients who donated skeletal muscle. The demographics of this population are consistent with the typical demographic of orthopaedic trauma patients. Most patients were males under 50 years of age. A greater proportion of the population were female as patient age increased, corresponding with an increased incidence of osteoporosis and risk of fracture following falls in this subpopulation.](image)
<table>
<thead>
<tr>
<th>Sample</th>
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<th>Gender</th>
<th>Muscle group</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>60</td>
<td>M</td>
<td>Vastus lateralis</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>M</td>
<td>Quadriceps femoris</td>
</tr>
<tr>
<td>3</td>
<td>69</td>
<td>M</td>
<td>Triceps brachii</td>
</tr>
<tr>
<td>4</td>
<td>80</td>
<td>M</td>
<td>Gluteus maximus</td>
</tr>
<tr>
<td>5</td>
<td>35</td>
<td>M</td>
<td>Vastus intermedius</td>
</tr>
<tr>
<td>6</td>
<td>18</td>
<td>M</td>
<td>Vastus lateralis</td>
</tr>
<tr>
<td>7</td>
<td>48</td>
<td>M</td>
<td>Tibialis anterior</td>
</tr>
<tr>
<td>8</td>
<td>25</td>
<td>M</td>
<td>Gastrocnemius</td>
</tr>
<tr>
<td>9</td>
<td>37</td>
<td>M</td>
<td>Pronator quadratus</td>
</tr>
<tr>
<td>10</td>
<td>78</td>
<td>F</td>
<td>Gluteus medius</td>
</tr>
<tr>
<td>11</td>
<td>19</td>
<td>M</td>
<td>Vastus lateralis</td>
</tr>
<tr>
<td>12</td>
<td>30</td>
<td>M</td>
<td>Biceps femoris</td>
</tr>
<tr>
<td>13</td>
<td>39</td>
<td>M</td>
<td>Vastus intermedius</td>
</tr>
<tr>
<td>14</td>
<td>28</td>
<td>M</td>
<td>Vastus lateralis</td>
</tr>
<tr>
<td>15</td>
<td>65</td>
<td>F</td>
<td>Soleus</td>
</tr>
<tr>
<td>16</td>
<td>19</td>
<td>M</td>
<td>Triceps brachii</td>
</tr>
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<td>17</td>
<td>21</td>
<td>M</td>
<td>Pronator quadratus</td>
</tr>
<tr>
<td>18</td>
<td>45</td>
<td>M</td>
<td>Triceps brachii</td>
</tr>
<tr>
<td>19</td>
<td>42</td>
<td>M</td>
<td>Tibialis anterior</td>
</tr>
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<td>25</td>
<td>M</td>
<td>Tibialis anterior</td>
</tr>
<tr>
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<td>46</td>
<td>M</td>
<td>Pronator quadratus</td>
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<td>27</td>
<td>M</td>
<td>Triceps brachii</td>
</tr>
<tr>
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<td>25</td>
<td>F</td>
<td>Triceps brachii</td>
</tr>
<tr>
<td>24</td>
<td>20</td>
<td>F</td>
<td>Quadriceps femoris</td>
</tr>
<tr>
<td>25</td>
<td>79</td>
<td>M</td>
<td>Gluteus medius</td>
</tr>
<tr>
<td>26</td>
<td>59</td>
<td>M</td>
<td>Gastrocnemius</td>
</tr>
<tr>
<td>27</td>
<td>23</td>
<td>M</td>
<td>Tibialis anterior</td>
</tr>
<tr>
<td>28</td>
<td>18</td>
<td>M</td>
<td>Pronator quadratus</td>
</tr>
<tr>
<td>29</td>
<td>23</td>
<td>M</td>
<td>Tibialis anterior</td>
</tr>
<tr>
<td>30</td>
<td>21</td>
<td>M</td>
<td>Pronator quadratus</td>
</tr>
<tr>
<td>31</td>
<td>19</td>
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<td>Pronator quadratus</td>
</tr>
<tr>
<td>32</td>
<td>24</td>
<td>M</td>
<td>Tibialis anterior</td>
</tr>
<tr>
<td>33</td>
<td>37</td>
<td>F</td>
<td>Pronator quadratus</td>
</tr>
<tr>
<td>34</td>
<td>19</td>
<td>F</td>
<td>Triceps brachii</td>
</tr>
<tr>
<td>35</td>
<td>59</td>
<td>F</td>
<td>Triceps brachii</td>
</tr>
<tr>
<td>36</td>
<td>59</td>
<td>F</td>
<td>Soleus</td>
</tr>
<tr>
<td>37</td>
<td>34</td>
<td>M</td>
<td>Rectus abdominis</td>
</tr>
<tr>
<td>38</td>
<td>34</td>
<td>M</td>
<td>Pronator quadratus</td>
</tr>
</tbody>
</table>

Table 13: Summary of the age, gender and muscle group of each patient who contributed skeletal muscle
5.1.2 Serum and cerebrospinal fluid (CSF)

5.1.2.1 Serum

Serum was collected from a total of 27 study subjects, who were divided into three groups based on the presence of traumatic brain injury (TBI) or traumatic spinal cord injury (SCI). The injured groups consisted of a total of 17 patients, of which 11 had TBI, defined as a Glasgow Coma Scale (GCS) on admission of less than nine. 6 patients had SCI, defined as a complete lesion resulting in quadriplegia or paraplegia. No patient had combined TBI and SCI. The normal control group was comprised of 10 healthy blood donors. The mean ages of the groups were 35.4 years for the TBI group (range = 18-76, SD = 16.5), 29.5 years for the SCI group (range = 18-55, SD = 13.1) and 27.6 years for the control group (range = 21-44, SD = 6.5) (Figure 18). 91%, 83.3% and 80% of the TBI, SCI and control group were male, respectively. There was no statistically significant difference in the mean age ($p = 0.37$) or gender ($p = 0.77$) of each group. Table 14 summarises the age, gender and injury of each person who contributed serum.

![Figure 18: Age distribution of patients who donated serum. There was no significant difference in the mean age of each group. Like the skeletal muscle donors, serum donors in the TBI and SCI groups were predominantly young males. The normal controls were healthy colleagues selected to have similar age and gender distributions to the injured groups.](image-url)
<table>
<thead>
<tr>
<th>Donor</th>
<th>Age (years)</th>
<th>Gender</th>
<th>Injury</th>
<th>Donor</th>
<th>Age (years)</th>
<th>Gender</th>
<th>Injury</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBI</td>
<td></td>
<td></td>
<td></td>
<td>SCI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>43</td>
<td>M</td>
<td>Traumatic SDH</td>
<td>1</td>
<td>55</td>
<td>M</td>
<td>T6 burst fracture (paraplegia)</td>
</tr>
<tr>
<td>2</td>
<td>76</td>
<td>M</td>
<td>Traumatic EDH</td>
<td>2</td>
<td>30</td>
<td>M</td>
<td>T8 burst fracture (paraplegia)</td>
</tr>
<tr>
<td>3</td>
<td>34</td>
<td>M</td>
<td>Traumatic intraparenchymal haemorrhage</td>
<td>3</td>
<td>24</td>
<td>M</td>
<td>L1 burst fracture (paraplegia)</td>
</tr>
<tr>
<td>4</td>
<td>34</td>
<td>M</td>
<td>Traumatic SAH</td>
<td>4</td>
<td>18</td>
<td>F</td>
<td>C5/6 dislocation (quadriplegia)</td>
</tr>
<tr>
<td>5</td>
<td>18</td>
<td>F</td>
<td>Multiple contusions</td>
<td>5</td>
<td>25</td>
<td>M</td>
<td>C6/7 subluxation (quadriplegia)</td>
</tr>
<tr>
<td>6</td>
<td>21</td>
<td>M</td>
<td>Traumatic EDH</td>
<td>6</td>
<td>25</td>
<td>M</td>
<td>T10/11 displacement (paraplegia)</td>
</tr>
<tr>
<td>7</td>
<td>48</td>
<td>M</td>
<td>Traumatic SDH and EDH</td>
<td>7</td>
<td>21</td>
<td>M</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>18</td>
<td>M</td>
<td>Traumatic intraparenchymal haemorrhage</td>
<td>8</td>
<td>44</td>
<td>M</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>31</td>
<td>M</td>
<td>Traumatic SDH</td>
<td>9</td>
<td>31</td>
<td>M</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>34</td>
<td>M</td>
<td>Multiple contusions</td>
<td>10</td>
<td>29</td>
<td>M</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>32</td>
<td>M</td>
<td>Traumatic EDH</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Healthy controls

| 1     | 26          | F      |        |
| 2     | 26          | F      |        |
| 3     | 23          | M      |        |
| 4     | 23          | M      |        |
| 5     | 26          | M      |        |
| 6     | 27          | M      |        |
| 7     | 21          | M      |        |
| 8     | 44          | M      |        |
| 9     | 31          | M      |        |
| 10    | 29          | M      |        |

Table 14: Summary of the age, gender and injury of the serum donors. (TBI = traumatic brain injury; SCI = spinal cord injury; SDH = subdural haemorrhage; SAH = subarachnoid haemorrhage; EDH = extradural haemorrhage; C, T and L refer to cervical, thoracic and lumbar vertebrae, respectively)
5.1.2.2 CSF

Cerebrospinal fluid (CSF) was obtained from 34 patients, who were divided into two groups based on the presence of TBI. The injured group consisted of 6 patients with a GCS of less than nine on admission. The control group was comprised of 28 patients without identified traumatic CNS or bone pathology. The mean ages of the groups were 33.2 years for the TBI group (range = 18-47, SD = 10.4) and 44.5 years for the control group (range = 18-76, SD = 15.7). There was a tendency for patients in the uninjured group to be older, but this did not reach statistical significance ($p = 0.051$) (Figure 19). The TBI group consisted solely of male patients while 57.1% of the control group were male. There was no statistically significant difference between the mean age of the TBI group and the males within the non-TBI group ($p = 0.054$). Table 15 summarises the age, gender and injury of each patient who contributed CSF.

![Figure 19: Age distribution of patients who donated CSF. All donors with TBI were males under 50 years of age, while females were better represented in the non-injured group. The patients in the non-injured group also tended to be older, although this did not reach statistical significance.](image-url)
<table>
<thead>
<tr>
<th>Donor</th>
<th>Age (years)</th>
<th>Gender</th>
<th>Injury</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>37</td>
<td>M</td>
<td>Bifrontal contusions</td>
</tr>
<tr>
<td>2</td>
<td>47</td>
<td>M</td>
<td>Traumatic SDH</td>
</tr>
<tr>
<td>3</td>
<td>33</td>
<td>M</td>
<td>Traumatic SAH with multiple contusions</td>
</tr>
<tr>
<td>4</td>
<td>39</td>
<td>M</td>
<td>Traumatic SDH and EDH</td>
</tr>
<tr>
<td>5</td>
<td>18</td>
<td>M</td>
<td>Traumatic SAH</td>
</tr>
<tr>
<td>6</td>
<td>25</td>
<td>M</td>
<td>Traumatic SDH and SAH</td>
</tr>
<tr>
<td>Non-TBI controls</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>48</td>
<td>F</td>
<td>CIDP</td>
</tr>
<tr>
<td>2</td>
<td>53</td>
<td>F</td>
<td>SAH</td>
</tr>
<tr>
<td>3</td>
<td>62</td>
<td>F</td>
<td>Intracerebral bleed</td>
</tr>
<tr>
<td>4</td>
<td>45</td>
<td>F</td>
<td>SAH</td>
</tr>
<tr>
<td>5</td>
<td>41</td>
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<td>Intracerebral bleed</td>
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<td>6</td>
<td>47</td>
<td>M</td>
<td>SAH</td>
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<td>31</td>
<td>F</td>
<td>Brain tumour</td>
</tr>
<tr>
<td>8</td>
<td>30</td>
<td>M</td>
<td>No pathology detected</td>
</tr>
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<td>9</td>
<td>49</td>
<td>M</td>
<td>Ischemic CVA</td>
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<tr>
<td>10</td>
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<td>No pathology detected</td>
</tr>
<tr>
<td>11</td>
<td>21</td>
<td>M</td>
<td>No pathology detected</td>
</tr>
<tr>
<td>12</td>
<td>47</td>
<td>M</td>
<td>No pathology detected</td>
</tr>
<tr>
<td>13</td>
<td>58</td>
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<td>Brain tumour</td>
</tr>
<tr>
<td>14</td>
<td>24</td>
<td>F</td>
<td>No pathology detected</td>
</tr>
<tr>
<td>15</td>
<td>57</td>
<td>M</td>
<td>No pathology detected</td>
</tr>
<tr>
<td>16</td>
<td>47</td>
<td>F</td>
<td>No pathology detected</td>
</tr>
<tr>
<td>17</td>
<td>31</td>
<td>M</td>
<td>No pathology detected</td>
</tr>
<tr>
<td>18</td>
<td>76</td>
<td>M</td>
<td>No pathology detected</td>
</tr>
<tr>
<td>19</td>
<td>63</td>
<td>M</td>
<td>Old parietal lobe infarct</td>
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<td>F</td>
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</tr>
<tr>
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<td>Acute leukaemia</td>
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<td>M</td>
<td>No pathology detected</td>
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<td>M</td>
<td>No pathology detected</td>
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<tr>
<td>27</td>
<td>53</td>
<td>F</td>
<td>No pathology detected</td>
</tr>
<tr>
<td>28</td>
<td>52</td>
<td>M</td>
<td>Old cerebellar infarcts</td>
</tr>
</tbody>
</table>

Table 15: Summary of the age, gender and injury of each patient who contributed CSF.
(TBI = traumatic brain injury; SDH = subdural haemorrhage; SAH = subarachnoid haemorrhage; EDH = extradural haemorrhage; CIDP = chronic inflammatory demyelinating polyneuropathy; CVA = cerebrovascular accident)
5.2 Skeletal muscle cell culture isolation and characterisation

5.2.1 Isolation of skeletal muscle cells
The same isolation protocol was used on all 38 muscle specimens. Only one specimen did not generate cells. An error in the isolation process, such as inadvertent discarding of the cell pellet after centrifuge, is suspected, although an anomaly in the patient’s muscle that resulted in the muscle cells not adhering to culture flasks in the conditions employed cannot be discounted.

5.2.2 Maintenance of cell cultures
All cells were maintained by regular observation using bright field microscopy to assess their viability and state of confluence. After the initial isolation, cells typically required 4 to 5 days to adhere to the culture flask then another 7 to 10 days before reaching confluence. Medium was changed after the first week, then weekly.

5.2.3 Cell morphology
The morphology of the isolated cells was assessed by light microscopy and immunohistochemical methods after initial culture in standard medium.

5.2.3.1 Light microscopy
Each muscle population consisted primarily of two cellular morphologies: elongated, multinucleated cells and spindle-shaped cells [Figure 20 (a)]. With time in passage, there was a decrease in the number of elongated, multinucleated cells.

5.2.3.2 Immunohistochemistry
Immunohistochemical staining was used to visualise specific markers within the skeletal muscle cell population [Figure 20 (b)-(f)]. The myogenic tendency of the cell populations was demonstrated by desmin staining of elongated cells containing multiple nuclei. DAPI and SYTO Green No. 25 were used to identify nuclei in fixed and unfixed cells, respectively. The stromal nature of the cell population was exemplified by the high degree of positive staining for vimentin. However, there was no observed Stro-1 expression. Some of the spindle-shaped cells constitutively stained positive for ALP. β-Actin was used to outline the cytoskeletal framework and was found in nearly all cells.
Figure 20: The morphology of cells isolated from adult human skeletal muscle. After isolation, cells were viewed under bright field conditions. The cell populations consisted of two main morphologies: elongated, multinucleated cells and spindle-shaped cells (a). Immunohistochemical staining was used to further define specific markers within the cell populations. SYTO green was used in living cells to identify cell nuclei, some of which were present in close proximity to one another within a multinucleated cell (b). The myogenic capacity of the cell populations was highlighted by the desmin positivity (green) of these multinucleated cells (c). Within all tested populations, the majority of fibroblastic cells were positive for actin (red) (d) and vimentin (green) (e), while some were positive for ALP (blue) (f). DAPI-stained nuclei are blue or red. Images are representative of experiments on at least three different cell lines.
5.2.4 RT-PCR and post-PCR gel electrophoresis

Assessment of the mRNA expression of the skeletal muscle cells was undertaken to characterise the constitutive expression of several key genes of different lineages. Markers of the myogenic lineage were MyoD and Myosin heavy chain IIa (MyHCIIa). ALP, Cbfa1 and two Osterix splice variants (long and short) were used as osteoblastic markers. Three BMP receptor markers were included to indicate the potential for BMP signalling in the muscle cell populations. PPARγ was used as a marker of adipocytic cells.

There was a uniform expression of mRNA regardless of the cell line tested. The muscle cell populations strongly expressed ALP in keeping with their positive staining by immunohistochemistry. In addition, there was weak expression of Cbfa1, but absent expression of both osterix splice variants. Although MyoD expression was absent, MyHCIIa, a marker of mature myotubes, was detection. Expression of mRNA for all three BMP receptors was detected throughout the tested cell lines. PPARγ also had relatively strong expression (Figure 21).

![Figure 21: Expression of mRNA within the skeletal muscle cell populations as detected using the reverse-transcriptase polymerase chain reaction. β-actin was used as a positive control in all experiments.](image-url)
5.3 Assessment of proliferation

5.3.1 Determination of the positive control
Fetal calf serum (FCS) is regularly used as a positive control because of its powerful growth-promoting properties. A positive control consisting of the skeletal muscle cells in DMEM supplemented with 12.5% FCS was used in every proliferation experiment. The proliferation rate of muscle cells in response to FCS was maximal at a concentration of 12.5% FCS (Figure 22). Thus, this concentration was used as the positive control in further experiments to represent the maximal proliferation rate of the skeletal muscle cells.

Figure 22: FCS dilution series. A 2:1 dilution series involving skeletal muscle cells in varying concentrations of fetal calf serum (FCS) was performed to determine the maximal proliferative effect for the positive control. A concentration of 12.5% FCS was found to stimulate maximal proliferation of the skeletal muscle cells and was subsequently used as the positive control in future experiments.
5.3.2 Effect of serum on proliferation of the skeletal muscle cells

5.3.2.1 Study group analysis

Proliferation rates for each serum sample were averaged and standardised to the serum-free negative controls present on each plate. Differences in mean proliferation rates induced by sera from the different study groups were analysed statistically at the first three dilutions (6.25%, 3.13%, 1.56%). The mean proliferation rates of skeletal muscle cells exposed to these varying serum concentrations are shown in Figure 23. The mean proliferation rate of the skeletal muscle cells exposed to the highest serum concentration (6.25%) from patients with TBI was significantly increased compared to patients with SCI ($p = 0.014$) and normal control subjects ($p = 0.02$). Although the trend for the TBI serum to have an increased proliferative effect compared to the other serum study groups continued at the lower concentrations, this did not reach statistical significance ($p = 0.123$ and $p = 0.441$ versus the SCI patients, and $p = 0.10$ and $p = 0.108$ versus the normal controls). There was no significant difference between the mean proliferation rates of the SCI and normal control group at any dilution. Serum from patients with both TBI and SCI induced a significant increase in the rate of proliferation of the muscle cells compared to the serum-free negative control at all three serum dilutions. The normal control serum also caused a significant increase in proliferation rate compared to the negative control, but only at the first dilution. No 95% confidence intervals of any of the study groups included the mean positive control value (2.38) (Table 16).
Figure 23: Mean proliferation rates [plus standard error of the mean (SEM)] of skeletal muscle cells after exposure to sera from the different study groups at the three highest serum concentrations. Cells were exposed to the experimental conditions for five days before measurement of proliferation using a MTS cell assay. Means were standardised to the internal negative control such that the negative control has a calculated value of one. The mean positive control value was 2.38. The TBI serum group (n=11) had a significantly higher proliferation rate than the other study groups at the highest serum concentration. Serum from patients with TBI and SCI (n=6) had a significantly increased proliferative effect on the skeletal muscle cells compared to the serum-free negative control at all serum concentrations ($p < 0.05$). The control group (n=10) proliferation rate was significantly elevated compared to the negative control only at the highest serum concentration ($p < 0.05$).

(a = sig. diff. to serum-free negative control; b = sig. diff. to normal controls; where sig. diff. is $p < 0.05$)

5.3.2.2 Dilution

The proliferative effect of the serum on the skeletal muscle cells was further analysed at the different dilutions. Two-way repeated-measures ANOVA showed that dilution had a significant effect ($p <0.001$) on mean proliferation rates in all serum study groups. This dose dependent decrease in proliferation with decreasing serum concentrations is better appreciated in the form of a line graph (Figure 24).
Figure 24: Line graph of mean proliferation rates of the serum study groups at three different serum dilutions (plus SEM). There was a significant decrease in mean proliferation rate with decreasing serum concentrations.

5.3.2.3 Time point analysis

An analysis of the proliferative effect of the TBI patient serum at different collection time points after injury was performed using the highest serum concentration (6.25%). This revealed no significant difference between the mean proliferation rates at the 6, 24 and 72-hour time points (Figure 25).

Figure 25: Mean proliferation rates (6.25% serum concentration) for the TBI group (n=11) 6, 24 and 72 hours post-injury were 1.85 (SD = 0.58), 1.86 (SD = 0.73) and 1.90 (SD = 0.57), respectively. None of these mean proliferation rates were significantly different to one another ($p = 0.92$).
5.3.3 5-bromo-2-deoxyuridine (BrdU) assay and stain

5.3.3.1 BrdU assay

The proliferation effects measured using the MTS assay were corroborated by the use of a BrdU assay. One 96-well plate was constructed, containing triplicated serum dilution series from two TBI patients, one SCI patient and a normal control subject. Quadrupliclated positive and negative controls were also included (Figure 26). The absorption of each well on the 96-well plate was measured compared to the results obtained for the same samples using the MTS assay (Table 17; Figure 27). A visual representation of the graphical data was obtained by a light microscopic analysis of each well (Figure 28). Characteristic complexes were found within isolated nuclei or aggregations of nuclei. An increased number of spindle-shaped cells and the formation of cellular condensations were also visible by light microscopy in the cell populations that underwent proliferation [Figure 35 (A)].

Figure 26: BrdU corroboration plate. A 96-well plate was constructed, containing triplicated serum dilution series from two TBI patients, one SCI patient and a normal control subject. Quadrupliclated positive and negative controls were also included. This figure shows the effect of serum dilution on the colour within each well. More intense colour indicates higher proliferation rates of the cells within the well. Demonstrated vividly is the decline in proliferation with subsequent serum dilutions.
5.3.3.2 5-bromo-2-deoxyuridine (BrdU) stain

Several attempts were made to determine the immunohistochemical morphology of the cells that underwent proliferation by co-staining the cells with BrdU. Several methods of BrdU staining were trialled, as mentioned in the Materials and Methods section. Unfortunately, despite the success of the BrdU assay, no cells were detected that were stained by BrdU. Many efforts resulted in disrupted cells with nuclear extrusion from the cells. Although cell morphology improved with modification of the fixation and staining techniques, no positively stained cells were observed.

Figure 27: BrdU corroboration of the proliferation rates measured by the MTS assay. The mean absorption for each serum sample on the BrdU 96-well plate was compared to the results obtained for the same samples using the MTS assay. Again, the proliferation rates measured by the BrdU method were standardised to the negative control such that the negative control had a value of one. For comparison, the positive control value on the BrdU plate was 2.43, while on the MTS plate it was 2.38. On all samples tested, the proliferation rates were higher using the BrdU assay compared to the MTS assay. Furthermore, the absolute differences between proliferation rates of the TBI samples and the SCI and normal samples were also greater.
Figure 28: Microscopic view of wells during the 5-bromo-2-deoxyuridine (BrdU) assay. Each black dot (arrows) represents a site of BrdU incorporation into DNA. The cells containing BrdU complexes underwent proliferation during the experimental period (3 days). Characteristic complexes were found within isolated nuclei or aggregations of nuclei. Cells exposed to 6.25% serum from patients with severe TBI (b) had a similar number of complexes as the positive control (a). The number of complexes decreases as the concentration of serum decreases (c-e). Serum from patients with SCI and normal control subjects (g), like the serum-free negative control (h), showed little evidence of proliferation by the BrdU method.
5.3.4 Muscle validation: proliferation independent of the muscle donor

A total of 6 serum samples from TBI patients, 2 from SCI patients and 2 from control subjects were tested in the muscle validation experiment. The three muscle samples were derived from the triceps brachii of a 69-year-old male, the gluteus medius of a 78-year-old female and the vastus intermedius of a 39-year-old male. There was no statistically significant difference in the proliferation rates induced by the same serum when tested with muscle samples from different patients. That is, the patient origin of the muscle cells did not have a significant effect on the measured proliferation rate (Table 18).

<table>
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<tr>
<th>Serum</th>
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<th>Muscle B</th>
<th>Muscle C</th>
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<td>2.38</td>
<td>2.40</td>
<td>2.43</td>
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</tr>
</tbody>
</table>

Table 18: Consistency of the proliferative effect of ten different sera samples on skeletal muscle cells from three different sources. Using repeat measures ANOVA, no significant difference was found in the serum-induced proliferation rate irrespective of the origin of the skeletal muscle (p=0.42). [Results to 2dp]

5.3.5 Lack of a proliferative effect of CSF

Proliferation rates for each CSF sample were averaged and standardised to the serum-free negative control present on each plate. For analysis of the difference in mean proliferation rates induced by CSF from the different study groups, the first three CSF dilutions (6.25%, 3.13%, 1.56%) were examined (Table 19). The mean proliferation rates of skeletal muscle cells exposed to the varying CSF concentrations from the different study groups are graphically shown in Figure 29. No significant difference between the mean proliferation rates of the TBI and uninjured study groups was found at any dilution (p = 0.20).
The TBI group consisted of only males. When the mean proliferative effect of CSF from the TBI group was compared to the mean proliferation rate of the males from the control group, no significant difference was found ($p = 0.07$). CSF from neither the TBI patients nor the uninjured control group resulted in a statistically significant change in the proliferation rate of the skeletal muscle cells compared to the negative control ($p > 0.05$).

![Figure 29: Mean proliferation rate (plus SEM) of skeletal muscle cells after exposure to CSF from TBI patients (n=6) or uninjured control subjects (n=28) at the three highest CSF concentrations. Cells were exposed to the experimental conditions for five days before measurement of proliferation using a MTS cell assay. Means were standardised to the internal negative control such that the negative control had a calculated value of one. The mean positive control value was 2.38. There was no significant difference between the mean proliferation rates of the study groups at any dilution ($p = 0.20$). A significant effect was not found when the subgroup of males in the control group (n=16) was compared to the TBI group, which was comprised of only males ($p = 0.07$). At no dilution did the CSF from any group have an increased proliferative effect compared to the negative control ($p > 0.05$).]
5.4 Assessment of differentiation

5.4.1 Determination of optimal mineralisation medium
A standard osteogenic differentiation medium contains β-glycerophosphate and ascorbic acid. These supplements provide the molecules for phosphate and collagen synthesis required for maturation of the bony extracellular matrix. The skeletal muscle cells did not tolerate the usually recommended concentrations of β-glycerophosphate and ascorbic acid employed (50µg/ml ascorbic acid and 10mM β-glycerophosphate). This was manifested by slow growth kinetics, lack of cell adherence and reduced cell viability (Figure 30). Hence, a dilution plate was constructed to determine the most effective concentration of each of the β-glycerophosphate and ascorbic acid. It was found that the highest doses tolerated by the cells were 2mM of β-glycerophosphate and 12.5 µg/ml of ascorbic acid.

Figure 30: Effect of different concentrations of supplementary β-glycerophosphate and ascorbic acid on skeletal muscle cells. A concentration of β-glycerophosphate in excess of 2mM and ascorbic acid greater than 12.5 µg/ml resulted in retarded cell growth and increased numbers of non-viable cells [(a), arrows]. The concentrations used in mineralisation experiments (2mM β-glycerophosphate and 12.5 µg/ml ascorbic acid) allowed cell growth (b).
5.4.2 Effect of serum and CSF on skeletal muscle cell osteoblastic differentiation

The osteoblastic differentiation of the skeletal muscle cells after exposure to serum and CSF was assessed by measurement of ALP activity, osterix protein expression and elaboration of a mineralised matrix.

5.4.2.1 ALP activity

Exposure of the skeletal muscle cells to serum from patients with CNS trauma and normal control subjects showed that all serum-treated populations, irrespective of the origin of the serum, exhibited an increase in supernatant ALP activity over the duration of the experiments (Figure 31). A significant difference in ALP activity was found between all serum-treated groups and the serum-free negative control at days 15 and 20, but there was no significant difference between the serum-treated groups at any time point. Exposure of the skeletal muscle cells to CSF from patients with TBI and control patients showed that both CSF-treated populations, irrespective of the origin of the CSF, exhibited a decrease in supernatant ALP activity from the onset of the experiments. No significant difference was found between the CSF-treated groups and the negative control (p = 0.20) (Figure 32).
Figure 31: ALP activity as a function of time in culture with serum. Two-way repeated-measures ANOVA was used to measure the effect of culture time (days) on ALP activity for each study group. A significant increase in ALP activity of serum-treated groups was found at Days 10 and 15 ($p < 0.001$). Taking into account all days of proliferation, significant mean differences in ALP activity were shown between the TBI high proliferating group and the negative control ($p = 0.019$), the TBI low proliferating group and the negative control ($p = 0.033$), and the normal control groups and the negative control ($p = 0.045$). The serum-free negative control showed a reduced ALP activity level over the first 10 days of the experiment and by day 15 there was no measurable ALP activity in these wells. (n=3 for all samples)

Figure 32: ALP activity as a function of time in culture with CSF. Two-way repeated-measures ANOVA was used to measure the effect of culture time (days) on ALP activity for each study group. The serum-free negative control showed a reduced ALP activity level over the first 10 days of the experiment, with a concurrent decrease in ALP activity within the CSF-treated groups. Days of incubation had a significant effect on proliferation. Significant decreases in ALP activity of the CSF-treated groups were evident between day 5 and days 15 and 20 ($p = 0.025$). No significant differences were found between the CSF-treated groups and the negative control at any time point ($p = 0.20$). (n=3 for all)
5.4.2.2  **Osterix (Osx) protein expression**

Skeletal muscle cells were treated with serum and CSF from CNS trauma patients and control subjects for one week before assessment of the protein expression of Osx, a marker of the mature osteoblast phenotype. Initially, Western Blotting was performed using fluorescent antibodies, but due to excessive background combined with a relatively weak Osx signal, the Enhanced Chemiluminescent (ECL) technique was trialled, and it was found to produce blots with greater clarity. Evidence of a band at the expected molecular weight (45kDa) for the Osx protein was detected in all serum treated cells but not the CSF-treated cells or the untreated negative control (Figures 33). A lane containing protein from hFOB 1.19 osteoblasts cultured in standard medium showed the same band.

![Figure 33: Non-quantitative Western blot with osterix antibody of serum study groups (upper row). 25µg of protein was loaded into each well. Lane 1 and 2 represent skeletal muscle cells treated with high proliferating serum from a TBI patient and low proliferating serum from a TBI patient, respectively. Lane 3 represents cells treated with serum from a normal control subject. Lane 4 is a serum-free negative control. Lane 5 is a positive control consisting of the osteoprogenitor cell line hfof1.19. The expected product is approximately 45kDa. The 50kDa molecular weight marker is indicated. Western blotting for β-actin protein is shown in the bottom row.](image-url)
5.4.2.3 Mineralised matrix

Villanueva staining of the skeletal muscle cells after three weeks culture with human serum from the study groups and a mineralisation medium of ascorbic acid and β-glycerophosphate showed the frequent condensations of spindle-shaped cells and a small number of positively staining bone nodules in serum-treated groups, which were not present in the CSF-treated cell populations or untreated negative controls (Figure 34). Each study group was tested in triplicate. At least two out of three plates representing each serum study group contained Villanueva-positive nodules. The mean number of Villanueva-positive nodules per well was 1.7 (SD = 1.5), 2.3 (SD = 2.5), 1 (SD = 1.7) and 1 (SD = 1) for the high proliferating TBI, low proliferating TBI, SCI and normal control group, respectively. There was no significant difference in the number of nodules between the serum groups ($p = 0.77$).
Figure 34: The spindle-shaped cell population proliferated and formed cellular condensations in response to serum (a). An increased number of these cells were observed after incubation of the skeletal muscle cells with serum from TBI patients compared to cells incubated in serum from other study groups or serum-free medium alone. Villanueva staining of muscle cells infrequently revealed positive staining nodules after three weeks culture in ascorbic acid, β-glycerophosphate and serum from TBI patients, SCI patients and normal controls (b,c). Many cells cultured in CSF and the serum-free negative control were not viable and there was no evidence of nodule formation under these conditions (d,e). A positive control consisting of hfob1.19 cells cultured in mineralisation medium is included for comparison (f).
5.5 Achievements

(1) Isolation of primary skeletal muscle cell cultures from different patients by a reliable and easily reproducible technique

(2) Characterisation of these primary skeletal muscle cell cultures, outlining the existence within them of multinucleated myotubes and spindle-shaped mesenchymal cells, some of which constitutively expressed the osteogenic markers ALP and Cbfa1, but not Osterix

(3) Validation of the muscle cell cultures to show that the origin of the muscle does not significantly effect the proliferative response to serum

(4) Demonstration of the dose-dependent proliferative effect of serum from patients with severe traumatic brain injury on the skeletal muscle cells in vitro in a manner that was significantly increased compared to normal control sera and the serum-free negative control

(5) Showing that the proliferative effect of the TBI sera was constant at six hours, 24 hours and three days after injury

(6) Illustration of the lack of an effect of serum from patients following traumatic spinal cord injury on the in vitro proliferation of the skeletal muscle cells compared to the normal control subjects

(7) Demonstration of the ability of human serum, from uninjured as well as injured subjects, to support an increase in alkaline phosphatase activity, the induction of osterix protein expression and the elaboration of mineralised nodules in the primary skeletal muscle cell cultures

(8) Illustration of the lack of an effect of cerebrospinal fluid from patients following traumatic central nervous system injury on the in vitro proliferation or osteoblastic differentiation of the skeletal muscle cells
Chapter 6: Discussion, Future Directions and Conclusions

The central aim of this in vitro study was to investigate the effect on adult human skeletal muscle cells of serum and cerebrospinal fluid (CSF) from patients with central nervous system (CNS) trauma. The hypothesis for this study was that skeletal muscle cells exposed to serum and CSF from patients with CNS trauma would proliferate and undergo osteoblastic differentiation. This hypothesis was based on the clinical observation of heterotopic ossification (HO) within the skeletal muscle of these patients and the evidence that some skeletal muscle cells have osteogenic ability. Previous in vitro studies have shown a mitogenic and osteogenic effect of serum and CSF from CNS trauma subjects on mesenchymal and osteoblastic cells, but none have specifically investigated primary cells from human skeletal muscle even though skeletal muscle is a common site of HO.

This study involved the isolation and characterisation of cell populations from human skeletal muscle followed by assessment of the effect of serum and CSF from patients with brain or spinal cord trauma on the proliferation and osteoblastic differentiation of these cells. Ultimately, this study showed that human serum, but not human CSF, was able to support the development of a bony phenotype within human skeletal muscle in vitro and, furthermore, serum from patients with traumatic brain injury (TBI) induced an accelerated rate of proliferation of the skeletal muscle cells. These findings may have implications in the understanding of the development of HO within skeletal muscle and the treatment of skeletal disorders.
6.1 Main Discussion

6.1.1 Skeletal muscle cells

6.1.1.1 Patient selection

The skeletal muscle used in this study originated from patients who underwent an orthopaedic trauma procedure at Royal Perth Hospital (R.P.H.). An analysis of the gender and age distribution of this population of patients revealed that they were predominantly males younger than 50 years old. The other major group of patients operated on routinely in R.P.H. orthopaedic trauma department is the elderly population, more often women, with fractures that are the result of falls. This demographic profile is consistent with the published epidemiology of orthopaedic trauma, which shows that fractures affect a wide variety of persons, but is especially prevalent in young males and elderly women (Maravic et al. 2005; Court-Brown and Caesar 2006; Urquhart et al. 2006).

6.1.1.2 Isolation of skeletal muscle cells

The skeletal muscle obtained was processed to produce a primary cell culture. The technique employed to isolate cells from adult human skeletal muscle was based on previously described methods of obtaining primary cell cultures from skeletal muscle (Lecoeur and Ouhayoun 1997; Levy et al. 2001; Bujan et al. 2005; Mastrogiacomo et al. 2005; Sakaguchi et al. 2005). This technique has been shown to produce a uniformity of cell morphology, behaviour and phenotypical markers across multiple cell lines. Morphologically, primary cultures of skeletal muscle consist of a population of fibroblastic cells interspersed with multinucleated myotubes. These cells are usually positive for vimentin, smooth muscle actin, HOP-26, collagen type I, fibronectin and variable amounts of desmin (Mastrogiacomo et al. 2005; Levy et. al, 2001). Interestingly, they also express markers of the osteogenic lineage, including ALP, osteonectin and osteopontin (Mastrogiacomo et al. 2005; Levy et al. 2001). There is much heterogeneity within this cell population; they consist of various amounts of fibroblasts, myoblasts and adipocytes, in addition to multipotent cells (Qu et al. 1998; Hwang et al. 2004). Alessandri et al. (2004) performed clonogenic studies on these cells to better reveal the potential of individual cells. Interestingly, a skeletal muscle cell population with two major
phenotypes was generated: fibroblast-like cells that attached to the culture flask, and round floating cells. By observing the progress of individual cells within the population, it was found that a small proportion of them could self-renew, whilst the majority had limited clonogenic ability. Other techniques to increase the number of putative mesenchymal stem cells from primary skeletal muscle cell cultures include the use of various growth factors, serum-free medium, trituration of the cell supernatant using micro-filters, freeze-thaw cycling and transfer of the initial cell supernatant to a collagen-coated flask (Williams et al. 1999; Jankowski et al. 2001; Romero-Ramos et al. 2002; Alessandri et al. 2004; Mastrogiacomo et al. 2005). In addition to mesenchymal cells, other cells within skeletal muscle have been implicated in osteogenesis, including satellite cells, “side population” cells derived from flow cytometric cell sorting, preplated skeletal muscle cell cultures and cells of the vascular wall (Uezumi et al. 2006; Collett and Canfield. 2005; Tavian et al. 2005; Bouleterau et al. 2002; Jankowksi et al. 2002; Musgrave et al. 2002; Deasy et al. 2001; Levy et al. 2001; Bosch et al. 2000; Doherty et al. 1998; Decker et al. 1995; Diaz-Florez et al. 1992). Thus, skeletal muscle cell populations contain many cells that are potentially capable of osteogenic differentiation.

6.1.1.3 Characterisation results

The morphology and mRNA content of the isolated skeletal muscle cell populations was characterised by bright field microscopy, immunohistochemistry and RT-PCR. These characterisation techniques were performed within the first three passages after the initial isolation of cells from freshly extracted skeletal muscle specimens. This protocol was developed to produce consistent results because the phenotype and behaviour of cultured cells may change during repeated passaging. For example, the desmin, MyoD and Pax7 content of skeletal muscle derived cell populations has been shown to decrease with each passage, along with the number of proliferating cells and number of nuclei within myotubes (Machida et al. 2004). Similar results have been found in other cell lines, such as osteoblastic cells and mesenchymal stem cells (Huang et al. 2001; Sakaguchi et al. 2005; Vacanti et al. 2005). In keeping with these findings, a reduction in the number of myotubes with time in culture was observed in this study. Thus, characterisation of passaged cells should ideally be made immediately upon their isolation or at the passage
employed for experimentation (Machida et al. 2004). In this study, practicalities of cell number resulted in characterisation within three passages of isolation and further experimentation within three passages of characterisation.

- **Myogenic markers**

Morphologically, the primary cultures of skeletal muscle consisted of a population of fibroblastic cells interspersed with elongated, multinucleated cells. The intrinsic myogenicity of the cultured cells was exhibited in the desmin positive staining of the multinucleated cells, which were consistent with myotubes. Furthermore, the cell populations showed evidence of myosin heavy chain IIa mRNA expression, although MyoD mRNA was absent. MyoD is a myogenic determination factor responsible for the commitment of progenitor cells to the myogenic lineage in both the embryo and adult (Berkes and Tapscott 2005; Parker et al. 2003). Its expression is reduced in mature muscle, but satellite cells show upregulated expression following muscle damage (Dias et al. 1992; Benoyahu et al. 2005). Conversely, the myosin heavy chains are a family of proteins that are present in terminally differentiated muscle cells (Francis-West et al. 2003).

- **Vimentin and Stro-1**

A majority of muscle cells, specifically those with spindle-shaped morphology, stained positive for the intermediate filament vimentin. The expression of vimentin is associated with the mesenchymal phenotype, including fibroblasts and skeletal muscle stem cells (Alessandri et al. 2004; Romero-Ramos et al. 2002). The spindle-shaped cells present in skeletal muscle are thought to be of mesenchymal origin and some of these cells have been likened to mesenchymal stem cells, bone marrow stromal cells and pericytes, because of their similar morphological, immunohistochemical and mRNA profiles, and their capacity for osteogenic differentiation (Meirelles et al. 2006; Mastrogiacomo et al. 2005; Levy et al. 2001; Williams et al. 1999). Practically all adult tissues contain a population of mesenchymal cells with stem cell-like characteristics, although the exact identification of mesenchymal stem cells is lacking due to inadequate characterisation techniques (Reyes et al. 2006; Young et al. 2004). STRO-1 is used as a marker of mesenchymal stem cells from the bone marrow stroma, particularly those cells with osteogenic potential (Gronthos et al. 1994;
Gronthos et al. 1999; Minguell et al. 2001; Baksh et al. 2004; Aguila and Rowe 2005). However, some studies report absent or weak STRO-1 expression in bone marrow mesenchymal stem cells (Sakaguchi et al. 2005). For example, STRO-1 is not required for osteogenic potential in adipose stromal cells (Gronthos et al. 2001). Furthermore, STRO-1 expression is progressively lost with time in culture (Stewart et al. 1999), so that an absence of STRO-1, such as observed in this study, does not imply a lack of differentiation capacity of a cell population.

- Osteogenic markers
The osteogenic potential of the skeletal muscle mesenchymal cell population is emphasised by their spontaneous expression of markers of osteogenesis (ALP, core binding factor alpha1 [Cbfa1], osteonectin, and osteopontin) (Mastrogiacomo et al. 2005; Levy et al. 2001). Under osteogenic differentiation conditions, this cell population has increased ALP activity (Levy et al. 2001) and the ability to produce a mineralised matrix in vitro that stains positive for Alizarin Red (Mastrogiacomo et al. 2005). The isolated cells are also involved in ossification when implanted in immunodeficient mice (Mastrogiacomo et al. 2005). In this study, the osteogenic capability of the skeletal muscle cell populations was suggested by the constitutive high levels of ALP at the mRNA, protein and functional level, and weak expression of the transcription factor Cbfa1, both of which are early markers of the osteoblastic lineage (Karsenty et al. 1999). The early stage of osteoblastic maturation was further confirmed by absence of Osterix expression at the mRNA or protein level in untreated cells.

- Bone morphogenetic protein receptors
A key family of molecules in osteogenesis – the bone morphogenetic proteins (BMPs) – bind to type I and type II membrane receptor serine/threonine kinase receptors to initiate intra-cellular cascades. BMPs have pleiotropic functions in a variety of tissues, including the stimulation of mesenchymal cells into the osteoblastic lineage (ten Dijke et al. 2003; Varga and Wrana 2005; Yanagita 2005). Detection of mRNA for the BMP receptors (BMPR) IA, IB and II in the skeletal muscle cells indicates the potential for BMPs to have an effect on these cells. BMPR-IA appears to play a more significant role than BMPR-IB in bone and cartilage development from mesenchymal progenitors and is involved at an
earlier stage of differentiation (Kaps et al. 2004). BMPR-IB mRNA is detectable by RT-PCR in a pre-myoblastic mouse cell line at lower levels than BMPR-IA mRNA (Namiki et al. 1997). However, forced expression of both BMPR-IA and BMPR-IB in C2C12 cells leads to expression of ALP and osteocalcin (Akiyama et al. 1997). In keeping with these studies, both BMPR-IA and BMPR-IB mRNA was detected in the skeletal muscle cells with BMPR-IA mRNA being present at greater levels. BMPR-II has also previously been detected in skeletal muscle cells, although osteogenic BMPs have weak affinity for this receptor unless type I receptors are also present (Kawabata et al. 1998).

- **PPARγ**
  
The expression of mRNA for PPARγ, a key transcription factor of the adipocytic lineage, by the skeletal muscle cells raises the possibility of the presence of adipocytes or cells with adipocytic potential. No specific stain, such as Nile Red or Oil Red O, was used to look for the existence of mature adipocyte cells in culture, but there was no evidence of vacuole-containing cells consistent with adipocytes. Cells with a fibroblast-like morphology similar to those isolated from skeletal muscle have previously been isolated through enzymatic digestion of adipose tissue (Ogawa et al. 2004; Peptan et al. 2006). Under osteogenic conditions, these cells generated osseous nodules with upregulation of osteogenic markers. In light of this evidence, although efforts were made to remove any adipose tissue from the original muscle extracts, it is possible that these cells could contribute to ossification. The expression of PPARγ mRNA may also be another indication of the presence within the cell cultures of progenitor cells capable of differentiation into mesodermal lineages (Wada et al. 2002; Chang et al. 2006). In addition, PPARγ may also be involved in skeletal muscle metabolism (Mahoney et al. 2005). PPARγ agonists have anti-inflammatory, anti-atherogenic, proliferative and anti-oxidant effects through actions on many cell types, including macrophages, endothelial cells, epithelial cells, vascular cells and smooth muscle cells (Cuzzocrea 2006; Shimizu et al. 2006; Touyz and Schiffrin 2006). PPARγ is also implicated in the control of cell cycle progression and apoptosis (Theocharis et al. 2004), indicating that it is involved in a multitude of cell functions in addition to adipocytic lineage determination.
6.1.1.4 Necessity for validation of muscle lines

In this study, two apparently independent variables arose because the skeletal muscle cells and the serum or CSF used in the same experiment originated from different subjects. The observed effects of the serum and CSF on the skeletal muscle cells may have been attributable to inter-sample differences in responsiveness of the muscle cells. Aside from the consistency of cell isolation and the morphological and behavioural similarity of cell lines from different patients, it was necessary to show that any effect induced by the serum or CSF was not dependent on which muscle cell lines were used. This was accomplished by a statistical analysis (ANOVA) of the proliferation rates of three muscle cell populations in response to the same ten serum samples. The muscle samples were selected to be representative of the gender and age demographics of the patients who contributed skeletal muscle and the sera were inclusive of subjects from all study groups. This analysis revealed no significant difference in the proliferation rate induced by a serum sample regardless of the muscle cell line used. Hence, it was concluded that the inter-patient variation in muscle specimens was not responsible for any significant effects observed in response to serum or CSF.

6.1.2 Serum and cerebrospinal fluid as putative vehicles for osteoinductive factors

6.1.2.1 Patient selection

An adult population was selected as serum and CSF donors because of the patient demographics at R.P.H. and the distinctive development of HO in children. In paediatric populations, HO occurs less commonly, with delayed onset, affects different sites and is more likely to resolve spontaneously than in adults (Garland et al. 1989). Donors in this study were representative of the typical demographic of patients with TBI and SCI, namely predominantly young males. For the serum experiments, an attempt was made to match the healthy control group to the CNS trauma groups with respect to age and gender. Statistically, this aim was achieved, as there was no significant difference in the age or gender distribution between groups. Identical selection criteria were used for inclusion into the CSF arm of this study to provide a standardised comparison between serum and CSF experiments, although ultimately only patients with TBI were enrolled, as CSF is not routinely obtained from patients.
with SCI. For the CSF experiments, the control group was formed by patients without CNS trauma but who had lumbar punctures performed as part of their management. The collection of appropriate human CSF controls is difficult as patients often have disease, some involving non-traumatic CNS conditions or protein abnormalities that could potentially influence results (Terry and Desiderio 2003). The control patients in this study had a variety of diagnoses, such as spontaneous subarachnoid haemorrhage, brain tumour and leukaemia, whilst some had no detectable pathology. However, even those patients with no detectable pathology were still unwell enough to warrant a lumbar puncture, indicating the presence of an unclassified disease.

This study was focussed on the effect of serum from patients with CNS trauma but without fractures of the pelvis or long bones. This comparison of the effect of CNS trauma in isolation removes the influence of significant fractures on the osteogenic process. HO has been documented to occur more often at the site of fracture in those with concurrent limb or pelvis fracture, especially when the fracture has been stabilised surgically (Garland et al. 1982; Garland et al. 1985; Garland 1988; Garland 1991; Pape et al. 2001). During fracture healing, alterations in local blood flow, tissue oxygenation, acid-base balance and electrolyte levels may predispose to HO (Fujimura et al. 2001; Lotta et al. 2001; Pape et al. 2001; Comarr et al. 1962). Furthermore, the amount of circulating factors associated with ossification is affected by the presence of fracture (Wildburger, Zarkovic, Egger et al. 1994; Wildburger et al. 1995; Wildburger et al. 1996; Wildburger et al. 2001; Trentz et al. 2005). Subsequently, with the eradication of bias as a result of concurrent significant fractures, any differences between groups are more likely to be attributable to the CNS injury.

6.1.2.2 Serum

It has been postulated that factors are released from traumatised CNS tissue and may be detectable in the systemic circulation following traumatic disruption of the blood-brain barrier (BBB) (Bidner et al. 1990; Karmani and Compson 2000; Boes et al. 2006). Consistent with this idea, various factors originating within the CNS, such as putative biomarkers of CNS injury, can be found in the serum following injury associated with BBB dysfunction (Hayakata et al. 2004; Ringger et al. 2004; Savola et al. 2004; Raabe et al. 2003; Pike et al. 2001).
Given previous *in vitro* data involving mesenchymal and osteoblastic cells and the incidence of HO within skeletal muscle, it was important to define the effect of serum after TBI and SCI on skeletal muscle cells. For these reasons, serum was investigated as a vehicle for putative osteoinductive factors. Fortunately, blood is easily collected as phlebotomy is routinely performed during the management of trauma patients.

6.1.2.3  *CSF*

Like serum, CSF is a potentially useful fluid to analyse as a putative vehicle for osteoinductive factors following CNS trauma. Given the intimate relationship of CSF to CNS tissues, it was proposed that should a suspected factor be released from the damaged CNS, it might be detectable within the CSF given sufficient trauma. Indeed, protein changes observed in the CSF are directly related to events within the CNS (Ottens et al. 2006). The collection of CSF is more complicated and invasive than the collection of serum, but excess volumes are collected during lumbar puncture and significant quantities can be drained from intracranial catheter devices.

6.1.2.4  *Other possibilities for specimen collection*

Other possibilities for specimen collection, besides serum and CSF, were considered. Brain and spinal cord parenchyma and urine represent other sources for detection of the effect of a systemically distributed factor potentially derived from the CNS. However, there are restrictive ethical constraints when dealing with experimentation on human CNS tissue from living patients, and CNS tissue from deceased individuals deteriorates quickly. In any case, this tissue may not be indicative of the systemic release of factors found within the CNS. It was also possible to collect urine, a waste fluid, and analyse it for osteogenic factors. Because urine is formed as a filtrate of blood by the kidneys, it may be considered to be representative of the blood contents. However, it is also possible that filtration and reabsorption may prevent the presumptive factors of interest from being excreted in the urine (Ottens et al. 2006).
6.1.3 Proliferation results

6.1.3.1 Assessment of MTS validity using BrdU

To assess proliferation of the skeletal muscle cells in response to serum and CSF, the colorimetric MTS assay was used. This assay involves the metabolism of a MTS salt into a coloured formazan product by mitochondrial enzymes, thus giving an indication of mitochondrial activity and cell growth (Twentyman and Luscombe 1987; Gieni et al. 1995; Pabbruwe et al. 2005). The MTS and related MTT assays have been used on a variety of cell types in similar proliferation experiments. For example, Boes et al. (2006) used this method for determining the proliferation rate of a mesenchymal stem cell line, a committed osteoblastic cell line and a fibroblast cell line after exposure to serum from rats with TBI. It has also been successfully employed in a non-fusing myoblast cell line to test porcine serum mitogenic activity (Zhou et al. 1994). However, several chemical and biochemical factors influence the reliability of these results, including the volume of medium and the concentration of serum (Sieuwerts et al. 1995; Zhang and Cox 1996). Hence, a BrdU-incorporation assay was used on one plate, including serum samples from all study groups, to validate the MTS results. Both the BrdU and the MTS methods have high correlation to the radioactive thymidine assay (Zolnai et al. 1998; Wagner et al. 1999). In fact, formazan-based colorimetric assays have been shown to have greater sensitivity than radioactive thymidine incorporation in measuring proliferation of lymphocytes and chondrocytes, particularly at lower concentrations (Pabbruwe et al. 2005; Gieni et al. 1995). In this study, the BrdU assay confirmed the proliferation induced by serum from the different groups. In fact, it revealed a greater difference between the study groups than the MTS assay, suggesting that if the BrdU had been used throughout this study instead of the MTS assay, further significant differences may have been elucidated. The fact that the MTS assay is an easier test to perform and the demonstrated comparability to the BrdU confirms its suitability for large-scale assessment of proliferation, as conducted in this study.

6.1.3.2 Study group results

- Negative and positive controls

The serum-free negative control was chosen as the comparison value for the proliferation rates of the serum and CSF-treated groups to determine the
relative effect of the proliferative agents above a medium without any anticipated proliferative effect. A positive control, containing 12.5% foetal calf serum (FCS), was also used to determine the maximal possible proliferative effect of the skeletal muscle as an indicator of the extent of any serum or CSF-mediated proliferation. FCS is commonly used as an in vitro growth stimulant as it contains a milieu of factors that provide significant growth-stimulating properties in a variety of cell types. Because the age and species of the serum donor influence the growth factor content of serum, the use of serum derived from foetal calves may not be appropriate for investigations with adult human cells (Yonezawa et al. 1992; McAlinden and Wilson 2000; Shahdadfar et al. 2005; Alexander et al. 2006). Although FCS has an enviable ability to expand cell populations and is often more accessible than human serum, its relevance to adult human cells in vivo is limited compared to adult human serum. This is particularly true when cells are cultured for clinical use in transplantations (Chachques et al. 2004; Nakamura et al. 2006).

- **Effect of study groups**

The first three serum and CSF dilutions (6.25%, 3.13% and 1.56%) were used for analysis of the effect of study group on proliferation rate based on the study design, previous studies on the proliferation rate of cells exposed to different serum concentrations and the unlikely physiological relevance of smaller concentrations. The results from this study indicate a relationship between severe TBI and serum-induced proliferation of skeletal muscle cells. The MTS assay showed a significant increase in the proliferation rate of skeletal muscle cells exposed to serum from the TBI patients compared to the serum-free negative control at all serum concentrations and the SCI patients and normal controls at the highest serum concentration (6.25%). Although the mean proliferation rate of the TBI serum group at the lower concentrations (3.125% and 1.56%) was higher than the other serum study groups, this difference did not reach statistical significance. The importance of the lack of a significant effect between severe TBI and the normal controls at lower serum concentrations is questionable given the small concentrations investigated. It is evident that there is a trend for greater differences between study groups with greater serum concentrations. Even the first three concentrations may be inadequate given that serum from the TBI patients only had a significant
increased effect on proliferation rate at the highest concentration. The upper extreme of the 95% confidence interval for the mean proliferation rate of the TBI serum did not include the maximal proliferation rate indicated by the 12.5% FCS positive control, indicating that higher concentrations of serum may have increased proliferation further. Previous studies have used serum concentrations ranging from 1 to 20% (Boes et al. 2006; Renfree et al. 1994; Kurer et al. 1992; Bidner et al. 1990). Boes et al. (2006) showed a significant increase in mesenchymal stem cell proliferation using 5% serum from rats with TBI. A significant increase in ALP secretion and proliferation by human femur cells also occurred following exposure to sera from SCI patients at 20% dilution. A significant difference between SCI patients with HO and those SCI patients without HO and normal controls was evident at all tested serum concentrations between 1 and 20% (Kurer et al. 1992). Conversely, Renfree et al. (1994) found no effect on neonatal rat calvarial cells of 10% serum from SCI or TBI patients. This may have been due to an inability of human factors to act on rat cells because of inter-species differences in key aspects of the signalling pathways, such as receptors or ligands. Bidner et al. (1990) investigated the effect of serum concentrations of 0.01%, 0.15, 1% and 10%, but only described significant effects of TBI at the 10% concentration.

- Effect of dilution

The dilution dependent reduction in proliferative effect of the TBI serum is consistent with decreasing concentrations of putative humoral factors. Interestingly, although the normal control group showed a significant decrease in effect with dilution, the average proliferation for this group was only statistically above the serum-free negative control at the 6.25% dilution. This is in keeping with previous studies that tested the proliferative effect of normal human sera. Serum from human controls, like FCS, has been shown to support the proliferation and differentiation of human osteoblasts (Bidner et al. 1990; Kurer et al. 1992; Yamamoto et al. 2003). Some studies have even shown that human serum is more effective at these functions than FCS (McAlindden and Wilson 2000; Hankey et al. 2001). Physiologically, this is understandable given the presence of species-specific serum growth factors. The SCI patient group, although not possessing a mean proliferation rate that was significantly different to the normal controls at any dilution, maintained a mean proliferation rate that
was significantly above the negative control value, indicating an effect of the SCI serum above a baseline proliferation rate at these dilutions, but still lower than the mean proliferation rates of the TBI serum group.

6.1.3.3 Serum collection time point analysis
The temporal evolution of the proliferative effect induced by serum from the TBI patients was analysed at the different time points after injury. The demonstration of equivalent mean proliferation rates of the TBI serum taken at 6, 24 or 72 hours post-injury suggests that the presence of any humoral factor is not appreciably different at these times. Thus, the processes underlying the observed serum-mediated affects appear to begin at least as early as 6 hours after injury and they continue at a statistically constant rate throughout the first three days after injury. No previous study has obtained three serum samples within the first three days of injury. Samples obtained early after injury (within two or three days) have been followed by samples taken after a week or even up to seven months after injury (Boes et al. 2006; Klein et al. 1999; Renfree et al. 1994; Kurer et al. 1992; Bidner et al. 1990). Bidner et al. (1990) found maximal thymidine incorporation in fetal rat calvarial cells from patient serum extracted at 35-37 days after injury. Conversely, Renfree et al. (1994) found a decrease in neonatal rat calvarial cell number exposed to serum extracted within 48 hours of injury, but a constant cell number thereafter. The evidence from this study of a sustained serum effect early after CNS injury is consistent with previous clinical observations that the development of HO following TBI is a rapid process, with bony deposits visible radiologically by ten days in exceptional cases, and typically by three weeks (Cope 1990).

6.1.3.4 TBI versus SCI
Two previous studies have reviewed the effect on the proliferation and ALP generation of osteoblastic cells exposed to serum from patients with SCI. Kurer et al. (1992) showed that SCI serum induced a statistically significant increase in ALP compared to control sera in cells extracted from human femurs. Conversely, Renfree et al. (1994) showed that sera from humans with SCI did not increase the proliferation rate of one-day old rat calvarial cells. In agreement with the findings of Renfree et al. (1994), this study found no significant effect of SCI serum compared to normal controls or the serum-free negative control. It
has been speculated that SCI represents a different disease process to TBI and involves different mechanisms for increased osteogenesis (Karmani and Compson 2000; Cope 1990; Garland 1988). Although both SCI and TBI constitute trauma to CNS tissue, brain and spinal cord are different structures with theoretically different peripheral effects on osteogenesis. The pathogenesis of HO following SCI may be more reliant on interruption of autonomic fibres within the spinal cord, that results in autonomic dysregulation of neurohormonal, vascular and metabolic factors (van Kuijk et al. 2002). This is borne out by the different epidemiology of HO in TBI and SCI patients. SCI patients have a greater overall incidence of HO compared to TBI patients. They also vary with respect to the sites of occurrence of the bone deposits (Garland 1988; Garland et al. 1980). There is a strong relationship of HO to complete lesions of the spinal cord, lower cervical or thoracic lesions, and spasticity (Lal et al. 1989; Bravo-Payno et al. 1992; Wittenberg et al. 1992; Dai 1998). However, the presence of limb spasticity has not been linked to HO in patients with TBI (Karmani and Compson 2000). Moreover, the severity of TBI, determined by Glasgow Coma Scale and length of coma, has been shown to determine the development of HO in some studies (Hurvitz et al. 1992; Ebinger et al. 2000; Kluger et al. 2000), whereas other studies dispute the predictive value of clinical or radiological signs (Sazbon et al. 1981; Catz et al. 1992; Hurvitz et al. 1992; Flin et al. 2002), such that no universal correlation between the clinical or radiological severity of TBI has been established. Histological differences between experimental heterotopic bone from rats with TBI and SCI have also been identified. Specifically, SCI subjects had a marked reduction in the degree of osteogenesis as a result of weak cell reaction to osteogenic stimuli (Otfinowski 1993). The process of fracture healing in SCI patients may also be distinct from that observed in TBI patients. In SCI patients, fracture healing may actually be impaired by the injury, as a result of immobilisation and paralysis, which leads to disuse osteopenia (Cope 1990; Garland 1988). Indeed, there remains a lack of clinical data investigating this issue.

### 6.1.4 Proliferation versus differentiation

During the proliferation experiments, it was evident that serum from the TBI patients had a significant stimulatory effect on the proliferation rates of the skeletal muscle cell populations. Using a BrdU assay, widespread DNA
synthesis was detected predominantly in nuclei of single cells or nuclei of cell aggregates throughout the tested cell cultures. This was consistent with bright field observations of increased numbers of spindle-shaped mesenchymal cells and the formation of cellular condensations. Unfortunately, immunohistochemical counterstaining of cells with BrdU and other markers was not successful despite multiple efforts, so it was not possible to further identify the exact cell types involved in this process. In any case, the complete identification of multipotent cells within skeletal muscle has not been achieved, even using the most refined techniques. Although the proliferation of mesenchymal cells, as evidenced in the proliferation experiments, is linked to osteoblast development and accelerated fracture healing, in isolation it is insufficient to cause the development of a mature osteoblast and mineralised extracellular matrix. Hence, the next phase of this study was focussed on answering the question: Did the proliferative effect correlate with an effect on osteogenic differentiation?

Differentiation and proliferation are different cellular processes under the control of diverse genetic signals, and both are essential for osteoblast development (Stein and Lian 1993; Balint et al. 2003). Proliferation generates adequate numbers of osteoprogenitor cells to permit successful ossification. The progression of differentiation of an individual cell is marked by a decrease of proliferation capacity with shift towards functions of the mature cell (Baksh et al, 2004). This process of differentiation of skeletal muscle cells into mature osteoblastic cells was monitored through detection of the expression of functional ALP, induction of osterix (Osx) protein and elaboration of a mineralised matrix. The serum factors responsible for inducing the proliferation of the skeletal muscle fibroblastic cells did not appear to inhibit osteogenic differentiation, based on the observation that Osx expression and Villanueva-positive nodules formed in cell populations treated with serum from severe TBI patients at a similar or slightly higher rate to the normal control group. This implies that putative serum proliferation and differentiation factors might work in a coordinated fashion to promote osteogenesis.
6.1.5 Osteogenic effect of serum

6.1.5.1 A suitable environment for production of a mineralised extracellular matrix

The addition of β-glycerophosphate and ascorbic acid to culture medium is required to support complete osteoblast maturation with production of a mineralised matrix (Maeda et al. 2004). Deposition of a collagenous extracellular matrix by a variety of cell types is dependent on ascorbic acid, although it was shown to not be essential in the induction of ossification in mouse myogenic cells (Aronow et al. 1990; Wada et al. 2002). Similarly, β-glycerophosphate is required for elaboration of a hydroxyapatite-containing matrix (Maniatopoulos et al. 1988). Typically, 50μg/ml ascorbic acid and 10mM β-glycerophosphate are used, although lower amounts of each have successfully supported ossification in vitro (Mastrogiacomo et al. 2005; Wada et al. 2002). Interestingly, muscle cells cultured in this study did not tolerate levels of ascorbic acid or β-glycerophosphate higher than 12.5μg/ml and 2mM, respectively, with evidence of retarded cell growth, non-adherence and cell death. Although the mechanisms are unclear, it is suspected that the cultured cells were particularly sensitive to the acid or phosphate at the higher concentrations.

6.1.5.2 Discussion of results

Characteristic cell markers of osteoblastic differentiation were investigated during the differentiation experiments. Specifically, expression of ALP, Osx and a mineralised matrix, were used as representatives of different stages of osteoblast development. Induction of Osx expression in the skeletal muscle cells after exposure to serum or CSF from the patients with CNS trauma was analysed at the protein level by Western blotting. The immortalised human foetal osteoblastic cell line hFOB1.19 was used as positive control in the Western blotting experiments to ensure viability of the Osx protein antibody. These cells were originally extracted from a human foetal limb and transfected with temperature sensitive and neomycin expression vectors. The hFOB1.19 line was established from the colony with the highest ALP expression. They represent a good in vitro model of osteoprogenitor cells capable of differentiation into mature osteoblasts, although Osx expression has not previously been demonstrated in this cell line (Harris et al. 1995). Functional
ALP activity was investigated through the use of an enzyme assay. In addition, the presence of a mineralised matrix was investigated through the use of the Villanueva osteochrome bone stain.

- **Fluorescent Western analysis**

  The fluorescent Western blotting method utilises the property of fluorescent compounds, conjugated to antibodies, to emit light at a higher wavelength than the wavelength of light that it absorbs. Fluorescent immunoblotting has several benefits over ECL, such as the stability of the signal and the ability to detect multiple bands simultaneously (Gingrich et al. 2000; Martin et al. 2003). However, excess background using fluorescent antibodies is a common problem that can be attributable to a number of factors, such as membrane autofluorescence, excessive antibody concentrations and insufficient blocking or washing (Pierce Biotechnology Inc. 2005; Koticha et al. 2006). Testing of primary, secondary and tertiary antibodies, the nitrocellulose membrane and the plastic sleeve revealed a high degree of membrane autofluorescence that was unrelated to antibody concentration or use of milk as a blocking agent. Because of success using the same nitrocellulose membrane after reversion to the ECL method, further protein assessment was undertaken with ECL. Future studies could investigate the effect of different types of membranes and different antibody combinations to optimise the fluorescent system.

- **Osteoblastic differentiation and ossification**

  During extended culture, the supernatant ALP activity within all serum-treated skeletal muscle cell populations increased significantly, demonstrating the growth promoting effects of human serum on cells expressing functional ALP. Kurer et al. (1992) also showed that serum from control volunteers dose-dependently increased the ALP levels in cultures of cells extracted from human femurs, although they were able to show a significant increased ALP level in patients with SCI, with or without HO. An increase in ALP activity in rat bone marrow stromal cells exposed to serum obtained from rats 24 and 48 hours after TBI has also been demonstrated (Klein et al. 1999). The significance of raised ALP levels to the osteoblastic maturation of the cultured cells was further investigated in this study by assessment of the expression of the protein form of Osx and the elaboration of a mineralised matrix. Untreated skeletal muscle cell
populations did not show evidence of Osx mRNA or protein expression, consistent with an immature state of osteoblastic differentiation. After one week in culture, all serum-treated skeletal muscle cultures showed evidence of induction of a 45kDa protein by both fluorescence and ECL methods, consistent with the molecular weight of the Osx protein. The induction of the Osx protein indicates the commitment of potential osteoprogenitor cells within the skeletal muscle populations to become mature osteoblasts. Cells exposed to human sera also showed evidence of mineralisation when cultured for three weeks in a mineralisation medium containing ascorbic acid and β-glycerophosphate. Together with the generic increase in ALP activity and induction of Osx expression, this implies that normal serum contains factors that are capable of supporting osteoblastic maturation and mineralisation by putative skeletal muscle osteoprogenitors in vitro. Previous studies have shown that human serum from healthy subjects can support the growth and differentiation of bone marrow-derived mesenchymal cells grown in osteogenic conditions consisting of ascorbic acid and β-glycerophosphate (Klein et al. 1996; Rojansky et al. 1999; Kobayashi et al. 2005), however, a differentiation agent such as dexamethasone is usually added to enhance this process. In this study, dexamethasone was not added to isolate the osteoinductive effect of the human serum and to minimise multilineage differentiation (Williams et al. 1999).

A number of characteristics of the in vitro environment are likely to have contributed to the small number of observed Villanueva-positive nodules and the similar ALP activity levels in the serum-treated groups. Osteoinducible cells, osteoinductive factors and the local tissue environment interact to determine the amount of bone formed in soft tissues (Chalmers et al., 1975). Potentially short half-lives and inappropriate storage or culturing conditions may have reduced the amount of putative growth factor present in the sera, thus decreasing the ability of the serum to stimulate ossification. Furthermore, insufficient time in culture may have attenuated the degree of mineralisation, although three weeks is usually sufficient for elaboration of a mineralised matrix in similar cellular contexts (Mastrogiacomo et al. 2005). In addition, the influence of paracrine and autocrine factors operating locally within the cell cultures cannot be discounted, nor can the role of an influx of cells from the circulation in vivo (Rawadi et al. 2003; Eghbali-Fatourechi et al. 2005; Sata et al. 2005). A scarcity of cells within
the skeletal muscle populations that were responsive to the osteoinductive stimulus, perhaps due to the use of the primary cell culture isolation technique, may also help to explain the absence of more effective expression of the osteoblastic phenotype by the cell populations. Visible nodules are the end-result of the entire process of proliferation and differentiation constituting osteoblast development, and they rely on a supply of cells with osteogenic potential, which may be relatively rare in primary skeletal muscle cultures (Roman-Roman et al. 2003). Although primary muscle cell cultures are inclusive of the broad range of cell types present in vivo, putative osteoprogenitor cells may be present in reduced quantities compared to more refined isolation techniques such as those involving cryopreservation of primary cultures, preplating or flow cytometric cell sorting (Qu et al. 1998; Bosch et al. 2000; Lee et al. 2000; Young et al. 2002; Kalajzic et al. 2005). However, as no skeletal muscle stem cell-specific markers have been definitively established, even with the use of refined techniques, the complete identification and localisation of the putative multipotent cells is unclear of specific osteoprogenitor cells and, furthermore, the extent to which the cell phenotype is influenced by the act of processing is uncertain (Deasy et al. 2001; Zammit and Beauchamp 2001; Lee et al. 2000).

6.1.5.3 Possible relevance to the development of HO following CNS trauma

Villanueva staining nodules arose from the population of spindle shaped cells, as opposed to cells with the myotube morphology, implying that cells involved in ossification within skeletal muscle cells in vitro lie external to the myofibre. The involvement of the skeletal muscle interstitium in HO has been suspected for decades (Urist et al. 1973; Soren and Waugh 1983; Puzas et al. 1989; van Kuijk et al. 2002; Cohly et al. 2003). Histological analysis of HO within human skeletal muscle following spinal cord injury shows that the heterotopic deposits of bone are separated from the muscle fibres by a plane of fibrous tissue, which often dissociates the fibres and results in local muscle atrophy and sclerosis (Damanski 1961). Early authors theorised about the metaplasia of skeletal muscle connective tissue cells into bone. It was proposed that two distinct classes of “fibrocytes” exist: an ossifying type, comprising the connective tissues of striated muscle; and a non-ossifying type (Huggins 1931). It has since been shown that one of the first histological alterations in muscle undergoing
HO is the proliferation of intramuscular mesenchymal cells (Craven and Urist 1971; Urist et al. 1978; van Kuijk et al. 2002). The crucial role in osteoblast development of mesenchymal stem cells reiterates that they are an important source of osteoprogenitor cells in HO within skeletal muscle. The proliferation of mesenchymal cells has been linked to accelerated fracture healing in rats with TBI in vivo, indicating the importance of this process in the increased osteogenesis seen after TBI (Boes et al. 2006). Taken together, it is plausible that the additional proliferative effect of serum factors present after severe TBI on skeletal muscle mesenchymal and early osteoblastic cells shifts the balance towards the development of bone in a tissue in which bone is not normally found. This proliferative effect may be essential in the development of HO in these patients by producing an excess of cells with osteogenic potential within soft tissue and at fracture sites. Local (Fujimura et al, 2001; Lotta et al, 2001; Major et al, 1980; Comarr et al, 1962) and genetic factors (Shore et al. 2006; de la Pena et al. 2005; Goncalves et al. 2005; Marusic et al. 1999) are also likely to play a role in this process in vivo (Figure 37).
Figure 35: Schematic of possible contributing factors to the development of heterotopic ossification (HO) in skeletal muscle following CNS trauma. In vitro evidence suggests that serum from patients with TBI has a proliferative effect on mesenchymal cells, both from skeletal muscle (this study) and immortalised cell lines (Boes et al. 2006), as well as osteoblastic cells derived from fetal rat calvaria (Bidner et al. 1990) and immortalised cell lines (Gautschi et al. in press). This proliferative effect may be essential in the development of HO in TBI patients by producing an excess of osteoprogenitors within soft tissue and at fracture sites. The absence of a response to CSF following TBI increases the likelihood that the putative proliferative factor(s) within the serum do not originate directly from trauma to the brain. The lack of proliferative effect of serum from SCI patients may indicate the importance of alternate pathways in the development of HO in these patients. In addition, human serum appears to constitutively support the process of osteoblast development and mineralisation within primary skeletal muscle cell cultures cultured in a suitable mineralisation medium. Local and genetic factors may influence this process in vivo.

(hFOB 1.19 = immortalised human fetal osteoblast cell line; FRC = fetal rat calvarial; BMPs = bone morphogenetic proteins)
6.1.6 Serum versus CSF

Unlike the effects demonstrated with exposure of the skeletal muscle cells to serum, CSF from either TBI or control subjects had no significant effect on proliferation or differentiation compared to the negative control. The only previous study to report an osteogenic effect of CSF following CNS trauma investigated the effect on rat bone marrow stromal cells of pooled CSF from rats with TBI (Klein et al. 1999). The lack of a demonstrable effect in the CSF experiments in this study raises a number of interesting possibilities regarding the pathogenesis of HO. From a methodological perspective, the laboratory handling of CSF may have contributed to the lack of effect. The R.P.H. laboratory processed all CSF samples for clinical purposes before experimentation was performed. For this reason, the CSF was stored at +4°C at all times rather than -20°C, as per the serum samples. Furthermore, the CSF was only collected from the R.P.H. laboratory weekly. The effects of this storage and collection method on any potential compounds within the CSF are unknown. However, denaturation of proteins due to storage at inappropriate temperatures or with lengthy delays before analysis may have contributed to the lack of a proliferative or osteogenic effect. Also, the concentrations of CSF tested were low (≤ 6.25% of the total medium volume), but equal to the sera amounts used. Greater quantities of CSF might be required to have a measurable effect on cell growth as only small concentrations of putative serum factor may enter the CSF. For example, damage to specific parts of the brain, such as the pituitary gland, could result in the release of an osteogenic factor directly into the systemic circulation without entry of significant levels into the CSF. However, a CSF concentration of 2% has previously been shown to support mineralisation in rat bone marrow stromal cells (Klein et al. 1999). The amount of mineralisation induced by CSF from both the rats with TBI and the uninjured rats was higher than that of a dexamethasone control, although not significantly so, suggesting that CSF has an inherent ability to support ossification. A statistically similar decrease in cell number and higher ALP activity in both TBI and normal groups was also measured, which was not corroborated by this study (Klein et al. 1999). The differences between this study and that of Klein et al. (1999) may be due to a lack of adequate growth factors present in the CSF in this study to facilitate the maintenance of the cells...
in culture, let alone their proliferation or differentiation. In addition, the proliferation assay in this study continued for only 5 days as opposed to 11 days in the Klein et al study. However, even by day 5 there had been a drop in the ALP expression of cells exposed to CSF compared to those exposed to serum and, after one week, there was an absence of Osx expression. This indicates a lack of early effect on both proliferation and osteogenic differentiation of CSF while the cultured cells remained viable. Ultimately, the lack of CSF effect could be evidence that the humoral effect observed in this study is not directly related to CNS trauma. This study does not exclude the possibility of an indirect mechanism for release of a humoral factor after TBI. That is, the putative factor may arise from another source apart from the damaged CNS. Locations such as immobilised bony matrix, peripheral nerves or denervated tissues cannot be excluded as sources for serum-mediated factors following CNS trauma (Bidner et al. 1990; Mohan and Baylink 1991; Karmani and Compson 2000; Lane et al. 2002). Additionally, a decrease in an osteoinhibitory factor is possible (Calandriello 1964; Bidner et al. 1990; Andermahr et al. 2006). Hence, the source, as well as the identity, of the serum-mediated effects remains unidentified.

6.1.7 Potential implications for the clinical manipulation of osteogenesis

6.1.7.1 Skeletal muscle osteoprogenitors in HO and bone regeneration

Isolation of an adult skeletal muscle cell population capable of participating in ossification within a pathological context provides new understanding about the pathogenesis of HO. Clinically, a better understanding of the development of HO should educate clinicians about the most effective methods of prevention and management of the morbidity caused by this condition. The use of easily available cells within skeletal muscle for bone formation may also aid in future research into clinically applicable areas such as alternative bone graft sources.

6.1.7.2 Isolation and clinical use of serum osteoinductive factor(s)

Further evidence to support the existence of a humoral osteoinductive factor or collection of factors, as provided by this study, is expected to stimulate further investigation into the identification of such factors. The ultimate clinical aim is the therapeutic use of an agent that could accelerate fracture healing and assist in the treatment of other common skeletal conditions, such as osteoporosis. An
understanding of the pathomechanisms of the putative factors might also facilitate inhibition of signalling pathways in conditions of exuberant ossification. Thus, the isolation of a factor, such as that which may be released following TBI, would benefit countless numbers of patients.

6.1.7.3 Candidate serum factors

The candidates for the serum factors proposed by this and previous studies are numerous and the identity and source of such factors remain the topic of speculation. Many factors that may influence mesenchymal and osteoprogenitor proliferation and the development of HO within skeletal muscle are present in the serum in abnormal concentrations following TBI. For example, abnormalities in the levels of interleukin-6 (Beeton et al. 2004), leptin (Rigaux et al. 2005), fibroblast growth factor (Wildburger et al. 1994b; Wildburger et al. 1995, Wildburger et al. 1996), growth hormone (Wildburger et al. 2001), insulin-like growth factor-1 (Beeton et al. 2002; Wildburger et al. 2001), prolactin (Wildburger et al. 1998) and parathyroid hormone (Trentz et al. 2005), amongst others, have already been investigated without great success. It might be expected that the most promising candidate group of potential factors would be the BMP family (Goff & Reichard 2006). BMPs play a central role in the entrance of mesenchymal cells into the osteogenic lineage. Individual BMPs are capable of initiating the entire cascade of osteogenesis within responsive tissues in vitro and in vivo, at least in animal models (Wozney 2002). In adult rat brains, BMP receptors are upregulated following TBI, indicating the possible role of BMPs in neuronal plasticity pathways (Lewen et al. 1997). Similarly, BMP7 mRNA is found at significantly higher levels in glial cells after traumatic SCI (Setoguchi et al. 2001). A recent study found that rats with TBI expressed mRNA for BMP-2 and 4 in hip musculature, but rats without TBI did not (Scherbel et al. 2001). The suggestion that following TBI skeletal muscle, by an unclear mechanism, exhibits upregulated genetic expression of molecules directly capable of ossification is promising because it would help to explain the increased osteogenesis seen within skeletal muscle after CNS trauma. This study supported the possible role of BMPs in facilitating ossification within human skeletal muscle by the detection of BMP receptor mRNA within skeletal muscle cell populations. However, the relevance to human populations of findings in animals has not been determined. Several examples highlight that
BMP effects in animals may not accurately imitate *in vivo* events in humans. For instance, heterotopic bone produced by BMP implantation in animal skeletal muscle forms by endochondral ossification (Wozney 2002), but specimens retrieved from humans typically do not contain cartilage, chondrocytes or their precursors (Puzas et al. 1989; Damanski 1961). The direct relevance of BMPs to the serum-mediated hypothesis of HO following CNS trauma is also questionable due to their limited systemic viability (Termaat et al. 2005). Furthermore, work by this group has shown that rhBMP-2, -4 and -7 have an apoptotic effect in hFOB1.19 cells, an *in vitro* model of human osteoprogenitor cells (data not shown). In the end, it is unlikely that one known factor is individually responsible for HO following TBI; a novel factor or a combination of known factors acting together is likely to be necessary (Goff & Reichard 2006).
6.2 Future directions

This study has revealed key points in the pathogenesis of HO following CNS trauma. It has also highlighted some of the avenues of investigation that remain to be explored before a complete understanding of this enigmatic condition is achieved.

6.2.1 Animal models

One limitation of *in vitro* investigations is the inability to take into account the multitude of factors that dictate disease within living organisms. The serum-mediated stimulation outlined in this study plays only one role in HO development within the skeletal muscle of patients following CNS trauma. The interplay between genetic and acquired predisposition to HO produces the heterogeneity of clinically observable HO in patients that have experienced apparently similar injuries and treatments. In the absence of ethically acceptable human models of HO and CNS trauma, animals could be used to further investigate these conditions in the future. Animal models of TBI and HO are found throughout the literature and have met with variable success (O'Connor 1998; Cernak 2005). Animal models have several benefits over human studies, including availability and reproducibility of research subjects. They allow standardisation of the type and severity of CNS trauma, removing the heterogeneity of human populations and allowing more accurate comparison between groups. Brain, muscle and spinal cord parenchyma can be repeatedly extracted from genetically similar animals. The major disadvantage to the use of animals is the uncertainty about extrapolation of findings to human populations. A direct extension of this study could be the implantation of primary skeletal muscle cells exposed to serum or CSF from CNS trauma patients into immunodeficient mice to assess *in vivo* cell survival. Additionally, an animal model of HO following CNS trauma could be used in association with cell labelling techniques to identify the type and location of cells involved in HO within skeletal muscle.

6.2.2 Clinical correlation

Correlation between clinical risk factors and the development of HO would determine the true relevance of CNS injury to increased osteogenesis in human
populations. A concurrent clinical and radiological assessment of increased osteogenesis must ensure appropriate matching of patient populations, routine radiographs and similar degrees of follow-up for all patients. The clinical setting usually determines that certain patients will be assessed more regularly than others because of their conditions, so it may be difficult to avoid the inherent bias in this regard. It may eventually be shown that there is no definitive link between laboratory findings and clinical observations (Pape et al. 2001). In vitro, a focussed assessment of established compounds on a cell culture is undertaken, whereas in vivo many factors influence the ultimate degree of HO. Some patients, whether by genetic or other predisposition, may be more likely to develop HO with the same or reduced quantity of the putative serum factors. In any case, it is imperative that a clinical study involving patients with SCI accurately matched for fractures and other demographics with patients without SCI should be conducted to determine the rate of fracture healing in this group.

6.2.3 Proteomics - a way forward

Ultimately, a thorough analysis of serum, CSF and other body fluids and tissues, such as that offered by proteomics, may be most useful in revealing the factors underlying the serum-mediated effects following TBI observed in this study (Denslow et al. 2003; Romeo et al. 2005; Schuhmann et al. 2005; Wang, Ottens et al. 2005; Ottens et al. 2006). Proteomics is the systematic collection and identification of proteins, using 2D electrophoresis, mass spectrometry and corroboration of results with protein libraries (Ottens et al. 2006). By using proteomics, the effect on protein profile of inciting events, such as CNS trauma, can be determined. There are few published studies that have used proteomics to analyse blood or CSF following CNS trauma, although there has been earnest discussion as to the potential benefits of such an approach (Denslow et al. 2003; Romeo et al. 2005; Schuhmann et al. 2005; Wang, Ottens et al. 2005; Ottens et al. 2006). Future studies should be focussed on a proteomic analysis of both blood and, perhaps, CSF of CNS trauma patients to elucidate discrepancies in comparison to normal populations. In this way, a putative osteogenic factor may be isolated.
6.3 Conclusions

Heterotopic ossification following central nervous system trauma is a complex condition involving numerous cell types that produce *de novo* ossification within soft tissues under the influence of multiple factors. Through this study, a population of cells isolated from skeletal muscle and possessing a predominantly mesenchymal phenotype with expression of osteogenic markers could represent putative osteoinducible cells. These mesenchymal cells responded to factors present in the serum of patients with severe traumatic brain injury within the first three days after injury by exhibiting an increased rate of proliferation. It was speculated that this increased rate of proliferation is required to enable the expansion of osteoinducible cells within the skeletal muscle, thereby overcoming the influences that prevent the deposition of heterotopic bone in normal circumstances. Furthermore, skeletal muscle cell populations treated with serum from humans with and without central nervous system damage possessed an ability to support the development of a bony phenotype. These findings imply that skeletal muscle cells have the capability to be directly involved in the development of heterotopic ossification. This idea is biologically plausible because ossification within skeletal muscle has been documented clinically. The lack of a proliferative effect of serum from patients with spinal cord injury suggests that the underlying aetiology of heterotopic ossification in these patients may be different to that in patients with traumatic brain injury. Furthermore, the lack of an effect of cerebrospinal fluid from patients with central nervous system trauma on the proliferation or differentiation of skeletal muscle cells possibly suggests that the damaged central nervous system may not be the origin of putative osteogenic factors, however, alternative explanations for this lack of cerebrospinal fluid effect cannot be excluded. The factor or factors responsible for these effects are not currently known. Much work lies ahead to better define which factors lead to heterotopic ossification within skeletal muscle. With this knowledge, there is the potential to assist untold numbers of patients in the rehabilitation from central nervous system trauma, fractures and other bone disorders.
Chapter 7: References


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Chapter 8: Appendix