Understanding heterotopic ossification after spinal cord injury

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MD

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Mater Research Institute
Abstract

Neurological heterotopic ossification (NHO) is a frequent complication of spinal cord and traumatic brain injuries (15-25% of patients) and manifests as abnormal ossification of soft tissues near joints. NHO is very debilitating and further delays rehabilitation as it causes pain, joint deformation and ankylosis and vascular and nerve compression. In the absence of an animal model the mechanisms leading to NHO are unknown and consequently there is no preventive treatment. The only effective approach to eliminate HO is complicated and expensive surgical resection. However these surgeries are delicate and can be challenging as they are performed once the NHO are mature, often large and incapacitating entrapping large blood vessels and nerves.

To elucidate NHO pathophysiology, we have developed the first animal model of NHO in genetically unmodified mice. Mice underwent a spinal cord transection (SCI); muscular inflammation was induced by intramuscular injection of cardiotoxin in limbs (IM-CTX). Formation of NHO was followed by μCT and immunohistology.

SCI alone or muscular inflammation alone did not induce NHO in mice. The combination of both SCI and muscular inflammation was necessary to induce NHO. This is consistent with clinical observations as NHO incidence is higher in patients with severe trauma or concomitant infection. Abundant F4/80⁺macrophages, which can provide pro-anabolic support in bone formation, were detected within the inflamed muscle and associated with areas of intramuscular bone formation (confirmed by von Kossa and collagen type 1 staining). In vivo depletion of phagocytic macrophages with clodronate-loaded liposomes prevented NHO formation whereas Zoledronate treatment exacerbated NHO. This supports our hypothesis that macrophage-mediated inflammation is a key activator of NHO following SCI. To further identify the source and type of macrophages, involved in the bone formation after SCI and test some potential treatment options different knock-out mouse models were investigated. My data suggest that local muscle residential macrophages potentially play an important role in NHO.

In addition we identified several populations of muscle progenitor cells that are prone to osteogenic differentiation in vitro. These data suggest that the mesenchymal progenitors that differentiate into osteoblasts in HO following spinal cord injury are already present in healthy muscles and therefore can be derived from local muscle progenitor cells rather than being recruited from the remote site, such as bone marrow.
Finally we investigated why SCI was necessary for NHO development. My hypothesis was that SCI causes release of systemic factors priming NHO. In support of this, I showed that NHO developed in non-paralysed inflamed front limbs of mice with SCI. Also, blood plasma from mice with SCI and IM-CTX induced osteogenic differentiation of cultured muscle mesenchymal progenitor cells (mMPC) sorted from naïve mice. This suggests that systemic factors facilitating NHO, such as substance P and G-CSF are released following SCI. These factors were then specifically targeted them in our mouse model to trial different types of treatment for prevention of bone formation.

In conclusion, our model suggests that NHO is a 2-insult process with 1) SCI inducing the release of factors that sensitize muscle progenitor cells to abnormal osteogenic differentiation and 2) macrophages accumulating in inflamed muscles then triggering abnormal osteogenic differentiation of mMPC. This study represents a significant advance in the understanding of NHO, revealing two targetable pathophysiologic mechanisms.
Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Publications during candidature


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Contributions by others to the thesis

All members of the Stem Cell and Cancer and Stem Cell Biology groups and supervisory team contributed to this thesis. Associate Professor Jean-Pierre Levesque and Associate Professor Ingrid Winkler conceptualised this project. Associate Professor Jean-Pierre Levesque, Associate Professor Ingrid Winkler and Dr Allison Pettit assisted with the design and interpretation of experiments. Dr Francois Genet introduced the animal model used in the project. Mrs Bianca Nowlan, Dr Susan Millard provided technical assistance with PCR work and immunohistochemical analysis. Dr Cerdyck Vaquette from the Queensland University of Technology provided technical assistance with microCT scan of animals and 3D μCT reconstitution. Some technical assistance was also provided by Dr Dalia Khalil and Mr Robert Wadley in regards to Flow cytometry. Non-routine technical work was provided by Mr Lawrie Wheeler from the University of Queensland Diamantina Institute for performing the microarray service required for Chapter 5. Assistance with analytical interpretation of transcriptome research data (Chapter 5) was provided by Dr Gethin Thomas from the University of Queensland Diamantina Institute. The laboratory groups in the Biological Therapies program at the Mater Research Institute also assisted with intellectual input into this project.

Statement of parts of the thesis submitted to qualify for the award of another degree

None
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<tr>
<td>α-MEM</td>
<td>Minimal Essential Medium, alpha</td>
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<tr>
<td>β2M</td>
<td>β-2-microglobulin</td>
</tr>
<tr>
<td>µCT</td>
<td>Micro-computerized tomography</td>
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<tr>
<td>3D</td>
<td>Three-dimensional</td>
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<tr>
<td>7AAD</td>
<td>7-aminoactinomycin D</td>
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<tr>
<td>Ab</td>
<td>Antibody</td>
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<td>APC</td>
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<td>APC-Cy7</td>
<td>Allophycocyanin-cyanine 7</td>
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<td>BM</td>
<td>Bone marrow</td>
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<tr>
<td>bmMSC</td>
<td>Bone marrow mesenchymal stromal cells</td>
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<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
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<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte-colony stimulating factor</td>
</tr>
<tr>
<td>gDNA</td>
<td>Genomic DNA</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>h</td>
<td>Hour/s</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks' balanced salt solution</td>
</tr>
<tr>
<td>Hif-1α</td>
<td>Hypoxia Inducible Factor 1α</td>
</tr>
<tr>
<td>HO</td>
<td>Heterotopic ossification</td>
</tr>
<tr>
<td>hu</td>
<td>Human</td>
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<tr>
<td>IC</td>
<td>Interstitial cell</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>INF</td>
<td>Interferon</td>
</tr>
<tr>
<td>M</td>
<td>Mouse</td>
</tr>
<tr>
<td>M1</td>
<td>“Classically activated” Macrophage</td>
</tr>
<tr>
<td>M2</td>
<td>“Alternatively activated” macrophage</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cell</td>
</tr>
<tr>
<td>Min</td>
<td>Minutes</td>
</tr>
<tr>
<td>mln</td>
<td>million</td>
</tr>
<tr>
<td>MPC</td>
<td>Mesenchymal progenitor cell</td>
</tr>
<tr>
<td>MRI</td>
<td>Mater Research Institute</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killers</td>
</tr>
<tr>
<td>OCT</td>
<td>Optimal cutting temperature</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PBTX</td>
<td>Phosphate-buffered saline (Ca(^{2+}) and Mg(^{2+})-free), containing 0.1% (v/v) Triton X-100</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PE-Cy7</td>
<td>Phycoerythrin-cyanine 7</td>
</tr>
<tr>
<td>PenStepGlu</td>
<td>Penicillin Streptomycin Glutamine</td>
</tr>
<tr>
<td>PerCP</td>
<td>Peridinin chlorophyll protein</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>Poly I:C</td>
<td>Polyinosine:polycytidylic acid</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantative reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>R</td>
<td>Receptor</td>
</tr>
</tbody>
</table>
RNA  Ribonucleic acid
RO  Reverse osmosis
RT  Reverse transcriptase
RT-PCR  Reverse transcription polymerase chain reaction
rpm  revolutions per minute
SAV  Streptavidin
SC  Satellite cell
SCF  Stem cell factor
SCI  Spinal cord injury
SD  Standard deviation
SDF-1  Stromal cell-derived factor-1
SEM  Standard error of the mean
°  Temperature
TBI  Traumatic brain injury
TGF  Tissue growth factor
TNF  Tumour necrosis factor
TRI  Translational Research Institute
UQ  University of Queensland
Chapter 1: Introduction

1.1 Heterotopic ossification

1.1.1 Description of disease
Heterotopic ossification (HO) was first described in patients with spinal cord injury (SCI) by Dejerine and Ceilier in 1918, during the First World War with the advent of X-ray radiography, however it started being investigated more systematically around the 1950s. Heterotopic ossification is described as a formation of bone tissues in “abnormal” sites, usually in soft tissues. HO is associated with many well-known diseases such as Guillain-Barre [1], hypophosphataemic vitamin D resistant osteomalacia [2], fibrodysplasia ossificans progressiva [3], etc. Heterotopic ossification is also well described following hip arthroplasty/surgery [4]. In general this pathology can be divided into 3 groups on ethiology: genetically predisposed (e.g. fibrodysplasia ossificans progressiva), trauma associated (surgery, burns, multitrauma) and neurogenic (spinal cord injury, brain injury) [5].

It results in a wide array of complications, that reduce the quality of life, these include partial to complete joint ankylosis, regional pain syndrome, osteoporosis, vessel and nerve compression and soft-tissue infection, all of which result in higher morbidity and mortality.

1.1.2 Epidemiology
Neurogenic heterotopic ossification is reported to occur in 25% to 60% of adult patients with spinal cord injury [6-9], and in 10% of paediatric patients [10].

The main sites of heterotopic ossification after brain injury or spinal cord injury are hip, shoulder, elbow and knee, based on the trial performed by Garland et al. on 496 patients [11]. Hip was also reported to be the main ossified site after SCI in paediatric population [10]. Heterotopic ossification has also been described in posterior longitudinal ligament [12], yellow ligament [13] and vocal cords [14].

Male sex and young age have been suggested to be risk factors for HO [15, 16], possibly due to the association with military service and more risky behaviour, although to date there is no sufficient data about reliable association of HO with race, sex or age [17].

1.1.3 Pathogenesis
To date pathogenesis of NHO is unknown due to the fact that most research of NHO in patients is retrospective.
1.1.3.1 Insight to FOP

Neurogenic HO is in some aspects similar to a very rare (1 in 2000000) genetic autosomal dominant disorder, leading to massive ossification of the whole body - fibrodysplasia ossificans progressiva. It is caused by mutations in the ACVR1 gene that encodes the bone morphogenetic protein (BMP) type I receptor activin A receptor-1 (ACVR1 also called ALK2). These activating mutations of ACVR1 cause the abnormal sensitisation of mesenchymal progenitor cells (MPCs) to BMPs, and stimulate their abnormal osteoblastic differentiation [18] [19]. As a result of these receptor is constantly active which leads to uncontrolled growth of bone and cartilage in muscles and joints. Also there have been reports that overexpression of BMP2/4 [20] together with underexpression of their antagonists noggin, chordin and follistatin are responsible for the symptoms of FOP [20-22]. All these findings require further investigations as they could be potential mechanisms for NHO after SCI. In patients with FOP, HO generally starts in the first decade of life and progressively expands to completely ossify the body before 40 years of age. Small injuries or trauma such as immunisation, surgery, viral infections or even physical activity can flare up HO to dramatic proportions [23]. This suggests that innate immunity/inflammation is a triggering event of HO in FOP. Further supporting this notion is the beneficial clinical response to high dose corticosteroids within the first 36 hours of HO flare-up in FOP patients[24]. Taking together all aforementioned findings this is possible to suggest that two major pathways are causing HO in FOP: abnormally elevated BMP signalling combined with inflammation.

Few investigations also assessed BMP-4 antagonists (noggin) inhibition [25] and inhibition of BMP type I receptors ALK2 and ALK3 in FOP [26]. Fontaine et al. reported a new mutation of the Noggin gene FOP: a guanine to adenine change that leads to substitution of Alanine residue by a Threonine [25]. Yu et al. have discovered a small-molecule inhibitor of BMP type I receptors, dorsomorphin, which selectively blocks ALK2, ALK3 and ALK6, followed by a discovery of a selective inhibitor of BMP type I receptor kinases, LDN-193189. This research group showed efficacy of LDN-193189 in reducing HO in vitro and in vivo on caALK2–transgenic mice. The same group has also mentioned that similar to corticosteroid treatment LDN-193189 inhibits ossification, suggesting caALK2 expression and an inflammation are both required for the development of ectopic ossification in their mouse model [26].

1.1.3.2 Mechanism and pathological changes in NHO following SCI

The mechanisms of heterotopic ossification following SCI are still poorly understood. Pathological changes are mediated by formation of osteoblasts from the intramuscular cells source, which can be muscle mesenchymal stem cells or so called interstitial cells or
muscle satellite cells from the intramuscular fibers or alternatively migration from the bone marrow [27]. Chalmers et al suggested that NHO may be caused by the induction of muscle progenitor cells to differentiate into osteoblasts as a result of mitogenic and osteogenic factors in the serum of SCI patients, or to be precise by changes in balance-ratio between pro- and anti-osteoinductive mediators [28]. Histologically HO presents as local microvascular alterations, vascular stasis and oedema of surrounding tissues [29]. Although an attractive hypothesis, still very little is known about release of osteoinductive biochemical factors and their influence on osteogenic differentiation of MPCs.

In addition the mechanism of neurogenic heterotopic ossification appears to have a traumatic component or inflammation, such as invasive medical procedures or bone fractures. Shehab et al. have found that prostaglandin E2 release during inflammatory response can drive osteogenic differentiation of cells as a mechanism of HO [30]. Heterotopic ossification after spinal cord injury has also been shown to be associated with the presence of spasticity and pressure ulcers [31]. A case-control study of 264 patients performed by Citak et al. has revealed higher risk of HO based on ultrasound after higher lesion of spinal cord (thoracic level in comparison with lumbar), spasticity, tracheostomy, pneumonia and urinary tract infection. So inflammation appears to also play an important role in developing HO [32]. Bone fractures and mechanical ventilation have also been risk factors associated with a higher incidence of HO after traumatic brain injury in 176 patients [33]. The neurogenic factor (degree of palsy) was found to induce HO in patients with brain injury [34].

HO is reported to be associated with peripheral nerve system injury as well as CNS [35]. Salisbury et al. described the model of FOP in which HO are caused by injection of mouse fibroblasts transduced with adenovirus carrying BMP-2. Using this model the authors further proved that inflammation induced in sensory neuron contributes to HO [36]. These authors suggest that BMP2 stimulates release of substance P and calcitonin gene related peptide, both of which indicate induction of neuroinflammation, which contributes to HO. In other trials BMP2 also has been shown to induce the release of neuroinflammatory proteins from sensory neurons such as substance P and calcitonin gene related peptide [37].

NHO in traumatic brain injury (TBI) pathogenesis is a subject of a great interest nowadays and there are a few results, published recently. Several biochemical markers were found in human serum from patients with central nervous system injury, such as C-reactive protein, erythrocyte sedimentation rate, Interleukin-6, Parathyroid hormone, Alkaline Phosphatase, tumour necrosis factor-alpha (TNF-α), that could be potential biomarkers for NHO [38].
BMP2 was found to be elevated after muscle injury and released during bone injury [39, 40]. Increased expression of osteoblastic differentiation genes Runx-2, osterix and cathepsin-K has been reported in TBI patients [41]. BMPs in cerebrospinal fluid from TBI patients were not reported to be in high concentrations so is unlikely to be responsible for the osteogenic cell response that triggers HO [42]. Basic fibroblast growth factor (bFGFs) was shown to increase up to seven times above normal in serum of head injured patients with a concurrent local injury [43]. 24-hour urinary hydroxyproline, which plays important role in collagen stability, has been observed in spinal-cord injured patients, though its effect on HO has not been investigated [44]. Trentz et al have published a study of 80 patients with TBI, where he reported a role of parathyroid hormone (PTH) in HO as a regulator of homeostasis of calcium and phosphate [45]. Leptin has been found to be low in patients with neurogenic HO [38], although it is known to be a promoter of bone progenitors and osteoblasts activation and was found also to decrease osteoblastic activity by binding to hypothalamic neurons [29]. Osteocalcin is another biomarker of bone remodelling, which was found to be significantly lower in patients with TBI [45]. Capmos de Paz et al. have reported that in CNS injury the mechanism of HO could be due to dysfunction of proprioception, so MSCs would undergo osteogenic differentiation on ligaments and muscle as an answer to stimuli [6].

To sum up, neurodamage and inflammation may both be required for the heterotopic bone formation. There are a number of changes in the serum of patients with SCI; however it is still not clear how it affects the osteogenic differentiation of cells in muscles. Also the definite origin of osteoprogenitors responsible for NHO is to be defined.

1.1.4 Current mouse models for FOP and HO

There are currently three mouse models of FOP all involving the BMP signalling pathway. The first one is caused by BMP4 transgene under the control of the neuron-specific enolase (Nse) reporter driving BMP4 expression in neurons, osteoblasts and macrophages [46]. Although HO spontaneously occurs in ageing nseBMP4 mice with progressive leukocyte infiltration in muscles, additional inflammation by subcutaneous or intramuscular injection of cardiotoxin considerably accelerates HO of the muscles at the site of injection within 2-3 weeks [47]. The next two mouse models of HO utilise activating R20H and Q207D mutations in the ACVR1 gene that causes FOP in humans. The first mutation is knocked-in the mouse Acvr1 gene [48] while the second is Cre-inducible
mutant transgene [26]. In both models intramuscular injection of cardiotoxin dramatically accelerates HO of the muscle at the site of injection.

In summary all current models utilise mice that have been genetically modified to increase BMP signalling. This suggests BMP signalling is a strong candidate pathway involved in development of HO. All three genetic models of FOP support our hypothesis that muscular inflammation triggers SCI-induced HO in normal individuals. However these genetically driven models of FOP are not physiologically relevant to SCI induced NHO as its prevalence is quite high – 15-25% of SCI patients, whereas FOP is very rare (1 in 2000000). Also single nucleotide polymorphism (SNP) analysis has not revealed SNPs in BMP pathways in NHO patients.

We are the first who developed a model that more closely matches the patients setting using SCI in normal mice plus an inflammatory trigger and will look at BMP signalling in this model.

1.1.5 Treatment

In many patients heterotopic ossification is so severe and debilitating that it impacts their lives in significant ways. Patients experience severe pain. Joint deformation occurs that may lead to ankylosis or grow around neurovascular bundle, compressing nerves and blood vessels. In paralysed patients it can even lead to inability to use a wheel chair.

Diagnosis of HO is usually made by X-rays, using Brooker classification of HO, bone scintigraphy and clinical examination. Lack of standardisation in diagnosing HO and absence of long follow-up in many trials make hard to form guidelines about HO treatment and prevention.

Treatment of HO has been divided into three main groups in one of the last review articles [49]. There are pharmacological treatment, non-pharmacological and the combination of both.

1.1.5.1 Pharmacological treatment

Pharmacological treatment includes NSAIDs – non-selective COX inhibitors and selective COX-2 inhibitors (which showed an increased risk of myocardial infarction [50]), etidronate (which delays calcification of heterotopic bone formation), N-acetylcysteine, warfarin and BMP receptors inhibition, but none of them were chosen as a guideline for HO prevention or treatment [51]. The most popular treatment remains NSAIDs – indomethacin and rofecoxib, bisphosphonates such as etidronate, however these often cannot be administered longterm.
NSAIDs are a cheap option with simple way of administration, inhibiting PGE synthesis and bone formation. This treatment can be successfully used perioperatively and in early postoperative period. NSAIDs have some adverse effects. NSAIDs increase perioperative bleeding, have serious gastrointestinal adverse effect and can cause thrombocytopaenia in case of prolonged treatment. Selective NSAIDs like meloxicam have less side effects, though they have a risk of cardiovascular events. Rofecoxib is not used anymore in clinical practice due to cardiovascular adverse effects and gastrointestinal events. Furthermore prophylactic NSAIDs used to prevent HO can also cause delayed healing of fractures and decrease bone healing in other parts of the body which is undesirable effect in case of multiple trauma associated with SCI [51]. Banovac et al performed few randomised control trials studying effects of bisphosphonates and NSAIDs with positive results [52-55], while Garland found no evidence of improvement in patients with HO, who received etidronate [56]. Considering these differences Cochrane review was published that assessed these publications and did not find it convincing [57]. N-acetylcystein and allopurinol combination was compared with NSAID (indomethacin) in the prevention of bone formation. Significant difference between two types of treatment was found by day 32 [58, 59]. Authors reported the possibility to reduce HO by scavenging of free radicals, the amount of which increases markedly due to ischemia/reperfusion injury – which often accompanies SCI in severe combined trauma. Warfarin was used to prevent HO by one group of researchers, who suggested that it inhibits vitamin K activity, while osteocalcin production and maturation is associated with a vitamin K-dependent carboxylation [60]. However since then no one showed clinical benefit from warfarin use in heterotopic ossification, moreover a few reports demonstrated increase in vascular calcification and osteoporosis [61].

1.1.5.2 Non-pharmacological treatment
Non-pharmacological treatment includes slow intensity electromagnetic field therapy [62], radiotherapy and physiotherapy, passive range of motion. However surgical excision remains the most widely practised option despite of certain complications such as wound infection, dehiscence, risks associated with anaesthesia etc. Surgical excision of mature and often large and incapacitating HO sites is the main option in most rehabilitation centres, although besides usual aforementioned complications associated with anaesthesia and surgery such as pressure sores, osteomyelitis, infection and bleeding, the range of motion gained after surgery is reduced with time.
Radiation therapy was used to treat HO in many trials, and although in some publications it was found successful [63-66], it doesn’t prevent bone formation in about 20% of patients [67] while has serious adverse effects, such as increased carcinogenesis and delayed healing [65]. Physiotherapy to improve the range of motion combined with bisphosphonates and surgical removal of HO has been reported to have a great benefit, though mostly in patients with the largest restriction preoperatively, according to De Palma [68].

1.1.5.3 A combination of pharmacological and non-pharmacological treatments
The combination of treatment combines surgery with either NSAIDs or bisphosphonates. Another described option of combined treatment is NSAIDs and electromagnetic field treatment to increase oxygen level and blood supply as an aim to prevent primary and secondary HO accordingly [68].

One of the first examples of combined therapy of HO was suggested by Silver et al. who has reported that anticoagulants with passive movements can prevent developing of HO after SCI only in early implementation before contractures developed in joints as it prevents inflammation associated with microtraumas [69]. Unfortunately, in spite of the advances mentioned above, none of the treatments prevents or cures HO entirely. Besides all of the reviewed methods have adverse effects. As a result severe pain syndrome with restricted mobility and delayed rehabilitation due to NHO after SCI remains a major problem that causes increase in invalidity and dependance which in turn adversely influences quality of life and leads to earlier death. Besides, absence of definite treatment increases the costs of rehabilitation. In summary there are currently no effective methods to prevent bone formation resulting from SCI.

1.2 Cell types that may be involved in HO
Osteoblasts are required to formskeletal bones and presumably HO in muscles. These osteoblasts may derive from mesenchymal progenitors that may reside in muscles in healthy individuals or be recruited to damaged muscle via the blood from remote reservoirs such as the bone marrow. Here I review the potential all origins of osteoblasts during heterotopic ossification, their biology and potential mechanism of action.

1.2.1 Mesenchymal progenitor cells
The mesenchymal stem cells can differentiate to the cells representative of connective tissues of the body. True mouse MSCs have recently been described using serial transplantation experiments [70]. However, they are rare cells, with a frequency in bone
marrow of 1 in $10^4$ to 1 in $10^5$. To ensure sufficient numbers of mesenchymal stem cells to perform experiments or to transplant, most investigators expand them *ex vivo* in 2D cultures on plastic dishes. However the cells produced in culture are not true stem cells as they rapidly differentiate and lose multilineage potentials and markers of true MSCs [70, 71]. In literature they are usually termed mesenchymal stromal cells, mesenchymal progenitor cells or transit amplifying cells. For the sake of clarity, we’ll call them mesenchymal stromal cells (MStroC). Some parts of this thesis will deal with mesenchymal *stromal* cells and the abbreviation “MStroC” will be used to denote this.

### 1.2.1.1 Phenotype and morphology

MStroC are plastic-adherent cells defined by the expression of CD73 and CD105 and lack of expression of the pan-leukocyte marker CD45 in humans. A small proportion of MStroC have CFU-F (colony forming unit fibroblast) activity [72, 73] suggesting the presence of a small number of more multipotent cells. Minimal criteria for defining multipotent human MStroC have been published in The International Society for Cellular Therapy position statement [72], as shown in a table:

1. MStroC must be plastic-adherent when maintained in standard culture conditions using tissue culture flasks.
2. 95% or more of the MStroC population must express CD105, CD73 and CD90, as measured by flow cytometry. Additionally, these cells must lack expression (<2% positive) of CD45, CD14 or CD11b, CD79a or CD19 and HLA class II.
3. The cells must be able to differentiate to osteoblasts, adipocytes and chondroblasts under standard in vitro differentiating conditions.

However this multi-potential is rarely determined at a clonal level and may not always occur in vivo. In most studies it is impossible to say these MStroC cultures contain actual multipotent cells or are a mixture of different lineage restricted progenitors. Other cell surface antigens characteristic of, but not unique to, MStroC include CD49b, CD130, CD146, CD200. In mice these characteristic markers include nestin [70], αV integrin (CD51), PDGFRβ, etc [74, 75]. Stro-1 is present on freshly isolated bone marrow MSCs, as well as MStroCs from other tissues, and is lost from MSPCs during *ex vivo* expansion and cannot be considered a unique MSC marker [76].

The marker CD34 deserves a separate attention as there are contradictory results about its presence on the surface of human MSCs. In a recent review of MSCs markers authors have discussed 10 articles which reported absence of CD34 and 5 articles that reported its
presence [77]. It could be associated with different phases of cell cycle or the quiescent or activated state of MSCs.

Following Friedenstein’s original observation, rodent bone marrow-derived stromal cells have been reported as the common progenitors of mesenchymal tissues. Thus, the mesodermal germ layer is the origin of MSCs, which can give rise to connective tissues. There are different names for MSCs such as osteogenic stem cells (Friedenstein), and marrow stromal stem cells (Owen), as these cells have been shown to generate stromal cells in long-term cultures [78].

As indicated above, MStroCs are self-renewing multipotent fibroblast-like cells that can be isolated by plastic adherence and can differentiate into the three mesodermal lineages (osteocytes, chondrocytes and adipocytes) in bulk culture [79-81]. MStroCs are present in many, if not all, tissues and organs (bone marrow, placenta, cord blood, heart, lung, liver etc) [82, 83]. Furthermore, as indicated above, the existence of true mesenchymal stem cells with clonal ability has been reported in the mouse [82], although during ex vivo expansion very few mesenchymal stem cells are present and most are committed mesenchymal stromal (or progenitor) cells, otherwise known as transit amplifying cells. The only known cells that could be closer to the true MSCs in humans are CD146+ pericytes described by Bianco, Sachetti [84] and Peault’s [71] groups. Sachetti et al have reported the ability of CD146+ cells to self-renewal, also these cells appear to be osteoprogenitors able to maintain vascular integrity in hematopoietic microenvironment in vitro [84]. CD146+ MSCs are capable of reconstituting a bone in a mouse and even form bone marrow after in vivo transplantation. Recently Corselli et al demonstrated that human CD146+ cells, which represents a small fraction of MStroC, are pericytes, supporting maintenance of hematopoietic progenitors and express markers of the perivascular niche typical for MSCs [71].

Murine MSCs are positive for Sca-1, CD90, CD29, CD44, CD49e, CD51, CD81, CD24, CD105, and negative for CD45, CD11b, CD31, Ter 119, CD3, B220, Gr1 and CD117 [85]. Paul Frenette group has recently reported that in the bone marrow, a small population within this phenotype that expresses the intermediate filament protein nestin are true multipotent MSC capable at a clonal level to reconstitute osteoblasts, osteocytes, chondrocytes and adipocytes when ectopically transplanted, capable of serial reconstitution in serial transplant, and able to support HSC self-renewal in vivo and vitro [70].
1.2.1.2 MSC Migration
There is still controversy about the ability of MSCs to migrate to the sites of inflammation [86]. Although very few MSCs were found to be circulating in peripheral blood, there is no evidence of MStroC mobilisation after injury, unless various cytokines are administered. Moreover MSCs lack the functional homing receptors used by leukocytes and haematopoietic stem/progenitor cells (HSPCs) to home to sites of tissue injury. For instance MSCs do not interact with selectin expressed by the inflamed vasculature because they cannot synthesize fucosylated sialyl Lewis\(^x\) sugars necessary to selectin ligand binding activity [87, 88]. MSCs require ex-vivo pre-treatment with fucosyl transferase VI in order to be able to adhere to the endothelium and home to the inflamed tissues and the bone marrow [89]. Thus it is not clear if and how MStroC contribute towards the bone formation in damaged muscles after SCI, as they do not readily migrate and are unable to home to sites of damage.

1.2.2 Skeletal muscle mesenchymal progenitor cells
Another potential origin of osteoblasts to form bone in damaged muscles are mesenchymal progenitor cells (MPC) residing in skeletal muscle. Two types of MPC have been described in the muscle: satellite cells (SC) and interstitial cells (IC).

1.2.2.1 Phenotype and morphology
Satellite cells are located between the basement membrane and sarcolemma, in close association with the plasma membrane of myofibers [90]. They are characterised by large nuclear-to-cytoplasmic ratio, few organelles, small nucleus and condensed interphase chromatin [91]. Besides giving rise to new myocytes, these cells have been found to have the ability for self-renewal and differentiate into cells of different lineages - two main features that prove their stemness [92]. Kuang et al. have recently reported that only about 10% of satellite cells can reconstitute themselves, while the rest differentiate only towards myogenic lineage [93]. Adult skeletal muscle satellite cells are reported to be the proliferative stem cell population [94].

Under normal physiological conditions in adult muscle, satellite cells are quiescent and can be identified by the expression of the transcription factor Pax7 and cell adhesion receptor \(\alpha7\)-integrin, but not MyoD in the mouse. Upon injury or pathological (autoimmune) damage, satellite cells start to proliferate and activate expression of the myogenic regulatory factors Myf5 and MyoD, re-enter the cell cycle, differentiate into “myoblasts” or muscle progenitor cells, and ultimately fuse to form new fibers, thus fulfilling their role repairing damaged muscle. Myf5 in adult myoblast proliferation and MyoD are essential for
differentiation. MyoD and myogenin mRNA expression in satellite cells can be induced as soon as 12 hours after injury [95].

A few authors report heterogeneity of satellite cell population, based on the surface markers and their ability to differentiate towards myogenic lineage [91, 96]. The proposal is that Pax7 is required for satellite cell maintenance and self-renewal (SC niche in muscles) and is expressed both in quiescent and activated satellite cells [97]. Also their identification biomarkers include α7-integrin, Myf5, M cadherin, laminin A/C and emerin [98]. Earlier there were reports that not all satellite cells express CD34, M-cadherin and Myf5 [99], however more recent articles define satellite cells as a CD34+ population [100, 101]. Among other markers of skeletal muscle satellite cells it is necessary to mention Pax3 a close paralog of Pax7 is expressed by skeletal muscle satellite cells [101]. Recently the sialomucin CD34 expressed highly in quiescent satellite cells (SC) and reduced in activated satellite cells. CD34 may act as antiadhesive molecule to facilitate migration and promote proliferation of satellite cells at the very early stage of muscle regeneration [102]. According to Mitchell et al. [100] and Monterras et al. [101] Pax7+ SC are CD34+ Sca1-. Unable to rule out expression of CD34 marker by satellite cells, Lee et al. suggested that muscle satellite cells could be originally CD34 positive but during the selection they differentiate into CD34 negative cells [103].

Muscle mesenchymal stromal cells are also called in literature as interstitial cells, due to their location. Their profile is similar to well-known bone marrow MSCs, as Sca1+ CD45−, however they express CD34 surface marker. These interstitial cells have been reported to be able to self-renew and differentiate towards myocytes, gaining Pax7 and M-cadherin markers – those of myocyte progenitors in vitro [104]. Mitchel et al. have recently described interstitial cells in their paper [100]. These cells express PW1 - transcription marker and Sca1, but not Pax7 as muscle satellite cells, which will be described later.

Interest towards skeletal muscle satellite cells and interstitial cells has risen over the past decades as it is a potential source of cells for the treatment of chronic or hereditary muscle diseases. However there are some controversial data about this cell population, showing the need of further investigation of SC phenotype and properties.

1.2.2.2 Differentiation potential and possible role of muscle satellite cells and interstitial cells in heterotopic ossification after SCI

Besides repairing damaged muscle by differentiating towards myoprogenitors and fusion to form myocytes spontaneous adipogenic differentiation of muscle satellite cells and interstitial cells can occur in culture [105]. Several types of cell isolated from skeletal
muscle have been reported to possess adipogenic differentiation potential including satellite cells, side population cells and MPCs. However, it is not clear whether these cells have the ability to induce in vivo fat formation in skeletal muscle. It is not known whether satellite cell plasticity or multipotency is operative in vivo, and little is known about the in vivo features of muscle side population cells or MPCs, such as their anatomical localization and pathophysiological roles.

To determine whether human myogenic progenitor cells are able to act as osteoprogenitor cells, Seale et al. cultured both primary and immortalized progenitor cells derived from the healthy muscle of a healthy woman. The undifferentiated myogenic progenitors spontaneously expressed two osteoblast-specific proteins, bone-specific alkaline phosphatase and Runx2 in vitro, and were able to undergo terminal osteogenic differentiation without exposure to exogenous inductive agents such as bone morphogenetic proteins in vitro. They also expressed the muscle lineage-specific proteins Pax7 (exclusive for muscle satellite cells) [106] and MyoD, and lost their osteogenic characteristics in association with terminal muscle differentiation. Both myoblastic and osteoblastic properties are possibly simultaneously expressed in the human myogenic cell lineage prior to commitment to muscle differentiation when cultured in vitro [107]. Hashimoto et at. have also found the simultaneous expression of the exclusively satellite cells marker Pax7 and bone-specific alkaline phosphatise on cultured muscle MPC. They cultured human muscle satellite cells with β-glycerophosphate (βGP) (10 mM) only in comparison with a combination of βGP with BMP2 and found that βGP alone induced calcification on the 6th day, while BMP2 alone did not have the same results. Muscle derived MPCs have higher potential for osteogenic and myogenic differentiation in comparison with adipose tissue-derived MPCs [108]. Skeletal muscle SC can differentiate into both myogenic and osteogenic lineage in vitro and in vivo after transfection with BMP2. Murine muscle satellite cells transduced with adenoviral vector encoding BMP2 injected into hind limbs induced ectopic bone formation within 14 days. Same cells have also repaired skull defect in mice at 2 weeks [103]. This suggests that muscle SC have the potential to generate bone in vivo when provided the right signal. Murine skeletal muscle satellite cells have also been found to differentiate into myocytes and osteocytes when cultured in Matrigel [109]. C2C12 mouse myogenic cells can differentiate into osteoblasts after supplementation with BMP-2, 4, 7 [110]. Supplementation of BMP7 in culture medium have also showed the osteogenic differentiation of muscle SC, proving their ability to turn into osteoblast in vitro [111]. In comparative analysis of adipose tissue derived stem
cells (ADSCs), muscle-derived stem cells exhibited higher osteogenic differentiation potential than ADSCs [108].

In vitro all human mesenchymal-like cell populations from skeletal muscle are reported to have equal osteogenic potential [112].

Of note in the single reports where the osteogenic activity of mouse/rat MPCs was directly compared to bone marrow MSCs or adipose tissue derived MSCs, the muscle MPCs had higher osteogenic activity in vitro [113].

Thus considering ability of muscle SC to differentiate towards all mesenchymal lineages and their location within muscle fibers it is likely that they can contribute towards to bone formation in muscles after SCI without the need for the recruitment other MPCs from distant tissue (such as the bone marrow) via the blood.

1.3 Macrophages are essential regulators of bone formation

Macrophages are plastic multifunctional cells and can adopt many different phenotypes and functions depending on the tissue of residence, and the state and mode of activation. While inflammatory macrophages are derived from circulating monocytes, there is controversy on the origin of tissue resident macrophages as to whether they are regenerated from circulating monocytes or from locally dividing tissue macrophages [114, 115]. Tissue resident macrophages are generally non-inflammatory. However upon activation, macrophages can be activated toward one or two broad range of behaviours: M1 classically activated pro-inflammatory macrophages or M2 alternatively activated tissue-reparative macrophages [116]. Freytes et al. have also investigated M1 and M2 macrophages and their effect on human MSCs survival and proliferation. They found that M2 macrophages and their associated cytokines support the growth of MSCs while M1 macrophages inhibited the growth of therapeutic MSCs [117]. M2 macrophages were associated also with immunoregulation, matrix deposition, remodelling and graft acceptance [118]. Macrophages M2 are also known to promote angiogenesis activating fibroblasts and endothelial cells.

Chang et al. have reported the presence of macrophages in endosteum, periosteum in trabecular and cortical bones of both mice and human [114, 119]. They have also suggested that macrophages contribute towards differentiation of osteoblasts by increasing maturation and mineralisation. These macrophages - called osteomacs - form a canopy over bone-forming osteoblasts in both the periosteum and endosteum of skeletal bones. Macrophage depletion using macrophage Fas-induced apoptosis (MAFIA) transgenic mice or using clodronate-loaded liposomes in wild-type mice causes a rapid loss of osteoblasts and arrest in bone formation proving that osteomacs are necessary to maintain osteoblast function and bone formation[120]. Likewise depletion of macrophages from cultures of
MSCs prevents their subsequent differentiation into osteoblasts, whereas re-addition of purified macrophages rescues osteogenic differentiation of these cultured MSCs. Thus a specialised population of macrophages is necessary to both osteoblast differentiation and function, and bone formation in skeletal bones.

It was previously shown that both macrophage types are recruited in bone fracture repair with M1 macrophages in the early stage and M2 macrophages supporting bone anabolism at later stages. It is possible that in HO, only one or both types of macrophages (M1, M2) are involved. Interestingly, osteomacs are also necessary to maintain the function of haematopoietic stem cell niches in the bone marrow. Indeed macrophage depletion causes the collapse of the haematopoietic stem cell niches that require MSCs and osteoprogenitors to function properly, leading to the mobilisation of haematopoietic cells from the bone marrow into the blood [120]. This is consistent with the observation that HO in patients with SCI contains a functional haematopoietic bone marrow with haematopoietic stem and progenitor cells (unpublished observation).

In similar disease discussed earlier FOP in humans heterotopic ossification begins in childhood after an inflammatory trigger – trauma or viral illnesses [19]. FOP was found be associated with mutations in the activin A receptor, type I (ACVR1), also known as activin receptor-like kinase 2, (ALK2), one of the bone morphogenetic protein (BMP) type I receptors (BMPR-I) [121]. Group of researchers suggested that the mutant ACVR1/ALK2 receptor in FOP is responsive to receptor stimulation by inflammation and possible macrophages play an important role.

Taken together, these data suggest that macrophages recruited into inflamed soft tissues could be important players of abnormal osteogenic differentiation of MSCs after SCI.
Chapter 2: Material and Methods

This chapter contains Material and methods common to all aims. Specific methods are described in details in the appropriate result chapters.

2.1 Ethics and cell source

All experiments were performed on C57BL/6J wild type mice, unless specified. Wild type C57BL/6J mice were purchased from the Animal Resource Centre (Perth, Australia). All mice were housed within the Annex building, MMRI, and Animal facilities, TRI, and were maintained in conventional cages, with passive air exchange. Mice were fed ad libitum. For each cell harvest, MSCs were harvested from six adult female mice, 5-6 weeks of age. This project was undertaken with prior approval from Animal Experimentation Ethics Committee of the University of Queensland (number 470/11, 054/14 – Appendices A,B). Mouse osteoblastic cell line MC-3T3 was a kind gift from Dr Allison Pettit's group.

2.2 Mouse Model of SCI induced HO

Mice were anesthetized by intraperitoneal injection of 75mg/kg ketamine, 10 mg/kg xylazine, diluted in sterile saline for injections. A laminectomy was practiced on the dorsal spine and the spinal cord trans-sectioned with a scalpel blade between T7 and T8. Muscles and skin were sutured. Control animals were sham-operated with anesthesia and incision of the dorsal skin. After the surgery while mice were still anesthetized, inflammation in the right leg hamstring muscle was induced by intramuscular injection of 12.5µg cardiotoxin (CDTX) from Naja mozambica (SIGMA-ALDRICH; C0759-1MG) diluted in 100µL phosphate-buffered saline (PBS). As control, the left leg hamstring muscles were injected with 100µL PBS without CDTX. At the end of the surgery, mice received a subcutaneous injection of 20mg/kg ciprofloxacin. Mice were then left to recover from the anesthesia on a heat pad at 37°C and then returned to their cages.

As operated mice were paraplegic due to the SCI, their bladders were emptied manually by gentle squeezing of the bladder between fingers twice daily during the whole experiment. As prophylaxis for bladder infections, mice were given antibiotics in their drinking water with a weekly alternating of 125 mg/L ciprofloxacin, or 400 mg/L sulfamethoxazole, 80 mg/L trimethoprim and 10 mg/L fluconazole. Each mouse was then monitored twice daily, body weight measured and urine tested with a urinary strip. A health score sheet adapted for paraplegic mice was used each day during the first 3 days and weekly after for the monitoring of these mice. Score sheet is shown in Appendix C.

At the
end of the experiments mice were euthanized with CO$_2$ between day 3 and week 3 after surgery.

2.3 Micro-computerized tomography imaging (µCT)

2.3.1 Whole mouse body harvest for computerized tomography

At specified time-points after surgery and CDTX injection, mice were euthanized by CO$_2$ asphyxiation. Skin was removed and the whole mouse body was put in a 50ml tube filled up with freshly made 4% paraformaldehyde in PBS. After 24hr fixation on a rotator in the cold room at 4°C, tubes were emptied, mouse carcasses rinsed once in PBS and preserved in a tube full of PBS containing 0.1% NaN$_3$ until processing for µCT or histology.

2.3.2 Micro-computerized tomography analysis

Whole mouse carcasses were analyzed by µCT. The scans were performed in a µCT scanner (µCT40, SCANCO Medical AG, Brüttisellen, Switzerland) at an energy of 45 kVp and intensity of 177 µA, 8W with 300 ms integration time and average integration of 1, which resulted in resolution of 30 µm. To cover the entire thickness of the ectopic bone, the number of slices was set at 350. Three-dimensional (3D) images of lower part of the mice bodies were reconstructed from the scans by the µCT system software package. Quantitative assessments of bone volumes in the muscular mass by subtraction technique of orthotopic mouse skeleton (hip, femur, tibia and fibula) were measured to detect and quantify HO as previously described [122]. Briefly, from the reconstructed cross-sectional images, a series of 350 slices were used for the final analysis. Manual contouring was used to define the outer boundary of the ectopic mineralized tissue. To detect new mineralized formations bone density were identified using X-ray attenuation levels as defined by the software as >180. Bone volume and tissue bone mineral density were calculated using the scanner’s software. Total volume of ectopic bone was defined as the sum of volumes of all mineralized tissue within the region of interest. Bone volume was measured in mm$^3$; bone density was measured in mg/cm$^3$. After scanning, the murine carcasses were transferred in 50 ml Falcon tubes with saline and sodium azide and stored in +4°C. The area of etopic bone formation was manually defined for each cross-sectional image and 3D automatic registration method was applied.
### 2.4 Cell isolation, sorting and culture

#### 2.4.1 Isolation and sorting of muscle satellite cells (SC) interstitial cells (IC) and bone marrow mesenchymal stromal cells (bmMSCs)

Four to six mice, 5-6 weeks of age, were sacrificed by cervical dislocation. After decontamination of the fur with 70% ethanol, hamstring muscles from naïve mice were collected and dissociated into single cell suspensions by successive digestions with collagenase and dispase as previously described [96]. Briefly muscle tissue was cut into 1-2 mm$^3$ pieces and incubated in Collagenase I, 500u/mL (Worthington Biosciences) for 30 min. Then digested muscle tissue was retrieved with a 3mL syringe plunger and centrifuged at 800rpm for 5min. Supernatant was removed and the spin was repeated three times. The second digestion step was performed by incubation muscle tissue in Collagenase type I 1.5U/mL with dispase II neutral protease, grade II 2.4ml/U (4942078001, Roche) at 37°C for 1 hour. To collect bone marrow MSCs (bmMSCs) the femurs and tibias were removed, cleaned of excess tissues and transferred into α-MEM (GIBCO Invitrogen 12561-056) on ice. All bones were crushed in a sterile ceramic mortar with a pestle, and were digested in a freshly-prepared solution of PBS containing 3 mg Type I collagenase/mL (Worthington Biosciences) and 10 ug DNase I/mL (Roche; 04716728001) on an orbital shaker (220 rpm, 37°C; 40 min.). The supernatant was transferred through a 40 µm filter into a sterile 50 mL tube. The crushed bones were washed twice with α-MEM medium, and the suspension was filtered into the same 50 ml tube that contained the supernatant. The pooled suspensions were centrifuged (350 x g; 4C; 5 min). The supernatant was discarded and the cells were resuspended in 1mL of MSC culture medium (1X α-MEM containing 20 % (v/v) FCS (GIBCO Invitrogen, 10099-141), 10 U penicillin/mL, 10ug/ml streptomycin and 2mM glutamine (GIBCO Invitrogen, 10378).

Single cell suspensions isolated from muscles and bone marrow were then stained with biotinylated mouse CD45 and Ter119, CD31-APC, anti-Sca1-PECY7, CD34-FITC, streptavidin-PE following standard procedure [72, 123, 124]. Two µg/mL 7-aminoactinomycin D was added 10 minutes before sorting in order to exclude dead cells. Cells were sorted on an Astrios cell sorter (Beckman Coulter) directly into αMEM with 20% FCS. Once sorted, cells were immediately washed in the same culture medium and cultured.
All three types of cells isolated from 4-6 hind limbs were plated into T25-T75 flasks (5000 cells/cm²), containing culture medium and were incubated in a humidified incubator (37°C, 5 % CO₂).

### 2.4.2 Culture of muscle SC, IC and bmMSCs

Once sorted, cells were immediately washed in the culture medium and cultured as described previously [100, 123]. After 72 hours the cells were washed in sterile, 1x PBS (Ca²⁺-, Mg²⁺-free; Gibco Invitrogen 14190-250), to remove non-adherent cells, and were re-plated in fresh culture medium. Adherent cells were passaged by 1:4 split when 90% confluent. Bone marrow MSCs were cultured in 1X α-MEM containing 20 % (v/v) FBS (Gibco Invitrogen, 10099-141), 10 U penicillin/mL, 10ug/ml streptomycin and 2mM glutamine (Gibco Invitrogen, 10378). Muscle cells were cultured in 1X high glucose DMEM high glucose (Gibco Invitrogen 11960-044) containing 20 % (v/v) FCS, 10% horse serum, 10 U penicillin/mL, 10ug/ml streptomycin and 2mM glutamine. All cells were expanded up to, and including, passage 4. Medium was aspirated and flasks were rinsed in 1X PBS. The PBS was aspirated and the cells were detached enzymatically by the addition of TrypLE™ Select (Gibco Invitrogen 12563-029; 5 mins; 37°C; 5% CO₂), washed and centrifuged (350 x g; 5 mins; 4°C). The supernatant was discarded and the cells were resuspended in 1mL of MSC culture medium, diluted 1:1 (v/v) with 0.4% (w/v) Trypan Blue Stain (Gibco Invitrogen 15250-061), to determine cell viability and numbers. Cells were seeded into sterile T75 flasks at a density ranging between 4,000-5,000 cells/cm², and were cultivated in culture medium At passage 4 cells were detached from the flasks with TrypLE™ Select, centrifuged at 350 x g (5 mins; 4°C) and were resuspended in cryopreservation medium (90% (v/v) FCS; 10% (v/v) DMSO (Sigma D5879)). Cryovials were stored at -80°C for 24 hours in “Mr Frosty” with isopropanol and then were transferred to liquid nitrogen for long-term storage.

### 2.5 Osteogenic differentiation of harvested cells

The protocol for osteogenic differentiation was adapted from [123]. Cells were grown to 90% confluence trypsinized and seeded in the same medium in 96-well plates. Once confluence was reached, medium was removed in each well; wells were rinsed twice with PBS and replaced by osteogenic differentiation medium and cultured for 3 weeks in a humidified incubator (37°C; 5% CO₂) with a medium changed every week. Osteogenic differentiation medium was made of 1x α-MEM supplemented with dexamethasone (0.2 μM), β-glycerol phosphate (10 mM), L-ascorbate-2-phosphate (200 μM), calcium chloride (2 mM), 10% (v/v) FBS and 10U penicillin/mL; 10ug streptomycin/ml; 2mM L-glutamine.
To establish osteogenic properties of blood plasma from operated mice, cultures of sorted muscle cells were established in microwells as described above but osteogenic differentiation was performed for 3 weeks in DMEM with high glucose supplemented with 20% FCS and 10% horse serum and 10% mouse blood plasma but without phosphoascorbic acid, β-glycerophosphate or dexamethasone.

For quantification of mineralization, medium was removed, wells were washed once with PBS and then fixed for 30 minutes with PBS containing 4% paraformaldehyde. Wells were then rinsed with PBS and milliQ water. Dried wells were then stained with 10mg/mL Alizarin Red S solution (Sigma; 056K0745) in water buffered to pH 4.1-4.3 with NH₄OH. After 10 mins, wells were rinsed with milliQ water, dried and photographed.

For detection of calcification calcium deposited by cells in a 48 or 96 well plate was then destained by 10% cetylpyridinium chloride dissolved in water containing 10mM sodium phosphate for 15 mins and absorbance was measured at 562nm.

2.6 Adipogenic differentiation of harvested cells

Cells were cultured in adipogenic differentiation medium (α-MEM, 3-isobutyl-1-methylxanthine (0.5 mM), indomethacin (60 µM), insulin (5 µg/ml), dexamethasone (1 µM), 10 % (v/v) FCS, 10U penicillin/mL; 10ug streptomycin/ml; 2mM glutamine for 3 weeks, fixed with 4% (w/v) PFA (pH7.3), rinsed in 60% (v/v) isopropanol and stained with oil red O stain (Sigma; 101K3489) for 30 mins, rinsed again in 60% (v/v) isopropanol and finally in distilled water, as described [74, 96, 123].

2.7 Tissue harvesting

2.7.1 Blood collection

To harvest blood plasma, mice were anesthetized by inhaling isoflurane using anaesthetic machine VetEquip (Pleasanton, CA, USA), 0.5 to 0.8 mL blood was harvested in tubes containing of 225U/mL heparin and 0.014 TIU/mL aprotinin by cardiac puncture. Mice were then immediately euthanized by cervical dislocation and further processed as described above. Blood samples were centrifuged twice at 800xg at 4°C for 10 minutes to harvest blood plasma. Blood plasma samples were aliquoted and stored at -80°C until use.

2.7.2 Hind limb for immunohistochemistry

Fixed limbs (as per 2.3.1) were decalcified up to 4 weeks at 4°C in PBS containing 14% EDTA adjusted to pH 7.4 with weekly solution changes. Following bone decalcification, hind limbs were processed on the Tissue-Tek VIP 6 according to the protocol (Table 2.1),
embedded in paraffin and 5µm sections cut and placed on SuperFrost Plus® slides (Menzel, Germany). Sections were deparaffinized and re-hydrated with xylene and graded ethanol and washed in TBS (immunohistochemistry) or deionized water (histology). F4/80 (rat anti-mouse F4/80, AbD Serotec) and osteocalcin (rabbit anti-mouse, Alexis Biochemicals, San Diego, USA) immunohistochemical staining was carried out as previously described [114]. In brief, a 3 step procedure using species specific biotinylated F(ab′)² secondary antibodies (Santa Cruz, California, USA) and horse radish peroxidase conjugated streptavidin (Dako, Glostrup, Denmark) to detect primary antibodies. Diaminobenzidine (DAB, Dako, Glostrup, Denmark) was used as the chromogen and sections were counterstained with Mayer’s haematoxylin (Sigma Aldrich) and mounted using permanent mounting media. Specificity of staining was confirmed by comparison to serial sections stained with matched isotype control antibodies (Rat IgG2b, AbD Serotec and normal rabbit IgG, Santa Cruz, respectively). All sections were examined using a Nikon Eclipse 80i microscope with a Nikon D5-Ri1 camera and NIS-elements imaging software.

**Table 2.1** Protocol for tissue processing before paraffin embedding

<table>
<thead>
<tr>
<th>Step</th>
<th>Reagent</th>
<th>Temp</th>
<th>Pressure/Vacuum</th>
<th>Mixing Rate</th>
<th>12hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>70% Alcohol</td>
<td>Ambient</td>
<td>None</td>
<td>Slow**</td>
<td>1:00</td>
</tr>
<tr>
<td>2</td>
<td>90% Alcohol</td>
<td>Ambient</td>
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<td>Slow**</td>
<td>1:00</td>
</tr>
<tr>
<td>3</td>
<td>95% Alcohol</td>
<td>Ambient</td>
<td>None</td>
<td>Slow**</td>
<td>1:00</td>
</tr>
<tr>
<td>4</td>
<td>100% Alcohol</td>
<td>Ambient</td>
<td>None</td>
<td>Slow**</td>
<td>1:00</td>
</tr>
<tr>
<td>5</td>
<td>100% Alcohol</td>
<td>Ambient</td>
<td>None</td>
<td>Slow**</td>
<td>1:00</td>
</tr>
<tr>
<td>6</td>
<td>100% Alcohol</td>
<td>Ambient</td>
<td>None</td>
<td>Slow**</td>
<td>1:00</td>
</tr>
<tr>
<td>7</td>
<td>Xylene</td>
<td>Ambient</td>
<td>None</td>
<td>Slow**</td>
<td>0:40</td>
</tr>
<tr>
<td>8</td>
<td>Xylene</td>
<td>Ambient</td>
<td>None</td>
<td>Slow**</td>
<td>0:40</td>
</tr>
<tr>
<td>9</td>
<td>Xylene</td>
<td>Ambient</td>
<td>None</td>
<td>Slow**</td>
<td>0:40</td>
</tr>
<tr>
<td>10</td>
<td>Wax</td>
<td>60°C</td>
<td>Vacuum*</td>
<td>Slow**</td>
<td>1:00</td>
</tr>
<tr>
<td>11</td>
<td>Wax</td>
<td>60°C</td>
<td>Vacuum*</td>
<td>Slow**</td>
<td>1:30</td>
</tr>
<tr>
<td>12</td>
<td>Wax</td>
<td>60°C</td>
<td>Vacuum*</td>
<td>Slow**</td>
<td>1:30</td>
</tr>
</tbody>
</table>

*Vacuum setting = Default Vacuum setting = 90sec vacuum; 30sec ambient pressure

** Slow = complete drain and fill at 20min interval
2.8 RNA extraction from mouse muscle

Hind limb muscles from mice were collected into 1.5mL Eppendorf tubes with 1mL of PBS on ice. Each muscle sample (one at a time) was transferred into 12mL tube with 2mL of Trizol (Ambion 15596018) and homogenized with Tissue Ruptor (QIAGEN Hilden). The mix was put on dry ice every 30 seconds to prevent overheating. Then 2mL of Trizol was added and the process was repeated. After processing all samples they were left in room t° for 30 mins to maximize RNA extraction. The mix was divided into 4 Eppendorf tubes with 1mL in each, centrifuged at 12000g, 4°C for 10 min to remove all the debris and lipids. Clear pink liquid was transferred to new sterile Eppendorf tube.

Whole BM RNA was extracted from femur flushed with 1 ml of PBS followed by transfer of 200 μL of BM cell suspension into 1mL of Trizol (Ambion 15596018). Endosteal RNA was collected by flushing the empty femur cavity with 1ml Trizol to solubilise and extract RNA from cells attached to the bone at the endosteum. Chloroform was added at ratio of one fifth the volume of the Trizol and samples were shaken and rested for 5min at room t°. Samples were then centrifuge at 12,000g, 4°C for 15mins. Supernatant was removed, an equal volume of isopropanol was added and samples were rested for 10mins. Samples were then stored at -70°C until reverse transcription.

For reverse transcription a quarter of RNA sample stored in isopropanol was centrifuged at 12,000g, 4°C for 15mins, washed with 1mL of 75% ethanol, centrifuged at 12,000g, 4°C for 15mins, dried at 65°C for 10mins and resuspended in 10-20μL of RNAse/DNase free water. Reverse transcription was performed using iScript cDNA kit (BioRad) per manufacturer's instructions: 5x first strand buffer (250mM Tris-HCl, 375mM KCl, 15mM MgCl, pH 8.3; provided with enzyme) and iScript Reverse Transcriptase in a final volume of 20μl at 42°C for 30mins. After 5min enzyme inactivation at 85°C, cDNA was diluted 1/10. Primers used in this study did not detect genomic DNA (gDNA). qRT-PCR was performed using Taqman Universal PCR master mix (ABI) and SYBR Green PCR Master Mix (ABI). The protocol for the PCR consisted of one cycle of 50°C (2 min), followed by 95°C (10 min), followed by 50 cycles each of 95°C (10 sec) and 60°C (60 sec). Results were normalized relative to β2-microglobulin mRNA or β-actin mRNA.

2.9 RNA extraction from sorted cells

Cells were harvested and sorted as per 2.3.2. After sort on Astrios cell sorter (Beckman Coulter) all cell populations were collected directly in the 1.5 mL Eppendorf tubes with 1mL of Trizol (Ambion 15596018). The sort was paused every 10^5 cells to mix collection tubes.
containing Trizol phase and sorted cells in aqueous phase. Collection tubes were then left for 15 min at the room temperature and stored at -70°C before extraction of RNA for minimum of 72 hours. After 3 days the samples were then thawed and choloroform was added at one fifth the volume of the trizol and samples were shaken and rested for 5min at room t°. Samples were then centrifuged at 21,000g, 4°C for 15mins. Supernatant was removed, 0.5μg Linear Polyacrylamide for DNA/RNA Precipitation (GenElute™-LPA, 56575-1ML, SIGMA) was added followed by adding an equal to supernatute volume of isopropanol. Samples were rested for 10mins at the room temperature. Samples were then stored at -70°C until reverse transcription, but minimum for 48 hours. For reverse transcription a quarter of RNA sample stored in isopropanol was processed as described in the section 2.8 “RNA extraction from mouse muscle”.

**2.10 Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)**

qRT-PCR was used to determine gene expression using SYBR® Green PCR Master Mix (Applied Biosystems, Warrington, UK) or TaqMan® Universal PCR Master Mix (Roche, USA) on a 7900HT Fast Real-Time PCR machine (Applied Biosystems). Each reaction was analysed in a 10 μl reaction volume containing: for SYBR® Green 4μl of a 1 in 10 dilution of cDNA (section 2.8.5), 0.5μl of each 1.0 μM forward and reverse primers and 5μl of Master Mix, for TaqMan® 4.5μl of a 1 in 10 dilution of cDNA (section 2.8.5), 0.5μl of TaqMan primer and 5μl of Master Mix. Each sample underwent amplification with an initial activation and denaturation at 50°C for 2 mins and 95°C for 15 mins; followed by 50 cycles of denaturation at 95°C for 10 sec, annealing and extension at 60°C for 45 sec unless otherwise noted. Melt curve analysis was performed following every amplification to calculate mRNA copy number. For qRT-PCR analysis the relative to housekeeping gene expression as determined by ΔCt was compared between the groups.

**2.11 Protein extraction from mouse muscle**

Hind limb muscles from mice were collected into 5mL tubes and put immediately on dry ice. Protein lysis buffer ingredients included: TrisHCl pH7.4 (100mM), Sodium orthovanadate (2mM), Sodium chloride (100mM), 1%Triton-X-100 (v/v), 10% glycerol (v/v), 0.1% SDS (v/v), sodium fluoride (1mM), sodium deoxycholate 0.5% (w/v), 1xULTRA tablet, EDTA-free (Roche Diagnostic, 05892953001).

Each muscle sample (one at a time) was transferred into 10mL tube with complete protein lysis buffer, 2.5mL per gram of tissue and put on ice for 5 min. All samples were homogenized with Tissue Raptor (QIAGEN Hilden) for 20 seconds twice with 15 seconds
on ice in between to prevent overheating. Then samples were rotated for 20 min at 4°C and centrifuged at 11000rpm at 4°C for 20 min. After processing all samples clear supernatant was collected and stored at -20°C before used in assay.

2.12 Processing and analysis of microarray data

2.12.1 Illumina microarray hybridisation and labelling

Total RNA was extracted from mouse hind limb muscles as per section 2.8. RNA was purified and concentrated using the RNaseasy MinElute Cleanup Kit, (QIAGEN). The concentration and integrity of total RNA was evaluated using microfluidics analysis by the 2100 Expert Bioanalyzer and NanoDrop. The entire electrophoretic trace of the sample was used by the Bioanalyzer software (Agilent Technologies, Inc.) to derive a RNA integrity number (RIN), which is an index between 1 and 10. A RIN threshold above 6.5 has been chosen. All samples used had a RNA integrity number (RIN) that ranged from 6.8 to 9.5 (Table 2.4), demonstrating high quality starting material. Briefly, 600 ng of RNA was amplified using the illumina TotalPrep RNA Amplification Kit with biotin-16-UTP labelling, including a 14 hour in vitro transcription using T7 RNA polymerase. A total of 750 ng of cRNA was hybridised to MOUSEREF-8 BEADCHIP KIT (8 samples per chip) overnight for 16 hours at 58°C (Illumina). It was then scanned on to a BeadStation 500 System using Beadscan software Version 3.5.31 (Illumina).

All Illumina microarray hybridisation and labelling experiments were performed by Mr Lawrie Wheeler (Human Genetics Group, The University of Queensland Diamantina Institute).
Table 2.2 Mouse hind limb RNA samples used for microarray analysis.

<table>
<thead>
<tr>
<th>Sample number</th>
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<th>R.I.N</th>
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</thead>
<tbody>
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<td>358.51</td>
<td>8.50</td>
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<td>8.40</td>
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<td>3</td>
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<td>324.66</td>
<td>9.10</td>
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<td>1.4 SCI+CDTX</td>
<td>175.5</td>
<td>8.90</td>
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<td>5</td>
<td>2.1 SCI+saline</td>
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<td>3.2 SHAM+CDTX</td>
<td>242.7</td>
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<td>11</td>
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</tr>
<tr>
<td>12</td>
<td>3.4 SHAM+CDTX</td>
<td>397.2</td>
<td>8.20</td>
</tr>
<tr>
<td>13</td>
<td>4.1 SHAM+saline</td>
<td>379.91</td>
<td>7.80</td>
</tr>
<tr>
<td>14</td>
<td>4.2 SHAM+saline</td>
<td>302.39</td>
<td>7.90</td>
</tr>
<tr>
<td>15</td>
<td>4.3 SHAM+saline</td>
<td>473.64</td>
<td>8.20</td>
</tr>
<tr>
<td>16</td>
<td>4.4 SHAM+saline</td>
<td>331.14</td>
<td>8.40</td>
</tr>
</tbody>
</table>

2.12.2 Normalisation and Background Correction

The variance stabilization transformation (VST) of data, normalisation, intensity-depending filtering and clustering of samples were performed using the Lumi package developed by the North-western University. Centred correlation (Pearson correlation) was used as the metric to determine similarity between samples for the clustering. Dendrograms or tree diagrams were constructed to represent the result from clustering. The degree of similarity between samples was reflected in the length of the branch. Average linkage was used to determine the distance between clusters.

2.12.3 Microarray analysis and validation

Data analysis was performed in BRB ArrayTools developed by the National Cancer Institute. To identify difference in expression of genes between the groups of samples class comparison was performed. SOURCE annotations were used for the pathway analysis. The gene sets were defined based on Gene Ontology and Lymphoid signatures.
The candidate genes identified with differential expressions obtained from the microarray experiments were validated using quantitative reverse transcription-polymerase chain reaction (qRT-PCR) as per section 2.10.

2.13 Statistical analyses

Results were analyzed using the non-parametric Mann-Whitney test or the Exact Fisher’s test using GraphPad Prism 5 software (GraphPad Sofwares, La Jolla, CA). P values below 0.05 were considered significant.
Chapter 3: Establishment of a mouse model of NHO

3.1 Introduction

Dr Francois Genet who is working in the spinal cord injury clinic in France first approached our group in 2012 about the problem of bone formation in the muscle of his patients with SCI or TBI (Fig. 3.1).

Figure 3.1 (A) 3D CT image of NHO in a patient with SCI; (B) Fragment of resected mature NHO (I Kulina, F Genet, J Pathol 2015)

To date the etiopathology of NHO is poorly understood for two reasons. Firstly, as studies on humans are retrospective, there is no study on the earliest stages of NHO to identify the early cellular and molecular events leading to heterotopic ossification in muscles and joints. Secondly, there is no unmodified animal model of NHO after central nervous system lesions [125, 126]. The only existing animal models of HO are genetically modified mouse models of

1) progressive osseous heteroplasia, where HO is developed subcutaneously and caused by inactivating mutations of the GNAS gene [127], and
2) FOP in which activating mutations of ACVR1 are introduced as a transgene or knocked-in the endogenous mouse Acvr1 gene [48], or in which BMP-4 or BMP-2 are overexpressed by means of transgenes or recombinant adenoviruses [36, 125, 128].

As patients themselves are not genetically modified, this means to-date there is no animal model of NHO after CNS lesion that replicates the condition in patients. The availability of such a model would provide opportunity to investigate the mechanistic processes linking
the original neurological lesion to NHO, and ultimately provide a model for pre-clinical testing of treatments to prevent or reduce NHO in humans. Our group is the first to report the development of a model of NHO in genetically wild-type mice, and demonstrate that the combination of both SCI and macrophages recruited by concomitant muscular inflammation is necessary to initiate NHO. This more physiological model of NHO now allows us mice to mimic in mice the processes occurring after spinal trauma in patients.

3.1.1 Aims and Objectives
To understand etiopathology of NHO which will increase prognostic information, enhance diagnostic abilities and provide treatment targets we needed to develop animal model that would mimic NHO as it happens in patients. Firstly the aim of this chapter was to establish animal model of NHO in genetically unmodified mice, however the main aim was to make it more physiologically relevant to resemble the bone formation after SCI as it happens in patients. Considering that NHO develops more often in patients with severe trauma or concomitant infection on top of SCI we decided to combine spinal cord injury with trauma and mimicked bacterial infection by intramuscular injection of LPS.

3.2 Methods specific for this chapter

3.2.1 Muscle injury induced by cardiotoxin
Mouse model is described in details in Chapter 2, section 2.2. Schematically our mouse model is represented on Fig. 3.2. To compare spinal cord transection with spinal cord compression we collaborated with Dr Marc Ruitenberg, The University of Queensland.

Figure 3.2 Schematic image of our novel mouse model of HO after SCI
3.2.2 Muscle injury induced by LPS
Spinal cord injury was performed as described in Chapter 2, section 2.2. However instead of cardiotoxin injection the right hind limb was injected intramuscularly with LPS 2.5mg/kg from Escherichia coli 0111:B4 (L4391-10x1MG, Sigma-Aldrich)

3.2.3 Muscle injury induced by mechanical impact
Mechanical injury was performed with the device represented on Fig.3.3 with the impulse of 0.23 m*kg/s or 0.29 m*kg/s. For the impactor weight of 132 g, the total energy that was transferred onto the mouse legs from a height of 25 cm was 0.32 J, as calculated by Dr Roland Steck with an impact velocity of 2.21 m/s. At a height of 15 cm impact energy was 0.19 J, with a velocity of 1.71 m/s. The skin wounds were closed by Silk 6-0 sutures. Postoperative care was performed as described in Chapter 2 section 2.2. Analgesia included oral Tramadol diluted in drinking water at the dose 25mg/L for 5 days after surgery.

Figure 3.3 Device used to create mechanical crush injury in our mouse model.

3.3 Results
3.3.1 NHO requires both SCI and muscular inflammation
In order to establish a first model of NHO in unmodified mouse, we first performed SCI between T7 and T8 in a large cohort of genetically unmodified mice and followed them up to 4 weeks for NHO. µCT analyses revealed that none of these mice developed NHO despite hind limb paraplegia (Table 3.1).
Table 3.1 HO formation requires SCI and muscular inflammation

<table>
<thead>
<tr>
<th></th>
<th>SCI alone, n (%)</th>
<th>SCI+CDTX, n (%)</th>
<th>CDTX alone, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No HO</td>
<td>102 (100%)</td>
<td>1 (0.9%)</td>
<td>9 (100%)</td>
</tr>
<tr>
<td>With HO</td>
<td>0 (0%)</td>
<td>112 (99.1%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Total</td>
<td>102</td>
<td>113</td>
<td>9</td>
</tr>
<tr>
<td>P-value</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
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</table>

Thus we hypothesized that additional factors may be required to form NHO. We further hypothesized that muscular or peri-articular inflammation could drive NHO following SCI. This hypothesis was based on the following observations. Firstly, in patients with SCI or TBI, NHO is associated with peri-articular inflammation [5, 54, 55, 129]. This inflammatory state can be caused by the neurological lesion, bed sores, muscular lesions and bone fractures acquired in the initial accident, chronic bacteriuria, etc. Secondly, in patients with FOP, minor mechanical lesions (e.g. a needle injection, or minor injury) results in HO flares at the site of injury. Similarly, in the 3 genetically manipulated mouse models of FOP, additional muscular inflammation by intramuscular or subcutaneous injection CDTX considerably enhances HO at the site of CDTX injection [48, 130].

To test this hypothesis, cohorts of mice underwent SCI alone, SCI with CDTX intramuscular injection in the right hamstring muscles to induce inflammation, or sham operation with CDTX intramuscular injection. None of the mice with SCI alone or CDTX alone developed NHO detectable by µCT (Table 3.1). In sharp contrast, 112 out of 113 mice with SCI and CDTX injection developed NHO (Table 3.1). The only mouse in this group did not develop HO, which is associated with the learning curve as surgery on this mouse was performed in the very beginning of our model establishing process. Remarkably, within this experimental group, NHO always developed in the right hind limb injected with CDTX and never in the contralateral left limb injected with PBS. Exact Fisher’s statistic demonstrated that NHO was very significantly associated with the combination of both SCI and intramuscular inflammation with CDTX ($p<10^{-4}$).

We have also compared bone formation in mice with complete transection of the spinal cord and incomplete SCI (by compression) followed by CDTX injection (Fig.3.4) 10 days after surgery. There was no NHO in muscles of mice with compression injury while mice with completely transected spinal cord showed significant bone formation in the hind limb muscles ($p=0.0095^{**}$).
Figure 3.4 (A) Illustrative μCT of mouse right legs 10 days following complete or incomplete SCI and CDTX injection; (B) Quantification of HO volumes by 3D μCT reconstitution following complete or incomplete SCI and CDTX injection. Data are mean ± SD for each experimental group of 6 mice per group; p<0.01**

Interestingly osteoporotic changes (degradation pits) in the pelvic bones and vertebrae were observed in all mice with SCI. This was not correlated to CDTX injection (Fig.3.5).
Figure 3.5 μCT of a representative mouse with (A) SCI and CDTX in the right leg hamstring muscles and saline in the left hamstring muscles, (B) SCI and saline in the right leg hamstring muscles.

3.3.2 Kinetic of NHO
As soon as three days after surgery followed by CDTX injection, small foci of mineralization were detected by von Kossa staining and IHC analysis disseminated through the hamstring muscle by μCT. These small ossifications then continued to grow and merged into a large mass of bone that occupied most of the hamstring muscles at the end of a period of 2 weeks (Fig. 3.6A,B). Again NHO developed exclusively in the right limb injected with CDTX. Control mice that underwent SCI without CDTX injection did not develop NHO during this time period. The early emergence of patches of ossification in the muscle that grew to progressively fuse into larger mineralized masses was similar to growing ossifications in patients as illustrated in Fig. 3.6C, however in much longer time course in comparison with mice.
Figure 3.6 Kynetic of NHO in our mouse model. A) Illustrative μCT of right legs at indicated time-points following SCI and CDTX injection. B) Quantification of HO volumes by 3D μCT reconstitution at indicated time-points following SCI and intramuscular CDTX injection. Each symbol represents a separate mouse. Bars and errors bars represent mean ± SD for each experimental group. C) X-ray radiographs of a SCI patient at different stages of NHO proximal to the hip at indicated time post-injury ([1 Kulina, F Genet, J Pathol 2015])

To test whether HO required the central neurological lesion associated with the SCI or hind limb immobilization caused by the paraplegia resulting from SCI, Dr F Genet has performed bilateral sciatic nerve section with CDTX intramuscular injection in the right hind and PBS in the control left leg in a cohort of mice. None of these mice developed NHO detectable by μCT (results not shown) suggesting that hind limb immobilization is not sufficient to support HO following muscular injury.

3.3.3 Physiological model of NHO
Considering that incidence rate of NHO is higher in patients with associated trauma or sepsis such as infected war wounds or following a car crash [131-133] we decided to
replace intensive muscle injury caused by cardiotoxin injection by crush injury of 2 different mechanical loads, which is closer to mechanisms of multiple trauma in patients with SCI. However considering the effect of crush injury could not be sufficient to cause NHO we have also mimicked bacterial infection by intramuscular injection of LPS at the time of crush-injury. Thus to make our model more physiological we combined:

- SCI
- Trauma (crush-injury) and
- Sepsis/inflammation

All mice with both types of crush injury in combination with LPS injection showed significant bone formation (Fig.3.7) in comparison with crush injury alone (p=0.0286 for crush injury 1 and p=0.0131 for crush injury 2, Mann-Whitney test). Interestingly the higher load of injury caused significantly higher bone formation in the muscle (p=0.0428, Mann-Whitney test) (Fig.3.7 A,B). It was also obvious that LPS injection alone was insufficient to cause HO formation (p-value=0.0043**, Mann-Whitney test) (Fig.3.8 A,B).
Figure 3.7 (A) Illustrative μCT of mouse right legs 10 days following SCI, LPS injection and crush injury from the height of 15cm (Impact1) and 25cm (Impact2), p<0.05*; (B) Quantification of HO volumes by 3D μCT reconstitution following SCI, crush injury and intramuscular LPS injection. Data are mean ± SD for each experimental group of 4 mice per group, pooled from two different experiments.
Figure 3.8 (A) Illustrative \( \mu \)CT of mouse right legs 10 days following SCI, LPS injection alone or LPS injection and crush injury from the height of 15cm (Impact1), \( p<0.01^{**} \); (B) Quantification of HO volumes by 3D \( \mu \)CT reconstitution following SCI, crush injury and intramuscular LPS injection. Data are mean \( \pm \) SD for each experimental group of 6 mice per group.

3.4 Discussion
In this work, we are the first to establish a mouse model of NHO is genetically non-modified wild-type mice. This latter point is essential as to-date, all mouse models of HO involve a genetic manipulation (e.g. introduction of a mutant gene or overexpression of a gene involved in BMP signaling), which is not clinically relevant to model NHO which affects a relatively high proportion of humans suffering SCI (20-25%). Our mouse model of NHO displays similar features to NHO in human patients. In particular, mouse and human
NHO share similar growth patterns with development of separate HO that merge into larger ossified mass over time. In this respect, our mouse model clearly establishes that SCI alone is not sufficient to induce NHO and requires additional local muscular inflammation caused by CDTX injection. Importantly, whilst CDTX-induced muscle inflammation alone was not sufficient to elicit HO, it was necessary to induce HO in response to SCI. This suggest that SCI could prime mesenchymal or muscle progenitor cells towards osteogenic differentiation, similar to ACVR1 mutations in FOP patients, whilst inflammation triggers and drives osteogenic differentiation that leads to HO.

It must be noted that SCI-induced HO and FOP are likely caused by different mechanisms. Indeed while FOP is always caused by activating mutations in the ACVR1 gene that encodes a BMP receptor, ACVR1 is normal in victims of SCI-NHO. Single nucleotide polymorphism (SNP) analysis in SCI-HO patients has revealed three genes that alter the risk of developing NHO [134]. Mutation in ADRB2 gene (β2-adrenergic receptor) that is part of adrenergic system was shown to increase the incidence of NHO, while polymorphisms in toll-like receptor4 (TLR4) and complement factor H (CFH), involved in immune signaling and inflammation were associated with decreased risk of NHO formation [134].

It has been published in numerous articles that both acute and chronic SCI is associated with bone cortex loss, bone density and bone mass reduction and osteoporosis [135]. We have unintentionally confirmed these findings in mice following SCI resection by performing μCT. Furthermore this loss of bone cortex occurs in mice even without intramuscular inflammation. In their article McCarthy et al. [136] have also pointed out fast decreasing in porosity of the tibia bones after SCI and the presence of partially mineralised osteons. It can explain degradation pits we noticed in mice after SCI, that were independent to CDTX injection.

To date there is no information whether NHO is formed by intramembranous or endochondral bone formation. Sections of mouse right hind limbs injected with CDTX were harvested 7, 14, 21 days and 6 weeks after SCI and stained by Toluidine Blue or Safranin O by our collaborator (Fig.3.9). Toluidine Blue staining did not reveal acidic proteoglycans 7 days, 14 days and 6 weeks after SCI with CDTX-induced inflammation. Safranin O staining also did not detect any cartilage glycosaminoglycans at day 14 and 21. Thus our data suggest that NHO formation after SCI is not via endochondral bone formation and most likely is formed by intramembranouse pathway as occurs in POH patients [137].
Figure 3.9 Immunohistochemistry of mouse right hind limbs harvested from mice with SCI and CDTX-induced inflammation. Top row: Toluidine Blue stain for acidic proteoglycans at 1 week (A), 2 weeks (B), 6 weeks (C) and positive control – femur epiphyseal cartilage (D) from same tissue section as in (A). Bottom row: Safranin O stain for cartilage glycosaminoglycan at 2 weeks (E), 3 weeks (F) and positive control – femur epiphyseal cartilage (G) from the section F. Purple colour indicated hematoxylin counterstain of nuclei, light blue – fast green stain. White bars 100μm.

It has been published that degree of severity of NHO depends on the completeness of SCI [132, 133]. Our data confirmed these findings. SC compression that did not lead to permanent immobility in mice in our experiment was insufficient to cause NHO even in the presence of CDTX injection. On top of that we have also found that the size of NHO is directly dependent of the severity of concomitant trauma impact in increasing manner (Fig.3.7B).

In conclusion, we have established the first model of NHO following SCI on wild-type non-genetically modified animals. We find out model shares many clinical and histological features of NHO in patients suffering of SCI or TBI. This model suggests that NHO are caused by the combination of neurological damage and muscular inflammation. To make our model even more physiologically similar to NHO that develops in patients following battle-field injury, I successfully altered our model to combine trauma with components of bacterial inflammation. This more physiological model will help in the discovery of true mechanisms of NHO. Establishment of this model in the mouse will enable to use powerful genetic tools to further understand the molecular mechanisms that lead to NHO, and
provide a clinically relevant pre-clinical model in genetically unmanipulated animal, to test drugs for their ability to reduce or prevent NHO in patients with SCI or TBI.
Chapter 4: Role of Macrophages in Heterotopic Bone Formation after Spinal Cord Injury

4.1 Introduction

The severity of neurotrauma increases the prevalence of HO formation in patients [29, 138]. Incidence of NHO is known to be higher in patients with severe trauma and concomitant infection [139]. In the late 80s Michelsson et al. demonstrated a positive correlation between HO formation and traumatic and inflammatory component on a rabbit model of HO, where bone was formed in immobilised limb [140, 141]. It has been shown previously that inflammation in the CNS involves different cell types and mechanisms compared to inflammation in peripheral tissues [142, 143]. In injuries of the CNS there is lower leukocyte recruitment in comparison with an equivalent peripheral injury. However contusion-type injuries of the spinal cord still result in rapid microglial cell activation and neutrophil infiltration. This inflammatory response after CNS injury can lead to damage of other tissues and organs. For example it can indirectly damage liver via acute phase protein production causing hepatic injury [142]. SCI induces cellular and molecular inflammatory cascade which promotes systemic cytokines release that subsequently may trigger chronic pathology [144]. Moreover it has been recently discovered and published that SCI can cause aseptic systemic inflammatory response syndrome and lead to immune system depression including splenic involution [142, 145]. Patients with SCI have been shown to be prone to development of pulmonary and urinary infections [146].

Considering that macrophages play a central role in any inflammatory process we decided to have a closer look on these cells. Dr Pettit has shown that a population of residential macrophages is present in the mouse and human osteal tissue and was required for normal function and efficient mineralization of osteoblasts [114, 120]. These macrophages, called osteomacs were also required for in vitro expansion of osteoblasts, promoted intramembranous healing of bone in mouse tibia injury model and were shown to increase collagen deposition and enhance mineralization at the site of injury [147]. Macrophages have also been shown to produce BMP-2 to contribute to osteogenesis during bone healing [148].

4.1.1 Aims and Objectives

With the results from the previous chapter that NHO requires a combination of SCI and muscle inflammation further investigation on the role of inflammation was performed.
Firstly the aim of this chapter is to find out whether inflammatory macrophages are involved in the NHO pathogenesis. To explore this we

- tested the effect of macrophage depletion on our mouse model using clodronate-loaded liposomes, which deplete phagocytic cells;
- As osteoclasts can also be depleted by clodronate-loaded liposomes, zoledronate treatment which specifically depletes osteoclasts alone was then tested.

Secondly, our aim was to investigate inflammation that promote heterotopic bone formation in affected limbs. This was assessed by

- causing muscle inflammation in SCI-operated mice then administering different types of inflammatory and anti-inflammatory agents or by
- performing surgery on mice with specific knockout genes, in which immune response was altered.

Finally in this chapter I attempted to reveal the origin of inflammatory cells that may prime osteogenic differentiation of muscle mesenchymal progenitor cells (MPCs). The source of inflammatory cells was tested by

- performing surgery in CCR-2 knockout mice;
- combining SCI with inflammation together with splenectomy as mobilisable pool of monocytes resides in spleen [149].

Also RNA expression was tested via qRT-PCR on whole muscle tissue from the hind limbs of SCI and SHAM operated mice with and without muscle inflammation on wild-type and CCR-2 knockout mice.

4.2 Methods specific for this chapter

4.2.2 SCI surgery and mouse strains used in this chapter

SCI surgery was performed as described in Chapter 2, section 2.2. Strains used in experiments include:

- CCR2 knockout mice: B6.129S4-Ccr2<sup>tm1Ifc</sup>/J were purchased from Jackson Laboratory and bred as homozygous;
- E-selectin knockout: B6.129S2-Sele<sup>tm2Hynt</sup>/J were obtained as homozygous from Paul Frenette and maintained at the TRI animal facilities
- Conditional deletion of Hif-1 in LysM-Cre mice: LysM<sup>Cre/WT;Hif1a<sup>fl/fl</sup></sup>. The strain was bred as B6.129P2-Lyz2<sup>tm1(cre)Ir</sup>/J x B6.129-Hif1a<sup>tm3Rsjo</sup>/J (control for this strain was -LysM<sup>Cre/WT;Hif1a<sup>WT/WT</sup></sup>) and
• C57BL/6J as a wild-type control.

All mice were euthanized for analysis 10-14 days after surgery.

4.2.3 Splenectomy technique and sham surgery

Splenectomy was performed as described earlier [150]. Briefly mice were anaesthetized; fur was sprayed with 70% ethanol. After skin incision peritoneal cavity was opened and spleen was gently pulled to the surface. Both vessel bundles were ligated twice with silk 6-0 thread, followed by gentle severing the blood vessels between the sutures. Peritoneal cavity and skin were closed by silk sutures 6-0. Recovery period care was performed as described in SCI surgery section, Chapter 2. For the SHAM splenectomy surgery was performed in the same way, however sutures were not placed on the blood vessels, that remained intact and spleen was not removed.

4.2.4 Treatment options

4.2.4.1 Clodronate-loaded liposomes

Clodronate-loaded liposomes were purchased from VU medisch centrum (product code 283539, Amsterdam, The Netherlands) [151]. Empty liposomes were prepared in the same conditions in PBS without clodronate. Phagocytic macrophages were depleted in vivo by retro-orbitally injecting 100µL/20g body weight clodronate-loaded liposome suspension immediately after surgery and injection of CDTX and then every second day for 2 weeks. Control mice were injected with an equivalent volume of saline or PBS-loaded liposomes prepared in the same conditions but without clodronate.

4.2.4.2 Zoledronate

Zoledronate (Zometa, Novartis Pharmaceuticals Australia, North Ryde, Australia) was injected intraperitoneally at 50 µg/kg daily for the first five days after surgery and then at 100µg/kg every third day from day 6 to day 12.

4.2.4.3 G-CSF

G-CSF (filgrastim (G-CSF) 6mg/0.6mL syringe, Neulasta) was injected subsutaneously twice daily at a dose of 125µg/kg. Treatment was started upon recovery after surgery and anaesthesia and was continued for the whole period of experiment (10-14 days).

4.2.4.4 Meloxicam

Meloxicam (Enzo Life Science, BML-EI292-0100) was injected subsutaneously twice daily at 6mg/kg starting when mice recovered after surgery and anaesthesia and was continued for the whole period of experiment (10-14 days).
**4.2.4.5 Harvest of inflammatory peritoneal macrophages**

Inflammatory macrophages were produced by naïve C57BL/6J mice. Lipopolysaccharides (LPS) from Escherichia coli 0111:B4 (L4391-10x1MG, Sigma-Aldrich) was injected intraperitoneally in a dose of 2.5mg/kg. Mice were sacrificed 48 hours later by cervical dislocation and peritoneal cavity was lavaged with 10mL of saline. All liquid was collected into 50mL Falcon tubes and centrifuged at 4°C for 5 min at 340g. Supernatant was discarded and cells were counted and resuspended in saline 1million cells per 100μL. Cell suspension was then injected into hamstring muscles of SCI operated mice. Saline was injected as a negative control.

**4.2.5 G-CSF quantification in mouse plasma**

G-CSF was quantified in mouse plasma harvested from SCI and SHAM-operated mice using R&D Mouse G-CSF Quantikine ELISA Kit (MCS00, R&D Systems). Mouse plasma was diluted with Assay Diluent RD1-54 in proportion 1:2. ELISA procedure was performed as per manufacturer's instructions and generation of standard curve and assessment was performed using GraphPad Prism 5 software (GraphPad Sofwares, La Jolla, CA).

**4.3 Results**

**4.3.1 Macrophage depletion considerably reduces NHO in mice**

We have already shown that muscular inflammation is necessary for NHO formation following SCI in our mouse model (Chapter 3, section 3.3.1). To further investigate the importance of macrophages, mice with SCI and intramuscular CDTX injection were administered intravenously with clodronate-loaded liposomes in order to deplete phagocytic macrophages [147, 152]. Control mice underwent SCI and intramuscular injection of CDTX but received PBS-loaded liposomes instead. Clodronate-loaded liposomes reduced the volume of HOs and in 3 out of 11 mice (~28%) prevented their development entirely (Fig.4.1). On average, the heterotopic bone volume was 11-fold lower in the clodronate liposome-treated group compared to the control PBS-liposome group (p-value: 0.0003*** by Mann-Whitney test) (Fig.4.1).

Previously it was found that clodronate-loaded liposomes can deplete both macrophages and osteoclasts [153], therefore we tested the effect of osteoclast depletion alone by injecting zoledronate in combination with SCI and CDTX as this treatment reportedly eradicates osteoclasts in the mouse. Efficacy of this zoledronate regimen was confirmed by the absence of bone resorption pits on iliac crest and vertebrae of mice with SCI. Interestingly we found that zoledronate administration actually increased the average
bone volume of NHO 3-fold compared to SCI+CDTX mice injected with saline when measured at Day14 (Fig 4.2). Thus osteoclast ablation by zoledronate has an opposite effect to combined macrophages and osteoclast depletion. Collectively, these results suggest that phagocytic macrophages, not osteoclasts, recruited in the inflamed muscle trigger the development of NHO subsequent to SCI.

Figure 4.1 Effect of macrophage depletion on NHO. (A) Illustrative μCT of right hind limbs after SCI with injection of CDTX and treatment with clodronate-loaded liposomes or vehicle; (B) Quantification of HO volumes by 3D μCT reconstitution following SCI, with injection of CDTX and treatment with clodronate-loaded liposomes or vehicle; bars and error bars represent mean ± SD of 4 mice per group pooled from two different experiments.
Figure 4.2 Effect of osteoclast inactivation on NHO. (A) Illustrative μCT of right hind limbs after SCI with injection of CDTX and treatment with zoledronate; (B) Quantification of HO volumes by 3D μCT reconstitution following SCI with injection of CDTX and treatment with zoledronate, clodronate-loaded liposomes or vehicle; bars and error bars represent mean ± SD of 5 mice per group.

4.3.2 Effect of G-CSF on NHO
To further test role of macrophages in SCI-induced NHO, mice that had undergone SCI and intramuscular injection of CDTX, were injected twice daily with G-CSF (125µg/kg) with and without meloxicam or vehicle alone from immediately after SCI surgery and CDTX injection. It was reported previously that G-CSF treatment enhances recovery of blood monocytes and increases numbers of macrophage and granulocyte-macrophage progenitors [154]. Also our group has previously shown that G-CSF treatment can lead to
the depletion of osteoblast and alter endosteal bone formation [120]. The NSAID meloxicam was used in this experiment as clinical application of NSAIDs show modest efficacy has been shown in treatment of patients with HO [155-157]. Mice were sacrificed 14 days after surgery, and microCT was performed. Surprisingly mice injected with G-CSF developed significantly larger bone masses in hind-limb muscles in comparison with saline-injected mice (p-value: 0.0051** by Mann-Whitney test). In our mice meloxicam alone had no effect on HO formation, however when G-CSF was injected together with Meloxicam there was no significant increase of heterotopic bone volume compared to vehicle injected mice (Fig.4.3), showing that Meloxicam successfully negates the G-CSF-mediated boost in NHO.
Figure 4.3 (A) Illustrative μCT of right hind limbs 14 days after SCI with injection of CDTX and treatment with G-CSF/Meloxicam; Quantification of HO volumes (B) and density (C) by 3D μCT reconstitution following SCI, intramuscular CDTX injection and treatment with G-CSF/Meloxicam; bars and error bars represent mean ± SD of 6 mice per group pooled from two different experiments.
As G-CSF enhanced HO in our mouse model we next tested whether administration of G-CSF together with LPS to mimick bacterial infection could cause HO in our mouse model. For that purpose mice that had undergone SCI and intramuscular injection of LPS on day 0, 2 and 4, were injected twice daily with G-CSF (125µg/kg) or saline from the day of surgery. The other control group received saline as intramuscular injection into right hind limb and G-CSF treatment as described above. We did not observe any NHO formed in any of the groups 14 days after surgery (Fig.4.4).

**Figure 4.4** (A) Illustrative μCT of right hind limbs 10 days after SCI with injection of LPS and treatment with G-CSF; (B) Quantification of HO volumes by 3D μCT reconstitution following SCI, intramuscular LPS injection and treatment with G-CSF; bars and error bars represent mean ± SD of 4 mice per group.

G-CSF was also measured in plasma of wild-type C57BL/6 mice with SCI and inflammation induced by CDTX injection to identify whether mice that develop NHO have an increase in plasma concentration of G-CSF that could drive abnormal bone formation. It was compared with concentration of G-CSF in plasma harvested from SHAM operated mice with and without inflammation and plasma harvested from mice with SCI alone at different time points. Time points included day 1, 3 and 7 after surgery.
Plasma standard curve and equation are presented on Fig.4.5A. In all 4 groups we noticed significant increase in the G-CSF concentration in plasma at Day 1 post-surgery (Fig.4.5B,C,D,E). Interestingly although major decrease in G-CSF concentration was observed in SCI and SHAM groups by Day 3, it remained high in group of SHAM-operated mice injected with CDTX (p-value <0.01**, Two-way ANOVA with Bonferroni posttest). This increase however was not observed in SCI+CDTX group where SCI-associated immune system changes might lead to slightly dampened inflammatory response. However subsequent decrease of G-CSF concentration was observed in all four groups by the day 7 including the CDTX group (Fig.4.5F).
Figure 4.5 Quantification of G-CSF in mouse plasma harvested from mice with and without NHO
Taken results together from these findings can be drawn 2 conclusions:

1) G-CSF worsens NHO and
2) We observe increased concentration of G-CSF in the blood plasma of all mice after surgery, whether it is SHAM or SCI.

4.3.3 Inflammatory peritoneal macrophages do not induce HO after SCI in mice

To test inflammatory macrophages involvement into NHO after SCI we injected inflammatory peritoneal macrophages suspension into right hind limb of SCI-operated mice. Macrophages were harvested from C57BL6/J mice treated with LPS 2 days prior harvest as described in Methods. Control group was injected with the same volume of PBS. At 14 days we did not observe any heterotopic bone formation in either group of operated mice (p-value: 0.68\textsuperscript{ns} by Mann-Whitney test).
4.3.4 Local muscle macrophages may play an important role on NHO
To test whether monocytes are recruited from remote reservoirs (e.g. bone marrow and spleen), we performed SCI following by CDTX injection on CCR2-knock-out mice with wild type C57BL6/J as a negative control. Mice lacking CCR2 chemokine receptor are known to have impaired monocyte/macrophage infiltration in response to inflammation and in general decreased inflammatory response. After 10 days mice were sacrificed and microCT analysis showed significant increase in bone volume in CCR2 deficient mice (p-
value: <0.0001*** by Mann-Whitney test). This shows that CCR2 is not necessary for macrophages to invade inflamed muscle and promote NHO development.

Figure 4.7 (A) Illustrative μCT of right hind limbs harvested 10 days after SCI with injection of CDTX in CCR-2ko mice; (B) Quantification of HO volumes by 3D μCT reconstitution following SCI and intramuscular CDTX injection in CCR-2ko mice; bars and error bars represent mean ± SD of 4 mice per group (experiment 1) and 8 mice per group (experiment 2) pooled from two different experiments.

To further investigate the differences in CCR2 knockout mice vs wild type control qRT-PCR for osteogenic and inflammatory markers was performed on mRNA extracted from
hind limb muscle at Day2 after SCI and CDTX injection. Interestingly M2 (anti-inflammatory macrophage) markers such as Arg-1, Chi3l3 and Mrc1 were significantly downregulated in CCR-2 knock-out mice (Fig.4.8A,B,C). However CCR-2 knockout mice expressed higher level of IL1β and CSF-1RNAs, however the difference was not statistically significant (Fig.4.8D,E).

**Figure 4.8** Quantification of macrophage markers in hind limb muscles of CCR-2 knockout and wild type mice. Quantification of (A) Arginase 1, (B)Chitinase 3l3, (C)Mrc1, (D)CSF1, (E)IL-1β mRNA by qRT-PCR on from whole hamstring muscle group harvested from wild-type C57BL/6 mice or CCR-2 knockout. All expression levels were relative to the housekeeper gene β-actin. Each dot represents hind limb muscle mRNA from a separate mouse (4 mice per group).
4.3.5 Splenectomy does not affect NHO after SCI in mice

To further test the origin of macrophages that are involved in NHO formation we performed a series of experiments to identify whether splenic monocyte population could be involved. We performed splenectomy immediately before SCI injury and CDTX injection on a cohort of mice. Control group underwent SCI surgery, CDTX injection and sham operation where peritoneal cavity was opened but spleen remained untouched. MicroCT analysis performed at Day 10 after surgery did not show any difference in bone formation between two groups of mice (p-value: 0.84 ns by Mann-Whitney test) (Fig.4.9). Furthermore when we performed splenectomy/SHAM surgery on CCR2-knock-out mice with SCI and CDTX injection the results were the same (p-value: 0.53 ns by Mann-Whitney test) (Fig.4.10). Therefore macrophages responsible for NHO are not recruited from a splenic reservoir via CCR2.
Figure 4.9 Effect of splenectomy on NHO. (A) Illustrative μCT of right hind limbs harvested 10 days after SCI with injection of CDTX and splenectomy; (B) Quantification of HO volumes by 3D μCT reconstitution following SCI, intramuscular CDTX injection and splenectomy; bars and error bars represent mean ± SD of 8 mice per group.
Figure 4.10 Effect of splenectomy on NHO in CCR-2 knockout mice. (A) Illustrative μCT of right hind limbs harvested 10 days after SCI with injection of CDTX and splenectomy in CCR2-ko mice; (B) Quantification of HO volumes by 3D μCT reconstitution following SCI, intramuscular CDTX injection and splenectomy in CCR2-ko mice; bars and error bars represent mean ± SD of 4 mice per group pooled from two different experiments.

4.3.6 E-selectin influence on NHO in mice
As E-selectin is utilised by circulating monocytes to roll and arrest an inflamed vasculature and extravasate into inflamed tissue, we reasoned that macrophages homing to damaged muscles may be reduced in E-selectin knockout mice. We compared effect of SCI and
CDTX injection in E-selectin knockout and wild type mice. MicroCT analysis performed at Day10 after surgery showed a trend towards reduction in bone volume in E-selectin knock-out mice compared to wild type mice (p-value: 0.06 ns by Mann-Whitney test) (Fig4.11). Interestingly the absence of E-selectin resulted in a significant decrease in density of newly formed heterotopic bone in E-selectin knock-out mice in comparison with control group (p-value: 0.0002*** by Mann-Whitney test) (Fig.4.11). This result supports our hypothesis that lessening the number of monocytes recruited to the site of muscle injury may lead to reduced amount of NHO after SCI.

**Figure 4.11** Role of E-selectin in NHO. (A) Illustrative μCT of right hind limbs harvested 10 days after SCI with injection of CDTX in E-selectin-ko and wild-type mice; Quantification of HO volumes (B) and density (C) by 3D μCT reconstitution following SCI, intramuscular CDTX injection in E-selectin-ko mice and and wild-type mice; bars and error bars represent mean ± SD of 5 mice per group pooled from two different experiments.
4.3.7 Myeloid cell-derived Hif enhanced NHO after SCI

To determine whether macrophages responsible for NHO are of M1 type polarized we used mice with conditional deletion of the Hif1α gene in myeloid cells (LysM\textsuperscript{Cre/WT}/Hif1α\textsuperscript{fl/fl}). LysM\textsuperscript{Cre/WT} x Hif1α\textsuperscript{WT/WT} mice were used as a control cohort. Indeed it has been reported that Hif1α expression is a cardinal feature of M1 polarized macrophages [158]. MicroCT analysis performed at Day10 after surgery showed tendency towards increase in bone volume in mice with inactivated Hif1α, although statistically not significant (p-value: 0.09 ns by Mann-Whitney test). We also observed significant increase in density of newly formed bone in myeloid-specific Hif1α inactivated mice in comparison with control group (p-value: 0.04* by Mann-Whitney test) (Fig.4.12). This result shows that absence of M1 and possible switch of polarization to M2 macrophages increase density of NHO after SCI. To increase statistical power this experiment needs to be repeated with larger cohorts of mice.
Figure 4.12 Role of M1 macrophages in NHO. (A) Illustrative μCT of right hind limbs harvested 10 days after SCI with injection of CDTX in LysM<sup>Cre/WT/Hif1α<sup>fl/fl</sup></sup> and control groups of mice; Quantification of HO volumes (B) and density (C) by 3D μCT reconstitution following SCI, intramuscular CDTX injection in LysM<sup>Cre/WT/Hif1α<sup>fl/fl</sup></sup> and control groups of mice; bars and error bars represent mean ± SD of 4 mice per group pooled from 2 different experiments.
4.4 Discussion
As muscular inflammation is necessary to NHO following SCI, we further investigated the importance of macrophages. To deplete all phagocytic cells mice with SCI and intramuscular CDTX injection were treated with clodronate-loaded liposomes immediately after surgery and then every 2nd day for 8 days [120, 147]. The control mice underwent SCI and intramuscular injection of CDTX but received PBS-loaded liposomes instead. We found that macrophage depletion significantly reduced the volume of HOIs and in ~28% of mice prevented development of HO. On average, the heterotopic bone volume was 11-fold lower in the clodronate liposome-treated group compared to the control group without clodronate liposomes. Clodronate is a first generation bisphosphonate that is unable to cross cell plasma membranes to kill osteoclasts effectively. However when packaged into liposomes, it is uptaken by phagocytes such as macrophages, antigen-presenting cells and osteoclasts and rapidly kills these cells once digested [147, 152, 159]. To confirm this effect was via macrophage and not the osteoclast depletion we repeated this experiment with zoledronate, which specifically depletes osteoclasts. At day 14, zoledronate increased the average bone volume of NHO up to 3 folds compared to SCI+CDTX controls injected with saline. This effect was opposite to that of clodronate-loaded liposomes. Collectively, these results suggest that phagocytic macrophages, not osteoclasts, recruited in the inflamed muscle trigger the development of NHO subsequent to SCI.

The role of inflammation and macrophages in our mouse model of NHO is also consistent with the two types of observations. Firstly SCI and TBI patients that develop NHO are those which suffering most violent impacts with bruising, additional bone fractures, or infections [5]. Furthermore, the incidence of NHO in war casualties where infections, inflammation and multitrauma are presumably more prevalent, is much higher than in accidents involving civilians [35, 160]. Secondly, ossification can be reduced by NSAID in NHO patients [49, 52, 53]. Our hypothesis that macrophages infiltrating inflamed muscles are critical to NHO is also consistent with our previous observation that specific subsets of macrophages are critical to the maturation of osteoblasts and bone formation in skeletal bones [114, 120]. In long skeletal bones, macrophage depletion induces a rapid loss of mature osteoblasts on bone surface, arrests bone formation and mineralization in adult mice [114, 120], and impairs bone repair following fracture [147], yet the mechanisms by which these specific macrophages induce osteoblast maturation and bone formation remain unknown.
Interestingly NSAIDs treatment in our experiment did not cause reduction of NHO bone volume. This could potentially be due to the excessively strong inflammatory stimuli CDTX caused in the muscles. This potential limitation can be addressed in future by performing next series of experiments in our new more physiological model of NHO (developed in Chapter 3) where we combined muscle crush-injury with LPS intramuscular injection to mimic bacterial infection.

**G-CSF**

Hara et al. [161] have shown that G-CSF is required for myocyte proliferation during muscle regeneration after injury. In addition our laboratory has shown that G-CSF administration depletes osteoblasts at the endosteum but not at the periosteum of skeletal bones resulting in a transient arrest in endosteal bone formation whereas periosteal bone formation is not perturbed [120]. Indeed our model of SCI-NHO, G-CSF administration significantly increased bone formation in the muscles after CTDX induced injury. It is possible that following G-CSF administration enhanced muscle regeneration when combined with SCI could help force proliferating muscle progenitor cells to differentiate more along the osteogenic lineage. An alternative explanation is that increased activation or homing of neutrophils or monocytes to the damaged muscle in mice administered G-CSF (G-CSF stimulates neutrophil progenitors proliferation and neutrophil production) could exacerbate the proosteogenic effect. Interestingly that while the anti-inflammatory meloxicam on its own did not reduce bone formation, it diminished the exacerbated NHO caused by G-CSF treatment.

However despite exacerbating the effect of NHO in SCI-operated mice with CDTX injection, G-CSF was insufficient to cause NHO on its own or even in combination with mimicked bacterial infection.

We also observed an increase of G-CSF concentration in plasma of SCI- and SHAM-operated mice at day 1. This is most likely explained as part of the immune response to inflammation. Indeed systemic G-CSF levels were greater in mice treated with CDTX.

**CCR2 and splenectomy**

In attempt to determine whether monocytes reside locally in the muscles or are recruited from a remote location such as from spleen to the damaged muscles. Rationale for these experiments is that CCR2 is a chemokine receptor used by inflammatory monocytes to home to the sites of injury. While the spleen is the primary source of mobilisable monocytes we performed a few sets of experiments:
• SCI-injury in CCR-2 knockout mice (Fig.4,7)
• SCI-injury in CCR-2 knockout and wild type mice with and without splenectomy (Fig.4.9, 4.10).

Mader et al. [162] have shown that CCR2 knockout mice were less prone to bone loss after denervation or hormonal changes due to impaired osteoclast maturation and function, that was also reported earlier [163]. Despite a decrease in function of osteoclasts, bone formation at the fracture healing site was delayed in CCR-2 knockout mice at day 14, however the volume of bone 3 weeks after injury was higher than in wild-type mice. Muscle regeneration after CDTX induced injury was also impaired in CCR knockout mice due to defective monocyte recruitment [164]. Warren et al. [165] have also noticed worse regeneration of freeze-injured muscle, however they have also noticed calcifications in the muscles of CCR2 knockout mice at day 21 after injury. In our experiments NHO bone volume was increased in CCR2 knockout mice with and without splenectomy in comparison with wild type control, which is consistent with bone volume increase data from previous research. However we found increase in bone mass earlier than 21 days after injury, which might be explained by the enhancing effect of SCI. Splenectomy on its own, did not change the bone volume of newly formed ossifications suggesting that splenic monocytes most likely would not be involved in NHO after SCI or can easily be replaced by monocytes from other depots.

**E-selectins**

The endothelial selectins are cell adhesion molecules that are involved in normal leukocyte function and their homing and activation [166]. E-selectin deficient mice are more susceptible to infections and inflammatory changes due to defective leucocyte homing. Mice deficient in E- and P-selectins have shown increased bone destruction following infection in comparison with wild type control along with increase in bone resorptive cytokine, IL-1α [167]. E-selectin has been shown to be involved in homing of endothelial progenitor cells [168, 169] and its deletion impaired inflammation response. E-selectin has also been shown to accelerate haematopoietic progenitors differentiation towards granulocyte and monocyte lineages in vitro, and increase HSC proliferation in vivo [170, 171]. During inflammation leukocyte adhesion molecule E-selectin becomes up-regulated on endothelial cells and guides migration of pro-inflammatory cells to the site of injury [172]. We found a reduction of the bone volume of NHO after SCI in our E-selectin knockout mice. This is possibly due to a dampened inflammatory response with a
reduction in number or activation of monocytes recruited to the site of CDTX-induced injury. Macrophages are pleotropic cells with many different functions and can be skewed towards particular roles by the factor in their local environment. It is still not clear whether macrophage skewing could play the main role in NHO. However we found quite a few publications on the role of macrophages in muscle regeneration, such as seen in our mouse model with CDTX-induced muscle injury. Saclier et al. [173] have shown that M2-skewed macrophages increased myogenic differentiation when co-cultured with human myogenic progenitor cells. These authors have also shown that in vivo regenerating muscles contain both inflammatory M1 and M2 polarised macrophages, and M2 macrophages were located in the same sites as differentiating myogenic progenitors. However despite of obvious role of M2 macrophages, authors specifically assessed effect of TNFα amongst other factors stimulating myogenic proliferation, and confirmed its role in myogenic differentiation. This confirms that both M1 and M2 skewed macrophages are required for adequate muscle regeneration after injury. Arnold et al. [174] have also published that monocytes recruited to the site of muscle inflammation caused by notexin injection are also a mixture of both M1 and M2 subsets in affected muscle. These authors also suggested that pro-inflammatory cells initiate growth of myoprogenitors while anti-inflammatory macrophages promote myogenic differentiation and muscle fibre growth. Another group of researchers have later also confirmed that in mice with altered M2 macrophage skewing as a result of genetic absence of IL-4, muscle regeneration was very delayed [175]. Wang et al. [176] have recently provided similar results inducing muscle injury by CDTX injection in CD11b-diphtheria toxin receptor transgenic mice that have shown delayed regeneration in impaired muscles in absence of anti-inflammatory macrophages. Thus it appears that both M1 (inflammatory) and M2 macrophages play a role in muscle regeneration. Again it’s hard to say whether M2 play a crucial role in our mouse model as the major altering factor is SCI that on its own affects immune system response. Macrophages have also been shown to stimulate collagen production by fibroblasts in co-culture experiments following by phagocytosing of apoptotic cells [177] that was driven by up-regulation in TGF-β. This is important as collagen type I deposition guides bone matrix formation in the sites of injury [178, 179]. Chen et al. have also shown that with the induction of the osteoconductive biomaterial β-tricalcium phosphate that is used in clinical bone regeneration most macrophages expressed M2 markers [180].
Both Hif1α and iNOS have been shown to be important in M1 polarization of macrophages [181]. Our microCT results from mice with Hif1α inactivated in myeloid cells showed increased NHO, suggesting M1 skewed inflammatory macrophages may play a role in dampening NHO. This is the opposite of what we predicted. A possible explanation for this unexpected result is that following deletion of Hif1α gene inflammatory macrophages cannot undergo M1 polarization and by default polarize to an M2 function instead. This suggests that M2 polarized macrophages may be more likely to be involved in abnormal bone formation after SCI. In the next Chapter 5 I show by microarray on damaged muscles in mice following SCI with/without CDTX injection that this may indeed be the case.

An alternative explanation for the increased NHO observed in mice with Hif1α depleted myeloid cells is that Hif1α affects M1 macrophage skewing. Recently Colegio et al. [182] have published that transcription factor Hif1α is also involved in M2 polarization. As lactic acid accumulated during tumor homeostasis this promotes expression of M2 specific markers such as Arg1, Fizz1 and Mgl2 via Hif1α. Therefore our studies need to be confirmed by examining whether conditional deletion of Hif1α in macrophages alters distribution and function of M1 and M2 macrophages accumulating in the muscles developing NHO.

This chapter results were combined into the Table 4.1:

**Table 4.1** Effect of G-CSF, E-selectin, CCR2 and splenectomy on NHO after SCI.

<table>
<thead>
<tr>
<th></th>
<th>Bone volume</th>
<th>Bone density</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-CSF</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>E-selectin knock-out mice</td>
<td>↓*</td>
<td>↓</td>
</tr>
<tr>
<td>CCR2 knock-out mice</td>
<td>↑</td>
<td>ns</td>
</tr>
<tr>
<td>Splenectomy</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>LysMCReHif1α mice</td>
<td>↑*</td>
<td>↑</td>
</tr>
</tbody>
</table>

* The difference between control and treated groups is not significant, but there is a trend towards increase/decrease (↑/↓); ns: not significant

Our results suggest that NHO after SCI requires recruitment of macrophages, granulocytes or both via the vascular cell adhesion molecule E-selectin, which is critically involved in leucocyte recruitment to inflamed vasculature. Granulocytes contribution via G-CSF is an interesting possibility and is to be further investigated in G-CSF receptor knock-out mice. Data from CCR2 knock-out mice and mice with splenectomy are surprising. It suggests a role of tissue resident muscle macrophages in NHO development with a dampening effect of spleen and marrow macrophages recruited via CCR2 in case of altered migration of monocytes from bone marrow or spleen.
Chapter 5: The comparison of gene expression profiles of operated mice and local changes in damaged muscles

5.1 Introduction
My data from previous experiments show that SCI alone is not sufficient to induce NHO and that macrophages in the inflamed muscle are involved in NHO development after SCI. However, a muscular inflammation alone is not sufficient to promote HO either, therefore it appears to be the combination of the two insults, SCI and muscular inflammation which causes NHO. I hypothesize that in the context of a SCI, macrophages infiltrating the inflamed muscles are activated differently to what they would be in the absence of SCI. Since my findings are novel, there is no prior knowledge of how SCI could alter macrophage activation in an inflamed muscle. In order to gain insights into possible mechanisms and test whether macrophage activation is altered following SCI, I have performed the transcriptome profile of muscles following SCI and/or CDTX-mediated muscle injury. These experiments were performed to test my hypothesis that the transcriptome of muscle cells and macrophage would be different in mice with muscular injury mediated by CDTX alone versus mice with muscle injury and SCI.

5.1.1 Aims and Objectives
In the experiments described in this chapter, I performed gene expression microarray analyses on RNA extracted from whole hind limb muscles from mice in four experimental conditions as follows: 1) SHAM-operated, 2) SCI alone, 3) intramuscular CDTX alone, 4) SCI together with intramuscular CDTX. Muscles were harvested at day 2 following surgery, an early time point in NHO development. To validate microarray results RNA expression was tested via qRT-PCR on whole muscle tissue from the hind limbs of mice from the same experimental groups. I also used some inhibitors of inflammatory cytokines found to be overexpressed in mice undergoing both SCI and intramuscular CDTX injury to test their potential inhibitory effect on NHO. Finally, I have performed qRT-PCR for some genes of the BMP signalling pathway in order to compare my NHO model with FOP, a condition where muscles similarly become ossified caused by mutatious and abnormal signalling of BMP pathway.
5.2 Methods specific for this chapter

5.2.1 Processing and analysis of microarray data
Processing and analysis of Illumina microarray is described in Chapter 2, section 2.12 in details. Total RNA was extracted from hamstring muscles from four cohorts of mice at Day 2 after surgery. Cohorts included:

- SCI surgery + CDTX injection
- SCI alone
- CDTX injection + SHAM operation and
- SHAM operation

RNA was cleaned-up, concentrated and amplified as described in 2.12.1, Chapter 2. Analysis was performed using BRB ArrayTools software.

5.2.2 SCI surgery and mouse strains used in this chapter
SCI surgery was performed as described in Chapter 2, section 2.2. All experiments for this chapter were performed on C57BL/6J. All mice were euthanized for microCT analysis 10-14 days after surgery and at 2 days after surgery for microarray assay.

5.2.3 Treatment options

5.2.3.1 Kineret
Kineret (Anakinra 100mg, catalogue number EU/1/02/203/002, Swedish Orphan Biovitrum, Sweden) is recombinant human IL-1 receptor antagonist and competitively inhibits the binding of IL-1 to its cognate receptor. It was administered by intraperitoneal injection as described previously [183] at two doses: 25mg/kg and 10mg/kg body weight immediately after surgery and injection of CDTX and then once daily for 10 days. Control mice were injected with an equivalent volume of saline. To inject 25mg/kg: 50μl of commercial stock from the syringe (100 mg/0.67 mL) was diluted in 1.45mL of saline. For 10mg/kg 20μl of stock from the syringe was diluted in 1.48mL of saline.

5.2.3.2 Etanercept
Etanercept (Enbrel 25mg, Pfizer New Zealand Ltd. Catalogue number R90456) is a fusion protein between the extracellular domain of human TNF-α receptor 2 and human IgG1 Fc fragment. It works by binding directly to human or mouse TNF-α and prevents the binding of the human/mouse TNF-α to their cellular receptors. It was administered by intraperitoneal injection in a dose of 10mg/kg and 20mg/kg of body weight immediately after surgery and injection of CDTX and then daily for 10 days, doses previously shown to
be effective in mice [184, 185]. Control mice were injected with an equivalent volume of saline (100 μl). Etanercept powder (25mg) was diluted with 1 mL of water for injections as per manufacturer’s instructions. To inject 20mg/kg: 336μl of stock was diluted in 1.76mL of water for injections. To inject 10mg/kg 168μl of 20mg/kg solution was diluted in 1.76mL of water for injections.

5.2.3.3 GW2580 (CSF1R kinase inhibitor)
To make vehicle for oral gavage of GW2580, 10μl of Tween20 was diluted in 10mL of water for injections, then 50mg of hydroxypropyl methylcellulose (USP grade H3785, Sigma Aldrich) powder was added as described previously [186, 187]. The mixture was rotated on rotating wheel until powder was fully dissolved at room temperature. This mixture was stored in -80°C for 10 days. To make fresh stock 3.2mg of GW2580 (orb61037, Byorbyt) was diluted in 100μL of vehicle by gently mixing with syringe mounted with a 23G needle until the suspension was homogenous. Mice were gavaged with GW2580 160mg/kg (100 μL) or vehicle at the day of surgery after recovery and then daily for 10 days.

5.2.4 qRT-PCR analysis
For qRT-PCR analysis expression was determined relative to β-actin. For myogenic markers RNA extracted from whole hamstring group of muscle was used. RNA was extracted from whole hind limb muscle as described in Materials and Methods section 2.8, Chapter 2. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) and analysis the relative to housekeeping gene expression as determined by ΔCt was compared between the groups as described in Chapter 2, section 2.10. Primers used in this chapter are outlined in the Table 5.1.
Table 5.1 Primers used for qRT-PCR

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<tr>
<td>Arg1</td>
<td>Mm00475988_m1</td>
<td>65*</td>
</tr>
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<td>β2M</td>
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<tr>
<td>β-actin</td>
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<td>Chi33*</td>
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*for TaqMan Gene Expression Array Mix

5.3 Results

Sixteen RNA samples harvested from whole hind limb muscle 48 hours after surgery were used in this study coming from four mice per experimental groups. The four experimental groups were:

- SCI surgery + CDTX injection
- SCI alone
- CDTX injection + SHAM operation and
- SHAM operation.
The IlluminaBeadChip array was used as described in 2.12, Chapter 2 and the data set was filtered and normalised using the Lumi package developed by the North-western University. All subsequent analyses were performed with BRB Microarray software on the normalised data set and were performed using SOURCE annotations for the pathway analysis. The gene sets were defined based on Gene Ontology and Lymphoid signatures 2.12.3, Chapter 2.

5.3.1 Mouse groups with NHO, SCI only or inflammation only display a unique transcriptomic profiles

Principal component analysis was performed on RNA from whole muscle from the 4 experimental groups to determine whether samples clustered according to the mouse treatment. All four groups of mice were compared on a two dimensional plane, principal component 1 and principal component 2 as shown in Figure 5.1. Figure 5.1A represents raw data, while in Figure 5.1B normalised data are presented. Black arrow indicates the only mouse sample that did not cluster with other samples from the same group and was considered outliers. Therefore, this sample was removed from the subsequent analysis of differentially expressed sets of genes. However it was left for statistical analysis of genes that expression was validated by qRT-PCR. Figure 5.1B displays four distinct clusters that match the mouse treatment groups. Interestingly, following data normalisation, the 4 treatment groups clustered at the 4 different corners of the principal component 2 D plot suggesting that SCI and CDTX-mediated injury have very distinct effect of cellular transcriptome in the muscle.

Hierarchical clustering was also performed to determine if the surgical groups were clustering as a pure or homogeneous population according to the type of surgery and injection, or to determine if there was heterogeneity between these groups of mice. In this analysis, hierarchical clustering resulted in relatively stable groupings. It can be seen in Figure 5.2 that there were 4 main groups, according to the type of surgery performed. All samples in each group are clustering together and groups appear to be small and homogeneous clusters of 4 samples except for one outlier sample from SCI+CDTX group that was clustered with CDTX group. In the hierarchy of this clustering CDTX mediated injury split the experimental groups upstream of the SCI showing that the effect of CDTX induced injury is dominant over the SCI on the muscle transcriptome. This is not too surprising as CDTX injection causes major destruction of the muscle and infiltration of macrophages whereas the effect of SCI is due to the result of the paralysis, a more subtle effect probably to do to reduced muscle activity and metabolism.
Figure 5.1 Principle component analysis and whole muscle gene expression arrays in mice that underwent SCI and or CDTX-mediated muscular inflammation. (A) The original data set containing all samples from SCI and SHAM-operated mice. (B) The normalised data set. This is the final data set used for all subsequent analyses. The black arrow indicate mouse sample from SCI+CDTX group that did not cluster with other samples from the same surgical group-outlier. Dotted line – SCI+CDTX, Compound line – SCI, Long dash dot line – CDTX, Dash line – SHAM

Figure 5.2 Hierarchical clustering of mouse surgery groups based on their differentially expressed genes. This is the final normalised data set contained all mouse samples used for all subsequent analyses. 1.1-1.4 – SCI+CDTX, 2.1-2.2 – SCI surgery, 3.1-3.4 – CDTX+SHAM, 4.1-4.4 – SHAM surgery
### 5.3.2 Ingenuity pathway analysis confirms expression molecules of inflammation and cellular response signalling pathways

BRB ArrayTool Gene Ontology and BioCarta Pathway databases were used to identify the major biological gene sets that were significantly differentially expressed between the four surgical groups. Gene Ontology database identified 5518 total investigated gene sets, BioCarta Pathway identified 264 total gene sets. I first evaluated the effect of SCI on whole muscle transcriptome by comparing SCI mice to SHAM-operated mice. In SCI vs SHAM analysis, 497 out of 5518 investigated gene sets passed the 0.005 significance threshold based on Two-sample T-test. LS/KS permutation test that finds gene sets which have more genes differentially expressed among the phenotype classes than expected by chance found 270 significant gene sets. In SCI+CDTX vs CDTX analysis 570 out of 5518 investigated gene sets passed the 0.005 significance threshold based on Two-sample T-test. LS/KS permutation test that finds gene sets which have more genes differentially expressed among the phenotype classes than expected by chance found 277 significant gene sets.

According to BioCarta Pathway database analysis of SCI vs SHAM showed that 29 out of 264 investigated gene sets passed the 0.005 significance threshold. Analysis of SCI+CDTX vs CDTX showed that 34 out of 264 investigated gene sets passed the 0.005 significance threshold. These differentially expressed pathways are represented in Table 5.2 and 5.3. All gene sets could be classified as biological process, cellular component and molecular function.

#### Table 5.2 Pathway analysis of gene sets differentially expressed between SCI and SHAM groups.

<table>
<thead>
<tr>
<th>Pathway description</th>
<th>Number and list of genes included in pathway</th>
<th>Type of pathway</th>
<th>LS permutation p-value</th>
<th>KS permutation p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effects of calcineurin in Keratinocyte Differentiation</td>
<td>Cdkn1a, Prkca, Ppp3ca, Nfatc1, Nfatc4, Ppp3cb, Calm3, Marcks, Sp1, Ppp3cc, Sp3, Nfatc3, Calm1, Nfatc2, Gnaq, Plcg1, Prkcb</td>
<td>cell proliferation and metabolism</td>
<td>0.00001</td>
<td>0.01875</td>
</tr>
<tr>
<td>Pathway Description</td>
<td>Genes</td>
<td>Regulation/Function</td>
<td>p-value 1</td>
<td>p-value 2</td>
</tr>
<tr>
<td>-----------------------------------------------------------------------------------</td>
<td>----------------------------------------------------------------------</td>
<td>-----------------------------------------------</td>
<td>------------</td>
<td>----------</td>
</tr>
<tr>
<td><strong>Control of skeletal myogenesis by HDAC &amp; calcium/calmodulin-dependent kinase (CaMK)</strong></td>
<td>Map2k6, Myod1, Ppp3ca, Camk2a, Nfatc1, Ppp3cb, Calm3, Mapk14, Hdac5, Ywhah, Ins, Igf1r, Igf1, Avp, Ppp3cc, Mapk7, Pik3r1, Calm1, Nfatc2, Pik3ca, Pik3cg, Insr, Akt1</td>
<td>cell proliferation and metabolism</td>
<td>0.00001</td>
<td>0.0123</td>
</tr>
<tr>
<td><strong>p53 Signaling Pathway</strong></td>
<td>Cdkn1a, Gadd45a, Pcn, Rb1, Bax, Trp53, Mdm2, Ccnd1, Ccne1, Apaf1, Cdk2, Bcl2, Timp3, E2f1</td>
<td>cell proliferation metabolism and apoptosis</td>
<td>0.00001</td>
<td>0.03482</td>
</tr>
<tr>
<td><strong>fMLP induced chemokine gene expression in HMC-1 cells</strong></td>
<td>Map2k6, Map2k1, Ppp3ca, Nfatc1, Nfatc4, Camk2a, Ppp3cb, Calm3, Hras1, Map2k2, Nfkbia, Mapk14, Fpr1, Pak1, Pik3c2g, Ppp3cc, Nfatc3, Ncf2, Raf1, Calm1, Nfatc2, Map3k1, Mapk1, Nfkb1, D830050J10Rik, Mapk3, Rela, Ncf1, Gna15, Plcb1, Map2k3</td>
<td>inflammation</td>
<td>0.00002</td>
<td>0.00755</td>
</tr>
<tr>
<td><strong>Shuttle for transfer of acetyl groups from mitochondria to the cytosol</strong></td>
<td>Slc25a11, Cs, Acl, Pdha1, Mdh1, Slc25a1, Pcx</td>
<td>cell proliferation and metabolism</td>
<td>0.00011</td>
<td>0.01175</td>
</tr>
<tr>
<td><strong>Regulation of PGC-1a</strong></td>
<td>Ppp3ca, Camk2a, Esrra, Ppp3cb, Calm3, Slc2a4, Ppargc1a, Hdac5, Ywhah, Camk4, Ppp3cc, Ppara, Hdac5, Calm1</td>
<td>cell proliferation and metabolism</td>
<td>0.00012</td>
<td>0.00004</td>
</tr>
<tr>
<td><strong>p38 MAPK Signaling Pathway</strong></td>
<td>Daxx, Tgfb2, Creb1, Cdc42, Grb2, Hmgn1, Map3k1, Hspb2, Myc, Shc1, Max, Atf2, Tgfbr1</td>
<td>cell differentiation</td>
<td>0.00012</td>
<td>0.00924</td>
</tr>
<tr>
<td><strong>Erythropoietin mediated neuroprotection through NF-kB</strong></td>
<td>Cdkn1a, Jak2, Arnt, Nfkbia, Sod2, Arnt, Grin1, Epor, Grin1, Nfkbi1, Epo, Hif1a, Rela</td>
<td>cell proliferation and metabolism</td>
<td>0.00015</td>
<td>0.01822</td>
</tr>
<tr>
<td>Pathway</td>
<td>Genes</td>
<td>Inflammation</td>
<td>Cell Proliferation</td>
<td>Programmed Cell Death and Apoptosis</td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>----------------------------------------------------------------------</td>
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<td>-------------------</td>
<td>-------------------------------------</td>
</tr>
<tr>
<td>TNF/Stress Related Signaling</td>
<td>Map2k6, Tgfr, Cdc42, Tgfr1, Ddit3, Mapkapk2, Hspb1, Map2k4, Stat1, Hras1, Creb1, Map3k7, Atf2, Traf2, Mapk14, Ripk1, Shc1, Tgfb1, Myc, Tgfb3, Mnk1, Jun, Map3k14, Tnfrsf1a, Tank, Nfkbia, Map4k2, Ripk1, Ikbkg, Casp2, Ikbkb, Atf1, Map3k1, Nfkbia, Mapk8, Rela, Tnf, Chuk, Cradd, Map2k3</td>
<td>0.00016</td>
<td>0.00049</td>
<td>0.00049</td>
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<tr>
<td>Toll-Like Receptor Pathway</td>
<td>Map2k6, Ecsit, Jun, Map3k14, Ira1, Map2k4, Traf6, Map3k7, Nfkbia, Mapk14, Ikbkg, Eif2ak2, Ikbkb, Fos, Ppara, Myd88, Map3k1, Nfkbia, Mapk8, Rela, Chuk, Pglyrp1, Map2k3</td>
<td>0.00033</td>
<td>0.0904</td>
<td>0.0033</td>
</tr>
<tr>
<td>Cell Cycle: G2/M Checkpoint</td>
<td>Cdkn1a, Gadd45a, Trp53, Mdm2, Cdc25c, Ywhah, Rps6ka1, Prkdc, Wee1, Myt1, Cdkn2d, Cdc34, Cdc25a, Cdc25b, Ep300, Brca1, Chek1, Ccnb1, Plk1</td>
<td>0.00037</td>
<td>0.16096</td>
<td>0.00037</td>
</tr>
<tr>
<td>NFkB activation by Nontypeable Hemophilus influenzae</td>
<td>Map2k6, Tgfr1, Dusp1, Map3k14, Map3k7, Nfkbia, Mapk14, Mapk11, Crebbp, Tgfr2, Smad3, Ikbkb, I11b, Myd88, Nfkbia, Nrk3c1, Ep300, Rela, Tnf, Chuk, Map2k3</td>
<td>0.00037</td>
<td>0.00532</td>
<td>0.00037</td>
</tr>
<tr>
<td>Signal Dependent Regulation of Myogenesis by Corepressor MITR</td>
<td>Myod1, Camk2a, Ywhah, Hdac9</td>
<td></td>
<td></td>
<td>0.00052</td>
</tr>
<tr>
<td>Role of nicotinic acetylcholine receptors in the regulation of apoptosis</td>
<td>Musk, Chrnb1, Rapsn, Chrng, Fasl, Ywhah, Bad, Ptk2b, Pik3r1, Pik3ca, Foxo3, Pik3cg, Tert, Akt1</td>
<td></td>
<td></td>
<td>0.00054</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.00594</td>
</tr>
<tr>
<td>Pathway</td>
<td>Genes</td>
<td>Significance (p-values)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------------------------------</td>
<td>----------------------------------------------------------------------</td>
<td>------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malate-aspartate shuttle</td>
<td>Slc25a11, Mdh1</td>
<td>cell proliferation and metabolism 0.00088 0.08255</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATM Signaling Pathway</td>
<td>Lmna, Rb1, Jun, Tnfrsf1a, Map2k4, Arhgdib, Map3k7, Parp1, Traf2, Dffa, Ripk1, Prkdc, Lmnb2, Casp2, Casp3, Pak1, Bag4, Fadd, Map3k1, Cdkn1a, Gadd45a, Rpa1, Trp53, Mdm2, Nfkbia, Mre11a, Chek2, Rad50, Rad51, Chek1, Trp73, Nfkb1, Abl1, Mapk8, Nbn, Rela, Brca1</td>
<td>inflammation 0.0009 0.00008 cell proliferation metabolism and apoptosis 0.00099 0.3424</td>
<td></td>
<td></td>
</tr>
<tr>
<td>The role of FYVE-finger proteins in vesicle transport</td>
<td>Tfrc, Hgs, Egfr, Trf</td>
<td>cell proliferation and metabolism 0.0012 0.01193</td>
<td></td>
<td></td>
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<tr>
<td>Agrin in Postsynaptic Differentiation</td>
<td>Chrna1, Musk, Arhgef6, Rapsn, Cdc42, Jun, Agrn, Ctn, Utrn, Lama2, Dag1, Git2, Itga1, Pak3, Egfr, Mapk1, Pak1, Sp1, Dmd, Dvl1, Nrg3, Lama1, Pak4, Mapk8, Mapk3, Chrme1, Lama4, Acta1, Itgb1</td>
<td>muscle specific cell proliferation and metabolism 0.00123 0.12024</td>
<td></td>
<td></td>
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<tr>
<td>Keratinocyte Differentiation</td>
<td>Map2k6, Prkca, Ets1, Map2k1, Jun, Map3k14, Tnfrsf1a, Map2k4, Ppp2ca, Hras1, Map2k7, Fasl, Nfkbia, Traf2, Mapk14, Prkch, Prkcg, Ripk1, Casp8, Fas, Egfr, Ilkbob, Mapk1, Fos, Sp1, Egf, Daxx, Noxa7, Raf1, Mapk13, Bcl2, Map3k1, Tnfrsf1b, Nfkb1, D830050J10Rik, Mapk8, Ets2, Mapk3, Rela, Prkce, Prkcb, Tnf, Chuk, Cebpa, Prkcd, Map2k3</td>
<td>cell proliferation and metabolism 0.00154 0.06435</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Role of MEF2D in T-cell Apoptosis</td>
<td>Prkca, Ppp3ca, Ppp3cb, Calm3, Nfatc1, A430107P09Rik, Capns1, Hdac2, Ppp3cc, Mef2d, Calm1, Nfatc2, Ep300, Capn2, Prkcb</td>
<td>inflammation</td>
<td>0.00326</td>
<td>0.08504</td>
</tr>
<tr>
<td>Signaling Pathway from G-Protein Families</td>
<td>Prkca, Map2k1, Ppp3ca, Nfatc1, Nfatc4, Jun, Ppp3cb, Calm3, Prkar2a, Hras1, Creb1, Prkar1b, Fos, Prkar2b, Rps6ka3, Ppp3cc, Nfatc3, Raf1, Calm1, Nfatc2, Gnaq, Prkar1a, Plcg1, Gnaq, Prkacb, Adcy1, D830050J10Rik, Mapk3, Prkcb, Nfatc1</td>
<td>cell proliferation and metabolism</td>
<td>0.00351</td>
<td>0.181</td>
</tr>
<tr>
<td>BCR Signaling Pathway</td>
<td>Prkca, Map2k1, Ppp3ca, Nfatc1, Nfatc4, Jun, Ppp3cb, Calm3, Hras1, Mapk14, Shc1, Fos, Ppp3cc, Nfatc3, Sos1, Raf1, Calm1, Grb2, Nfatc2, Btk, Cd79b, Map3k1, Plcg1, D830050J10Rik, Mapk8, Mapk3, Vav1, Syk, Prkcb, Cd79b</td>
<td>inflammation</td>
<td>0.00783</td>
<td>0.22291</td>
</tr>
<tr>
<td>Polyadenylation of mRNA</td>
<td>Papola, Cstf3, Cstf1, Cpsf3, Cpsf1, Pabpn1</td>
<td>cell proliferation and metabolism</td>
<td>0.00862</td>
<td>0.00057</td>
</tr>
<tr>
<td>Signal transduction through IL1R</td>
<td>Map2k6, Irak2, Ecsit, Jun, Map3k14, Irak1, Traf6, Map3k7, Nfkbia, Mapk14, Ikkb, Tgfb1, Il1rap, Tgfb3, I1b, Irak3, I6, Tgfb2, Myd88, Ifna1, Il1rn, Map3k1, Nfkb1, Ifnb1, Mapk8, I11a, Rela, Tnf, Chuk, Il1r1, Map2k3</td>
<td>inflammation</td>
<td>0.01239</td>
<td>0.29512</td>
</tr>
<tr>
<td>Human Cytomegalovirus and Map Kinase Pathways</td>
<td>Map2k6, Map2k1, Rb1, Map2k2, Creb1, Mapk14, Mapk1, Sp1, Pik3r1, Pik3ca, Map3k1, Nfkb1, Pik3cg, Mapk3, Rela, Akt1, Map2k3</td>
<td>proliferation</td>
<td>0.01255</td>
<td>0.28157</td>
</tr>
<tr>
<td>Cell Cycle: G1/S Check Point</td>
<td>Cdkn1a, Rb1, Trp53, Tfdp1, Cdkn2b, Ccna1, Ccnd1, Smad3, Tgfb1, Cdkn1b, Tgfb3, Cdkn2a, Abi1, Dhfr, Ccne1, Tgfb2, Cdk2, Cdc25a, Cdk6, Skp2, Gsk3b, E2f1</td>
<td>cell proliferation and metabolism</td>
<td>0.01275</td>
<td>0.11727</td>
</tr>
<tr>
<td>--------------------------</td>
<td>---------------------------------------------------------------------------------</td>
<td>----------------------------------</td>
<td>----------</td>
<td>----------</td>
</tr>
<tr>
<td>Y branching of actin filaments</td>
<td>Psma7, Wasf1, Nckap1, Wasf2, Wasl, Wasf3, Nck1, Pira1, Abi2, Acta1</td>
<td>cytoskeleton structure</td>
<td>0.02186</td>
<td>0.30535</td>
</tr>
<tr>
<td>Influence of Ras and Rho proteins on G1 to S Transition</td>
<td>Cdkn1a, Rb1, Hras1, Tfdp1, Nfkbia, Ccnd1, Ikbkg, Ikbkb, Mapk1, Pak1, Cdkn1b, Ccne1, Pik3r1, Raf1, Cdk2, Pik3ca, Nfkbl, Rhoa, D830050J10Rik, Cdk6, Mapk3, Rela, Chuk, E2f1, Akt1</td>
<td>cell proliferation and metabolism</td>
<td>0.04878</td>
<td>0.43207</td>
</tr>
</tbody>
</table>

This table clearly shows that majority of differentially expressed pathways between SCI- and SHAM-operated groups are related to muscle biology and repair, cell metabolism, and cell proliferation with only a few related to inflammation. In majority of differentially expressed pathways up- and down-regulated genes are spread equally in both groups. However all genes in Polyadenylation of mRNA, p53 signaling pathway and role of nicotinic acetylcholine receptors in the regulation of apoptosis are up-regulated in SCI alone group. Indeed all three pathways are involved in apoptosis and as we mentioned earlier that could be associated with 21-28% loss of muscle mass in the limbs of mice with SCI [188]. Interestingly all genes were down-regulated in SCI group in the following pathways:
- Regulation of PGC-1a, which is involved in muscle specific cell proliferation;
- shuttle for transfer of acetyl groups from mitochondria to the cytosol, involved in glucose metabolism and as a result in the muscle function; and
- malate-aspartate shuttle, also specific for skeletal muscle tissue.

This down-regulation can possibly be explained by muscle paralysis and slower rates of muscle cells metabolism.

The pathway analysis of gene sets differentially expressed between SCI+CDTX and CDTX groups however presented a very different picture with the most of these pathways related to inflammation (Table 5.3).
**Table 5.3** Pathway analysis of gene sets differentially expressed between SCI+CDTX and CDTX groups.

<table>
<thead>
<tr>
<th>Pathway description</th>
<th>List of genes included in a pathway</th>
<th>Type of pathway</th>
<th>LS permutation p-value</th>
<th>KS permutation p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>mTOR Signaling Pathway</td>
<td>Tsc2, Mnk1, Eif4a2, Eif3a, Ppp2ca, Eif4ebp1, Eif4a1, Pdk2, Rheb, Pik3r1, Pten, Pdk1, Eif4e, Eif3a, Pik3ca, Rps6kb1, Akt1, Tsc1</td>
<td>cell proliferation</td>
<td>0.00001</td>
<td>0.01188</td>
</tr>
<tr>
<td>TNF/Stress Related Signaling</td>
<td>Map2k6, Map2k4, Rela, Map3k1, Jun, Tank, Traf2, Nfkb1, Casp2, Tnfrsf1a, Nfkb1, Map3k14, Mapk14, Ripk1, Tnf, Chuk, Ikbkg, Tank, Ikbkb, Map4k2, Cradd, Atf1, Mapk8, Map2k3</td>
<td>inflammation</td>
<td>0.00004</td>
<td>0.00007</td>
</tr>
<tr>
<td>Toll-Like Receptor Pathway</td>
<td>Pglyrp1, Map2k6, Map2k4, Rela, Map3k1, Jun, Nfkb1, Myd88, Traf6, Nfkb1, Map3k14, Mapk14, Chuk, Ecsit, Ikbkg, Ikbkb, Eif2ak2, Ppara, Fos, Map3k7, Ira1, Mapk8, Map2k3</td>
<td>inflammation</td>
<td>0.00014</td>
<td>0.00195</td>
</tr>
<tr>
<td>NFkB activation by Nontypeable Hemophilus influenzae</td>
<td>Map2k6, Rela, Ep300, Mapk11, Nfkb1, Myd88, Smad3, Tgfr1, Nfkb1, Map3k14, Mapk14, Il1b, Tnf, Chuk, Tgfr2, Nr3c1, Dusp1, Ikbkb, Crebbp, Map3k7, Map2k3</td>
<td>inflammation</td>
<td>0.00026</td>
<td>0.00011</td>
</tr>
<tr>
<td>Keratinocyte Differentiation</td>
<td>Ets2, Map2k6, Map2k4, Ppp2ca, Rela, Map3k1, Jun, Raf1, Traf2, Nfkb1, Tnfrsf1a, Daxx, Prkcq, Prkca, Nfkb1, Hras1, Map3k14, Mapk14, Fas, Ripk1, Tnf, Chuk, Map2k7, Mapk1, Bcl2, Hoxa7, Egf, Map2k1, Ets1, Sp1, Cebpa, Mapk3, Ikbkb, Prkcb, D830050J10Rik, Tnfrsf1b, Egrf, Map2k7, Prkce, Fas, Prkcd, Fos,</td>
<td>cell proliferation and metabolism</td>
<td>0.00044</td>
<td>0.00493</td>
</tr>
<tr>
<td>Pathway</td>
<td>Genes</td>
<td>Function</td>
<td>p-value</td>
<td>q-value</td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>----------------------------------------------------------------------------------------------------------</td>
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<td>---------------</td>
<td>--------------</td>
</tr>
<tr>
<td>Acetylation and Deacetylation of RelA in The Nucleus</td>
<td>Rela, Ep300, Fadd, Nfkb1, Nfkbia, Ripk1, Tnf, Chuk, Fadd, Ikbkg, Tnfrsf1a, Ikbkb, Tnfrsf1b, Crebbp, Traf6, Ikbkg</td>
<td>cell proliferation and metabolism</td>
<td>0.00058</td>
<td>0.00064</td>
</tr>
<tr>
<td>TACI and BCMA stimulation of B cell immune responses.</td>
<td>Rela, Tnfrsf13c, Traf2, Nfkb1, Traf6, Map3k14, Mapk14, Chuk, Tnfrsf17, Tnfrsf13b, Tnfrsf13b, Traf3, Mapk8</td>
<td>inflammation</td>
<td>0.00062</td>
<td>0.00015</td>
</tr>
<tr>
<td>TNFR2 Signaling Pathway</td>
<td>Rela, Traf3, Map3k1, Tnfaip3, Tank, Traf2, Nfkb1, Nfkbia, Map3k14, Ripk1, Tnf, Chuk, Ikbkap, Ikbkg, Dusp1, Ikbkb, Tnfrsf1b, Traf1, Ikbkg</td>
<td>inflammation</td>
<td>0.0008</td>
<td>0.00343</td>
</tr>
<tr>
<td>Internal Ribosome entry pathway</td>
<td>Eif4a2, Eif3a, Eif4a1, Ptbp1, Eif4e</td>
<td>cell proliferation and metabolism</td>
<td>0.00081</td>
<td>0.00433</td>
</tr>
<tr>
<td>NF-kB Signaling Pathway</td>
<td>Fadd, Rela, Map3k1, Tnfaip3, Nfkb1, Myd88, Tnfrsf1a, Traf6, Nfkbia, Map3k14, Ripk1, Tnf, Chuk, Ikbkg, Ikbkap, Tnfrsf1b, Ii1r1, Ii1a, Map3k7, Irak1</td>
<td>inflammation</td>
<td>0.00086</td>
<td>0.00125</td>
</tr>
<tr>
<td>Chromatin Remodeling by hSWI/SNF ATP-dependent Complexes</td>
<td>Actb, Smarca4, Smarce1, Smarcc1, Nr3c1, Nf1, Tbp, Smarcb1, Smarcd1, Arid1a</td>
<td>cell proliferation and metabolism</td>
<td>0.00096</td>
<td>0.00933</td>
</tr>
<tr>
<td>Ceramide Signaling Pathway</td>
<td>Fadd, Map2k4, Rela, Map3k1, Cycs, Raf1, Traf2, Nfkb1, Tnfrsf1a, Ripk1, Cycs, Aifm1, Tnfrsf1, Smpd1, Mapk1, Bcl2, Map2k1, Mapk3, Casp8, D830050J10Rik, Bax, Nsmaf, Bad, Mapk8</td>
<td>programmed cell death and apoptosis, inflammation</td>
<td>0.00137</td>
<td>0.00076</td>
</tr>
<tr>
<td>SODD/TNFR1 Signaling Pathway</td>
<td>Fadd, Birc2, Traf2, Bag4, Tnfrsf1a, Ripk1, Tnf, Casp8, Tnfrsf1b</td>
<td>inflammation</td>
<td>0.00175</td>
<td>0.00496</td>
</tr>
<tr>
<td>Free Radical Induced Apoptosis</td>
<td>Rela, Sod1, Xdh, Gss, Nfkb1, Tnf, Gpx1</td>
<td>programmed cell death and apoptosis</td>
<td>0.00243</td>
<td>0.00548</td>
</tr>
<tr>
<td>--------------------------------</td>
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<td>---------</td>
</tr>
<tr>
<td><strong>fMLP induced chemokine gene expression in HMC-1 cells</strong></td>
<td>Ppp3ca, Map2k6, Rela, Map3k1, Camk2a, Nfatc1, Raf1, Pik3c2g, Nfkib1, Map2k2, Nfkbia, Nfatc4, Hras1, Mapk14, Ncf1, Ppp3cb, Mapk1, Fpr1, Nfatc1, Map2k1, Ppp3cc, Nfatc2, Mapk3, D830050J10Rik, Calm3, Nfatc3, Pak1, Gna15, Ncf2, Plb1, Ncf2, Map2k3, Calm1, Pik3c2g</td>
<td>inflammation</td>
<td>0.0028</td>
<td>0.00458</td>
</tr>
<tr>
<td>Induction of apoptosis through DR3 and DR4/5 Death Receptors</td>
<td>Lmna, Fadd, Rela, Birc2, Cycs, Traf2, Nfkb1, Tnfrsf10b, Nfkbia, Map3k14, Ripk1, Chuk, Casp9, Apaf1, Bid, Casp6, Bid, Bcl2, Tnfrsf25, Cflar, Casp3, Casp8, Casp7, Dfia, Tnfsf12, Dfbb, Tnfsf10, Gas2</td>
<td>programmed cell death and apoptosis</td>
<td>0.003</td>
<td>0.00205</td>
</tr>
<tr>
<td>HIV-I Nef: negative effector of Fas and TNF</td>
<td>Lmna, Fadd, Rela, Map3k1, Birc2, Cycs, Rasa1,Traf2, Nfkb1, Bag4, Casp2, Tnfrsf1a, Daxx, Mdm2, Nfkbia, Map3k14, Fas, Mdm2, Ripk1, Tnf, Chuk, Map2k7, Rb1, Casp9, Apaf1, Bid, Casp6, Psen1, Bcl2, Cflar, Arhgdib, Casp3, Casp8, Tnfrsf1b, Map2k7, Lmnb2, Parp1, Traf1, Casp7, Psen1, Psen2, Prkcd, Dfia, Lmnb1, Gsn, Fas, Dffb, Cradd, Map2k7, Fasl, Prkdc, Mapk8</td>
<td>inflammation</td>
<td>0.0034</td>
<td>0.00221</td>
</tr>
<tr>
<td>Pathway</td>
<td>Genes</td>
<td>Function</td>
<td>p-value 1</td>
<td>p-value 2</td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>-----------------------------------------------------------------------</td>
<td>---------------------------------</td>
<td>------------</td>
<td>------------</td>
</tr>
<tr>
<td>p38 MAPK Signaling Pathway</td>
<td>Mknk1, Hspb1, Map2k6, Map2k4, Map3k1, Atf2, Tgb2, Traf2, Daxx, Tgfr1, Hras1, Mapk14, Mapkapk2, Hmgn1, Shc1, Ripk1, Myc, Cdc42, Stat1, Myc, Tgfr1, Ddit3, Creb1, Max, Hspb2, Grb2, Map3k7, Tgb3</td>
<td>cell differentiation</td>
<td>0.00353</td>
<td>0.03044</td>
</tr>
<tr>
<td>CXCR4 Signaling Pathway</td>
<td>Cxcr4, Crk, Rela, Gnaq, Raf1, Cxcl12, Pik3c2g, Nfk1b, Prkca, Bcar1, Hras1, Ptk2b, Pik3r1, Mapk1, Map2k1, Crk, Mapk3, Pik3ca, Prkcb, D830050J10Rik, Gnaq, Plcg1</td>
<td>inflammation</td>
<td>0.00398</td>
<td>0.00875</td>
</tr>
<tr>
<td>T Cell Receptor Signaling Pathway</td>
<td>Lat, Ppp3ca, Map2k4, Rela, Map3k1, Jun, Zap70, Rasa1, Nfatc1, Raf1, Cd3d, Nfk1b, Cd3g, Prkca, Nfkbia, Nfatc4, Hras1, Vav1, Shc1, Cd3e, Ppp3cb, Pik3r1, Lck, Map2k1, Ppp3cc, Fyn, Nfatc2, Mapk3, Pik3ca, Prkcb, D830050J10Rik, Calm3, Cd247, Nfatc3, Fos, Sos1, Pik3cg, Grb2, Zap70, Mapk8, Plcg1, Calm1, A430107P09Rik</td>
<td>inflammation</td>
<td>0.00608</td>
<td>0.10654</td>
</tr>
<tr>
<td>Control of Gene Expression by Vitamin D Receptor</td>
<td>Tsc2, Smarca4, Smarce1, Ep300, Smarcc1, Vdr, Carm1, Rpgrip1, Actl6a, Baz1b, Top2b, Ncor1, Snw1, Med1, Rxra, Crebbp, Smarcd1, Ncoa3, Arid1a, Kat2b, Chaf1a</td>
<td>cell proliferation and metabolism</td>
<td>0.00665</td>
<td>0.01182</td>
</tr>
<tr>
<td>Endocytotic role of NDK, Phosphins and Dynamin</td>
<td>Ppp3ca, Ap2a1, Epn1, Eps15, Ppp3cb, Synj2, Dnm1, Ppp3cc, Calm3, Nme2, Nme1</td>
<td>cell proliferation and metabolism</td>
<td>0.00789</td>
<td>0.11027</td>
</tr>
<tr>
<td>Polyadenylatio n of mRNA</td>
<td>Cstf1, Cstf3, Pabpn1, Cpsf3, Cpsf1, Papola</td>
<td>cell proliferation and metabolism</td>
<td>0.00892</td>
<td>0.0917</td>
</tr>
<tr>
<td>Pathway Description</td>
<td>Gene Symbols</td>
<td>Cell Proliferation</td>
<td>Inflammation</td>
<td></td>
</tr>
<tr>
<td>-----------------------------------------------------------------------------------</td>
<td>------------------------------------------------------------------------------</td>
<td>--------------------</td>
<td>--------------</td>
<td></td>
</tr>
<tr>
<td>Cadmium induces DNA synthesis and proliferation in macrophages</td>
<td>Rela, Jun, Raf1, Nfkb1, Prkca, Nfkb1, Hras1, Tnf, Myc, Mapk1, Myc, Map2k1, Mapk3, Prkcb, D830050J10Rik, Fos, Plcb1</td>
<td>0.00907</td>
<td>0.00681</td>
<td></td>
</tr>
<tr>
<td>The information-processing pathway at the IFN-beta enhancer</td>
<td>Irf3, Rela, Jun, Atf2, Irf1, Nfkb1, Crebbp, Kat2b, Hmgb1</td>
<td></td>
<td>0.01224</td>
<td>0.16993</td>
</tr>
<tr>
<td>Msp/Ron Receptor Signaling Pathway</td>
<td>Mst1r, Csfs1, Il1b, Ccl2, Tnf, Mst1</td>
<td></td>
<td>0.01693</td>
<td>0.00371</td>
</tr>
<tr>
<td>Role of Ran in mitotic spindle regulation</td>
<td>Kpnb1, Rangap1, Tpx2, Rcc1, Kpna2, Ranbp1, Kif15, Aurka, Ran</td>
<td></td>
<td>0.0263</td>
<td>0.00187</td>
</tr>
<tr>
<td>Deregulation of CDK5 in Alzheimers Disease</td>
<td>Ppp2ca, Capn1, Capns1, Cdk5r1, Cdk5, Gsk3b</td>
<td></td>
<td>0.04224</td>
<td>0.05334</td>
</tr>
<tr>
<td>NFAT and Hypertrophy of the heart (Transcription in the broken heart)</td>
<td>Calr, Ppp3ca, Prkar1a, Myh2, Camk2a, Nfatc1, Raf1, Mel2c, Ctf1, Nkx2-5, Nfatc4, Hras1, Mapk14, Hbegf, Agt, Ppp3cb, Pik3r1, Mapk1, Prkar2a, Map2k1, Ppp3cc, Csnk1a1, Pik3r1, Nfatc2, Mapk3, Fgf2, Igf1, D830050J10Rik, Calm3, Rps6kb1, Acta1, Camk4, Akt1, Crebbp, F2, Nfatc3, Gsk3b, Edn1, Hand1, Plk3cg, Nppa, Prkar2b, Prkar1b, Prkacb, Gata4, Hand2, Mapk8, Calm1</td>
<td>cell proliferation and apoptosis</td>
<td>0.0719</td>
<td>0.08844</td>
</tr>
<tr>
<td>IL-6 signaling pathway</td>
<td>Csnk2a2, Jun, Raf1, Cebbp, Hras1, Ptpn11, Il6st, Shc1, Stat3, Il6, Il6ra, Srf, Map2k1, Jak2, Ptpn11, Mapk3, Jak3, D830050J10Rik, Csnk2a1, Fos, Sos1, Jak1, Grb2</td>
<td>inflammation</td>
<td>0.09595</td>
<td>0.19796</td>
</tr>
</tbody>
</table>
Among all differentially expressed gene sets changes in Msp/Ron Receptor Signaling Pathway between SCI+CDTX and CDTX alone groups were of a high interest. It was the only differentially expressed pathway where all genes included in this pathway were highly up-regulated in SCI+CDTX group. All genes included in this pathway are inflammatory cytokines and the pathway includes macrophage stimulating protein (Fig.5.3).

**Figure 5.3** Clustered heatmap of significantly differentially expressed genes included in Msp/Ron Receptor Signaling Pathway that were up-regulated in SCI+CDTX group

MSP/RON signaling plays a role in inflammation and the response to tissue injury. It has been implicated in the functional regulation of mononuclear phagocytes [189]. Not much is known about this signalling pathway role in muscles, however Chanda et al. have published that MSP/RON signaling may regulate hepatic glucose metabolism [190], but what is more important MSP has also been shown to stimulate bone resorbing activity of osteoclasts [191].
5.3.3 Differentially expressed genes

After removing outlier sample 1.2 (SCI+CDTX group) cluster analysis of all groups was performed with set p-value of univariate test with p value below 0.005. I have got a number of genes significant at 0.005 level of the univariate test: 7548. Since SCI+CDTX and CDTX alone groups were clustered closer on the hierarchical cluster map, cluster analysis was then performed between these two groups with set p-value of univariate test 0.005. The number of genes significant at this level was 2851. I then sorted these genes on the fold change based on up-regulated genes (2 fold and more) and downregulated genes (0.5 fold and less) in SCI+CDTX group. Analysis revealed 135 up-regulated and 145 down-regulated genes.

Then new cluster analysis of all groups was performed with selected up-regulated and down-regulated gene subsets. Heatmaps are represented on Figure.5.4.
Figure 5.4 Clustered heatmap of significantly differentially expressed genes that were (A) down-regulated in SCI+CDTX group and (B) up-regulated in SCI+CDTX group.
Upregulated osteoblast and macrophage markers

My previous data from Chapter 4 show that macrophages play a crucial role in NHO after SCI. Numerous genes related to inflammation or macrophages were similarly highly up-regulated in SCI+CDTX group – the only group where NHO develops compared to muscles from mice with SCI injury alone (Fig 5.4 and table 5.4). Indeed some genes up-regulated in SCI+CDTX group are exclusive to osteoblasts or activated macrophages. Also numerous inflammatory markers are upregulated in SCI+CDTX group. Interestingly these markers were significantly higher in group, where CDTX-induced inflammation was coupled with SCI, suggesting that SCI may enhance muscular inflammation.

Table 5.4 Osteoblasts and macrophages expressed markers which are differentially expressed among classes and up-regulated in SCI+CDTX group compared to all groups

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Function</th>
<th>Expression pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd163</td>
<td>CD163 antigen</td>
<td>scavenger receptor for the hemoglobin-haptoglobin complex, M2 marker; acute-phase response, inflammatory response</td>
<td>bone, bone marrow</td>
</tr>
<tr>
<td>Slpi</td>
<td>secretory leukocyte peptidase inhibitor</td>
<td>secreted inhibitor which protects epithelial tissues from serine proteases released by granulocytes</td>
<td>strongly induced in macrophages activated by LPS or thioglycollate</td>
</tr>
<tr>
<td>Cd14</td>
<td>CD14 antigen</td>
<td>LPS binding, lipoteichoic acid binding; immune system process, inflammatory response, response to molecule of bacterial origin, response to tumor necrosis factor</td>
<td>LPS activated macrophages</td>
</tr>
<tr>
<td>Trap1</td>
<td>TNF receptor-associated protein 1</td>
<td>negative regulation of cellular respiration, negative regulation of oxidative stress-induced intrinsic apoptotic signaling pathway, protein folding, response to stress</td>
<td>Highly expressed in liver, kidney, brown adipose tissue</td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
<td>Function</td>
<td>Location</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
<td>-----------------------------------------------------------------------------------------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>Irf3</td>
<td>interferon regulatory factor 3</td>
<td>cellular response to LPS, immune system process, immune response, positive regulation of interferon-alpha and beta production</td>
<td>B-cells, live and spleen</td>
</tr>
<tr>
<td>Glycam1</td>
<td>glycosylation dependent cell adhesion molecule 1</td>
<td>Involved in cell adhesion, regulation of immune response</td>
<td>highly expressed in lymph nodes and mammary gland</td>
</tr>
<tr>
<td>Chi3l3</td>
<td>chitinase 3-like 3</td>
<td>Inflammatory response, M2 marker</td>
<td>Granulocytes; bone and bone marrow</td>
</tr>
<tr>
<td>Itgb5</td>
<td>integrin beta 5</td>
<td>part of VLA-5, the integrin receptor for fibronectin and VCAM-1</td>
<td>osteoblasts, LPS activated macrophages</td>
</tr>
<tr>
<td>Cxcl14</td>
<td>chemokine (C-X-C motif) ligand 14</td>
<td>platelet factor 4, immune response, chemoattractant and activator of monocytes, NK, dendritic cells</td>
<td>Brain tissue and cortex</td>
</tr>
<tr>
<td>Socs3</td>
<td>suppressor of cytokine signaling 3</td>
<td>inhibits the signal from the G-CSF receptor and IL6 receptor family</td>
<td>LPS activated bone marrow macrophages</td>
</tr>
<tr>
<td>P2ry6</td>
<td>pyrimidinergic receptor P2Y, G-protein coupled, 6</td>
<td>Regulator of immune response</td>
<td>macrophages, dendritic cells and microglia</td>
</tr>
<tr>
<td>Saa3</td>
<td>serum amyloid A 3</td>
<td>Acute inflammatory phase protein, recruitment of immune cells to the site of inflammation</td>
<td>LPS activated macrophages and microglia</td>
</tr>
<tr>
<td>Mfge8</td>
<td>milk fat globule-EGF factor 8 protein</td>
<td>Stimulates angiogenesis, chemotherapy resistance to cancer cells</td>
<td>LPS activated macrophages, spleen, lymph nodes</td>
</tr>
<tr>
<td>Plod3</td>
<td>procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3</td>
<td>synthesis of collagens, deficiency causes Ehlers–Danlos syndrome</td>
<td>Macrophages and osteoblasts</td>
</tr>
<tr>
<td>Ier3</td>
<td>immediate early response 3</td>
<td>Involved in apoptosis and specific immune response</td>
<td>mast cells, T-cells, macrophages and osteoblasts</td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
<td>Function</td>
<td>Tissues/Cells</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------------------------------------------------</td>
<td>--------------------------------------------------------------------------------------------</td>
<td>-----------------------------------------------------------</td>
</tr>
<tr>
<td>Srgn</td>
<td>Serglycin</td>
<td>Mediator of apoptosis, mast cell and T-cell secretory granule organization</td>
<td>mast cells, follicular B-cells, LPS activated macrophages</td>
</tr>
<tr>
<td>Hp</td>
<td>Haptoglobin</td>
<td>Acute-phase response, immune response to bacterial infection</td>
<td>macrophages, osteoblasts, bone, bone marrow, liver</td>
</tr>
<tr>
<td>Hyal2</td>
<td>Hyaluronoglucosaminidase 2</td>
<td>Monocyte activation, response to viruses</td>
<td>LPS activated macrophages, osteoclasts and osteoblasts</td>
</tr>
<tr>
<td>Col18a1</td>
<td>Collagen, type XVIII, alpha 1</td>
<td>Angiogenesis</td>
<td>LPS activated macrophages, osteoblasts, mast cells</td>
</tr>
<tr>
<td>Timp1</td>
<td>Tissue inhibitor of metalloproteinase 1</td>
<td>Inhibitor of many matrix metalloproteinases released by granulocytes/monocytes, tissues in repair, inhibits collagenase</td>
<td>Osteoblasts</td>
</tr>
<tr>
<td>Sema3f</td>
<td>Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3F</td>
<td>Role in vascularisation together with VEGF, immunoglobulin secretion</td>
<td>osteoblasts, osteoclasts, mast cells, macrophages</td>
</tr>
<tr>
<td>Txndc5</td>
<td>Thioredoxin domain containing 5</td>
<td>Expressed in response to hypoxia</td>
<td>Osteoblasts, follicular B-cells, bone marrow macrophages</td>
</tr>
<tr>
<td>Kcnab2</td>
<td>Potassium voltage-gated channel, shaker-related subfamily, beta member 2</td>
<td>Hematopoietic progenitor cell differentiation</td>
<td>LPS activated macrophages, NK-cells, osteoclasts, neuroglia</td>
</tr>
<tr>
<td>Igfbp7</td>
<td>Insulin-like growth factor binding protein 7</td>
<td>Involved in neurogenesis</td>
<td>Osteoblasts, spleen, kidney, lymph nodes</td>
</tr>
</tbody>
</table>

*Downregulated osteoblast and macrophage markers*

Surprisingly, some inflammatory markers were also down-regulated in SCI+CDTX group (Table 5.5). Surprisingly macrophage inflammatory type activation was reduced in the CDTX+SCI group compared to CDTX, which may be effect of SCI. Interestingly a few muscle specific genes like Enol3, Tpm2, Actc1, Myoz1, Vgl12, Myom1, 2310042D19Ryk (Perm1), Fh1 and Adipor1 were down-regulated in SCI+CDTX group.
exclusively. It may suggest that muscle affected by CDTX has worse regeneration abilities in the presence of SCI.

**Table 5.5** Osteoblasts and macrophages expressed markers which are differentially expressed among classes and downregulated in SCI+CDTX group compared to all groups

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Function</th>
<th>Expression pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Itm2b</td>
<td>integral membrane protein 2B</td>
<td>mutation is associated with familial dementia, T-cell development and functioning, repressed by CSF-1</td>
<td>mast cells, osteoblasts, LPS activated macrophages, spinal cord, cerebellum</td>
</tr>
<tr>
<td>Mlf1</td>
<td>myeloid leukemia factor 1</td>
<td>myeloid progenitor cell differentiation</td>
<td>Skeletal muscle</td>
</tr>
<tr>
<td>Ifit3</td>
<td>interferon-induced protein with tetratricopeptide repeats 3</td>
<td>anti-viral immunity</td>
<td>LPS activated macrophages</td>
</tr>
<tr>
<td>Ifit2</td>
<td>interferon-induced protein with tetratricopeptide repeats</td>
<td>apoptotic process, cellular response to interferon-alpha, anti-viral immunity</td>
<td>LPS activated macrophages</td>
</tr>
<tr>
<td>Clec4a1</td>
<td>C-type lectin domain family 4, member a1</td>
<td>regulate leukocyte reactivity</td>
<td>B-cells, dendritic cells and macrophages express C-type lectin receptors</td>
</tr>
<tr>
<td>Lgals9</td>
<td>lectin, galactose binding, soluble 9</td>
<td>T-cell immunoglobulin, overexpressed in Hodgkin’s disease, knock-out mice have increased susceptibility to collagen induced arthritis</td>
<td>LPS activated macrophages, osteoclasts</td>
</tr>
<tr>
<td>Ctsa</td>
<td>cathepsin A</td>
<td>degradation of glycosaminoglycans, proteoglycans, and glycoproteins</td>
<td>LPS activated macrophages, osteoclasts, microglia</td>
</tr>
<tr>
<td>Calr</td>
<td>calreticulin</td>
<td>regulates NF-Kb signalling, regulation of monocyte/macrophage compartment</td>
<td>LPS activated macrophages, osteoclasts, osteoblasts</td>
</tr>
<tr>
<td>Gbp3</td>
<td>guanylate binding protein 3</td>
<td>Anti-viral immunity, cellular response to interferon-beta</td>
<td>LPS thioglycollate activated macrophages</td>
</tr>
<tr>
<td>Lgals3bp</td>
<td>lectin, galactoside-binding, soluble, 3 binding protein</td>
<td>Macrophage scavenger receptor (M1 and M2), immune response associated with NK</td>
<td>LPS thioglycollate activated macrophages, osteoblasts, osteoclasts</td>
</tr>
<tr>
<td>Protein</td>
<td>Description</td>
<td>Function</td>
<td>Condition</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
<td>----------</td>
<td>-----------</td>
</tr>
<tr>
<td>Gns</td>
<td>glucosamine (N-acetyl)-6-sulfatase</td>
<td>Mutation causes mucopolysaccharidosi s</td>
<td>LPS thioglycollate activated macrophages, osteoclasts, mast cells, B-cells</td>
</tr>
<tr>
<td>Gna12</td>
<td>guanine nucleotide binding protein (G protein), alpha inhibiting 2</td>
<td>acetylcholine receptor signaling pathway, cell cycle</td>
<td>LPS thioglycollate activated macrophages, osteoclasts, mast cells, follicular B-cells</td>
</tr>
</tbody>
</table>

5.3.4 Validation of macrophage and inflammation associated markers differentially expressed among the groups by qRT-PCR

Considering that previous experiments described in Chapter 4 confirmed that macrophages are required for NHO. Two main types of macrophages have been identified previously: inflammatory (M1-like) and tissue regenerative (M2-like) [192], however their actual roles are complicated, especially taking into consideration phenotype skewing process when M2 replaces M1 phenotype in the site of inflammation. In this chapter I took the opportunity to utilise the microarray data to determine whether specific macrophage biases (either M1 or M2) I have compared expression of M1- and M2- related markers between the groups of operated mice. I discovered that M2 inflammatory macrophage markers Arg-1, Mrc1, Chi3I3, Ccl2, Lgals were all up-regulated in SCI+CDTX group compared to all other groups by the microarray results (Fig.5.5).
Figure 5.5 Differentially expressed genes of anti-inflammatory macrophage markers in muscle of SCI and SHAM operated mice with and without CDTX induced inflammation at Day 2 after surgery – microarray results. (A) Ccl2, (B) Chi3l3, (C) Arg1, (D) Lgals3, (E) Mrc1 mRNA from hind limb muscle from operated mice. Data are represented as mean ± SD. Data were analyzed by One-way ANOVA with Bonferroni’s multiple comparison test. Each dot represents hind limb muscle mRNA from a separate mouse (4 mice per group)
Inflammatory cytokines IL-6, IL-1β, TNFα and Csf1 were also highly upregulated in SCI+CDTX group in comparison with three other groups including one with combination of SHAM surgery and CDTX-induced inflammation (Fig.5.6).

**Figure 5.6** Differentially expressed genes of inflammatory cytokines in muscle of SCI and SHAM operated mice with and without CDTX induced inflammation at Day 2 after surgery – microarray results. (A) IL-1β, (B) Csf1, (C) Tnfα, (D) IL-6. Data are represented as mean ± SD. Data were analyzed by One-way ANOVA with Bonferroni’s multiple comparison test. Each dot represents hind limb muscle mRNA from a separate mouse (4 mice per group).

Microarray results were validated on the mRNA isolated from hind limb muscles of SCI- and SHAM-operated mice with and without CDTX injection. Endosteal and central bone marrow (CBM) mRNA was used as a reference control and were not used in statistical analysis. Ccl2 was expressed significantly higher in SCI+CDTX group in comparison with SHAM operated mice, however the difference was not statistically significant between other groups of operated mice (Fig.5.7 A). Same tendency was observed for Lgals3 and IL1β (Fig5.7D). Arginase 1 was upregulated in both groups with CDTX induced
inflammation, however the difference between groups with and without CDTX induced inflammation was not statistically significant (Fig.5.7C). Despite the differentially expressed Mrc1 accordingly to microarray results, qRT-PCR failed to show the difference between the groups (Fig.5.7 F). Difference in cytokines Csf1 and Tnfα expression (Fig.5.7G,H) also did not match microarray results, as Csf1 was significantly upregulated in SCI alone group, while Tnfα was not significantly differently expressed in all four groups of mice according to the qRT-PRC results, however in gene microarray results both Csf-1 and Tnfα were significantly upregulated in SCI+CDTX group. Comparative analysis of inflammatory markers expression at Day 2 and Day 4 after surgery has shown reduction in all markers throughout all groups by the Day 4 (Fig.5.8A,B,C,D,E). Interestingly that Csf1 and Tnfα remained up-regulated in SCI alone group by Day 4 (Fig.5.8F,G).
Figure 5.7 Inflammatory mRNA expression in muscle of SCI and SHAM operated mice with and without CDTX induced inflammation at Day 2 after surgery – qRT-PCR results. Quantification of (A) Ccl2, (B)Chi3l3, (C)Arg1, (D)Lgals3, (E)IL-1β, (F)Mrc1, (G)Csf1, (H)Tnfα mRNA by qRT-PCR on hind limb muscle from operated mice. Endosteal and central bone marrow mRNA from untreated naïve mice was used as a control. All expression was compared to the housekeeper gene β-actin. Data are represented as mean ± SD. Combined time course for all four experimental groups of animals. Data analysed by One-way ANOVA with Kruskall-wallis test. Each dot represents hind limb muscle mRNA from a separate mouse (4 mice per group).
Figure 5.8 Inflammatory mRNA expression in muscle of SCI and SHAM operated mice with and without CDTX induced inflammation at Day 2 (pale grey bars) and Day 4 (dark grey bars) after surgery – qRT-PCR results. Quantification of (A) Ccl2, (B)Chi3l3, (C)Arg1, (D)Lgals3, (E)IL-1β, (F)Csf1, (G)Tnfα mRNA by qRT-PCR on hind limb muscle from operated mice. Endosteal and central bone marrow mRNA from untreated naïve mice was used as a control. All expression was compared to the housekeeper gene β-actin. Data are represented as mean ± SD. Combined time course for all four experimental groups of animals. Data analysed by Two-way ANOVA with Bonferroni post-test.
In the next section, I utilized the microarray data to identify specific inflammatory agents for intervention with the aim of finding potential treatments that alleviate NHO following SCI. Mice were administered with drugs that inhibit cytokines involved in inflammation:

- IL-1
- TNF-α

as well as monocyte/macrophage development and M2 activation:

- CSF1R

5.3.5 IL-1 receptor antagonist does not improve NHO

Inflammatory cytokine IL-1β was found to be increased locally in the muscles of SCI-operated mice with CDTX injection by microarray and qRT-PCR (Fig.5.5). To inhibit IL-1 in vivo, we performed treatment of operated mice with Kineret at doses of 10mg/kg or 20mg/kg daily from immediately after the surgery. Mice in control group were injected with the same volume of saline. At 10 days we did not observe any changes in NHO volumes or density between the groups of operated mice (p-value: 0.2303\textsuperscript{ns} by Mann-Whitney test for Kineret dose 20mg/kg and, 0.5273\textsuperscript{ns} for Kineret dose 10mg/kg).
Figure 5.9 Effect of IL-1 receptor antagonist on NHO. (A) Illustrative μCT of right hind limbs after SCI with injection of CDTX and treatment with IL-1 receptor antagonist Kineret; Quantification of HO volumes (B) and density (C) by 3D μCT reconstitution following SCI with injection of CDTX and treatment with IL-1 receptor antagonist Kineret; bars and error bars represent mean ± SD of 6 mice per group.

5.3.6 Combination of IL-1 receptor antagonist and TNF-α inhibitor reduced bone formation in NHO
As TNF-α expression was also found to be up-regulated in muscle from mice with SCI+CDTX (Fig.5.5). We also inhibited TNF-α in vivo by administering Etanercept.
In mice that underwent SCI and intramuscular CDTX injection I administered TNFα inhibitor Etanercept alone or in combination with IL-1 receptor antagonist Kineret. Control group was injected with the same volume of saline - 100μL. Treatment groups included:

- Control group (saline)
- Kineret 10mg/kg
- Kineret 20mg/kg
- Etanercept 20mg/kg and
- Etanercept 10mg/kg + Kineret 10mg/kg.

At 10 days we observed a significant reduction of NHO volumes in group of mice injected with Etanercept 20mg/kg and combination of Etanercept and Kineret (10mg/kg each) (p-value: 0.0424* by Mann-Whitney test), but there was no difference between Etanercept alone group and Etanercept plus Kineret group. Of note, although the reduction of NHO volume by etanercept treatment was significant, NHO were still large.
Figure 5.10 Effect of IL-1 receptor antagonist and TNF-α inhibitor on NHO. (A) Illustrative μCT of right hind limbs after SCI with injection of CDTX and treatment with IL-1 receptor antagonist Kineret and TNF-α inhibitor Etanercept; (B) Quantification of HO volumes by 3D μCT reconstitution following SCI with injection of CDTX and treatment with IL-1 receptor antagonist Kineret and TNF-α inhibitor Etanercept; bars and error bars represent mean ± SD of 4 mice per group, data pooled from two experiments.
**5.3.7 CSF1R kinase antagonist reduced the bone density but not the volume of NHO**

My microarray analysis revealed that a macrophage stimulatory factor CSF1 was increased locally in the muscles of SCI-operated mice with CDTX injection. To test the effect of CSF1 inhibition we performed treatment with CSF1R kinase small antagonist GW2580. Mice with SCI and CDTX-induced inflammation were gavaged with GW2580 160mg/kg or vehicle at the day of surgery after recovery and then daily for 10 days. At 10 days we did not observe any changes in NHO volumes between the groups of operated mice (p-value: 0.9551 ns by Mann-Whitney test), however bone density was significantly reduced after treatment with CSF1 kinase antagonist (p-value: 0.0098**, One way Anova, Bonferroni’s multiple comparison test; p-value: 0.0428*, Mann-Whitney test) (Fig.5.11)

![Figure 5.11 Effect of CSF1R kinase inhibitor on NHO.](image)

(A) Illustrative μCT of right hind limbs after SCI with injection of CDTX and treatment with CSF1R kinase inhibitor; Quantification of HO volumes (B) and density (C) by 3D μCT reconstitution following SCI with injection of CDTX and treatment with CSF1R kinase inhibitor; bars and error bars represent mean ± SD of 4 mice per group pooled from two different experiments; p-value: **0.01-0.0001
5.3.8 BMP signalling and osteogenic mRNA expression in muscle of mice with and without NHO

RNA was extracted from hamstring muscle of 4 groups of mice, 4 mice in each group. The groups included:

- SCI+CDTX
- SCI alone
- CDTX + SHAM operation
- SHAM-operated mice
- Central bone marrow and endosteal RNA were also extracted from naïve mice in steady state to see how they compared to RNA extracted from muscles. The rationale for these 2 control tissues was to evaluate RNA abundance relative to a well-known site of bone formation (femoral endosteum) and a site with little bone formation despite the abundance of mesenchymal progenitor cells and haematopoietic cells (central bone marrow).

It has been documented that in FOP BMP signalling is abnormally activated due to activating mutations of the Acvr1 receptor [48]. I investigated a possible role of BMP signalling molecules in SCI-induced NHO by assessing mRNA expression of BMP family members in muscle of mice with SCI or SHAM with and without muscular inflammation. Interestingly at day2 after surgery Acvr1 mRNA was upregulated in mice with SCI alone in comparison with other groups of mice and CBM reference (Fig.5.12A). This upregulation remained consistent in this group at day4 after surgery, while Acvr1 mRNA from mice with CDTX injection remained the same by day4 (Fig.5.13A). This is interesting as Acvr1 (a part of BMP signaling pathway) is the most common driving mutation observed in patients with FOP [193]. BMP2, 4, 5 and 7 were slightly upregulated in SCI alone group and by day4 after surgery BMP2 has got increasingly upregulated in SCI alone group (Fig.5.12B). BMP4, 5 and 7 expression did not change markedly during the same time course in the SCI group (Fig.5.12 C,D,E) and were slightly downregulated in the other three groups by the day 4, however the decrease was not statistically significant (Fig.5.13 B,C,D,E).

Interestingly Noggin, the BMP receptor antagonist was also upregulated in SCI group at
day 2, however it reduced to the level of other 4 groups of mice by the day 4 (Fig. 5.12F, 5.13F). Muscle harvested from SCI operated mice have also expressed higher levels of markers for early osteogenic development, such as Runx2 and osterix/Sp7 at day 2 (Fig. 5.12 G,H), however both of these markers decreased in SCI group by day 4 (Fig. 5.13 G,H). Interestingly osterix mRNA significantly increased in CDTX and SCI +CDTX groups by the day4, while Runx2 decreased to the same level in all 4 groups by the day 4 after surgery.
Figure 5.12 Osteogenic mRNA expression in muscle of SCI and SHAM operated mice with and without CDTX induced inflammation at Day 2 after surgery – qRT-PCR results. Quantification of (A) Acvr1, (B) BMP-2, (C) BMP-4, (D) BMP-5, (E) BMP-7, (F) Nog, (G) Runx2, (H) Osterix/Sp7 mRNA by qRT-PCR on hind limb muscle from operated mice. Endosteal and central bone marrow mRNA from untreated naïve mice was used as a control. All expression was compared to the housekeeper gene β-actin. Each dot represents one sample of mRNA, harvested from one mouse. Data analysed by One-way ANOVA with Kruskall-Wallis test.
Figure 5.13 Osteogenic mRNA expression in muscle of SCI and SHAM operated mice with and without CDTX induced inflammation at Day 2 (pale grey bars) and Day 4 (dark grey bars) after surgery – qRT-PCR results.

Quantification of (A) Acvr1, (B)BMP-2, (C)BMP-4, (D)BMP-5, (E)BMP-7, (F)Nog, (G)Runx2, (H)Osterix/Sp7 mRNA by qRT-PCR on hind limb muscle from operated mice. Endosteal and central bone marrow mRNA from untreated naïve mice was used as a control. All expression was compared to the housekeeper gene β-actin. Data are mean ± SD. Combined time course for all four experimental groups of animals. Data analysed by Two-way ANOVA with Bonferroni post-test.
We then evaluated changes in TGF-β family in our microarray results (Fig.5.14). Interestingly, BMP4 and BMP-5 were significantly down-regulated in SCI+CDTX group (Fig.5.14C,D) while other markers did not differ much between the groups.

Figure 5.14 Differentially expressed genes of osteogenic markers in muscle of SCI and SHAM operated mice with and without CDTX induced inflammation at Day 2 after surgery – microarray results. (A) Acvr1, (B)BMP2, (C)BMP4, (D)BMP5, (E)BMP7, (F)Nog mRNA by qRT-PCR on hind limb muscle from operated mice. Data are represented as mean ± SD. Each dot represents hind limb muscle mRNA from a separate mouse (4 mice per group). Data were analyzed by One-way ANOVA with Bonferroni’s multiple comparison test. *0.05-0.01; **0.01-0.0001; ***<0.0001
5.4 Discussion

The importance of osteogenic factors including bone morphogenetic proteins in bone regeneration and formation is well known. However it is important to remember that inflammatory cytokines were also shown to be involved in bone tissue regeneration process [194]. As specified in previous Chapter 4, specific tissue residential macrophages osteomacs support osteoblast survival differentiation and function in vivo in steady-state as well as in bone fracture models [114, 147].

Chapter 5 microarray results show that a lot of genes which are macrophage specific or involved in macrophage activation and response to interferons were differentially regulated in inflamed muscles depending whether there was SCI or not. Therefore SCI changes macrophage activation profile in the inflamed muscle. Interestingly M2 related genes Cd163 and Chi3l3 were significantly up-regulated only in SCI+CDTX group, suggesting M2 involvement in the bone formation strongly supporting our results in Chapter 4. Amongst down-regulated genes in the same group a lot of them are markers and cytokines associated with LPS-activated macrophages, which predominantly are M1 polarised macrophages: Itm2b, Ifit3, Ifit2, Gna12 [192] and Lgals3bp, that was described as M1 and M2 marker [195]. Thus the data suggest that while CDTX promotes an inflammatory response in muscle, when CDTX is administered after spinal cord injury this inflammatory response is skewed more towards M2 phenotype. Based on those data we chose factors to investigate which are detailed below.

CCL2

Moreno et al. [196] have shown induction of CCL2 and axon loss in a multiple sclerosis model within months after CNS injury. Deletion of CCL2 chemokine by Spiegelmer-based inhibitor mNOX-E36 was shown to decrease CNS accumulation of M1 macrophages and M1 markers expression [197]. It is interesting, as we have found that mostly M2 markers are increased in the SCI+CDTX group together with CCL2 up-regulation. It supports our results from Chapter 4 about the possible role of M2 macrophages in NHO formation after SCI.

TNFα, IL-6 and IL-1

TNFα, IL-1 and IL-6 have been shown to play crucial role in bone regeneration and healing after fractures [198]. TNFα and IL-1β were shown to increase within the acute phase of inflammation after fracture – within the first 24 hours – which associated with intensive infiltration of injury site by inflammatory cells. The second peak in TNFα was observed
after 21-28 days, suggesting that during this period the cytokine is expressed by newly formed chondrocytes and osteoblasts [198, 199]. IL-1β knockout mice were shown to have increased bone volume and density associated with decreased osteoclast formation and maturation [199] while IL-1RI-deficient mice have decreased bone mass and increased number of osteoclasts [200]. Indeed TNFα, IL-1 and IL-6 were found to be significantly upregulated in SCI+CDTX group at Day 2 after surgery. However qRT-PCR results did not show significant difference in TNFα between SCI and SCI+CDTX groups which could be explained by bone damage during laminectomy to perform SCI surgery.

IL-1 was shown to be one of the most potent activators of osteoclastogenesis and also a dominant factor in osteoclast differentiation [201]. It has been shown to increase bone resorption in chronic arthritis, therefore IL1 blocking has been suggested as a potential treatment of RA. IL-1 receptor antagonist Kineret was developed for rheumatoid arthritis and Sjögren's syndrome treatment [202-205]. It has also been shown to reduce alcoholic liver injury [183]. Bresnihan et al. have shown that along with antiinflammatory effect Kineret markedly reduced bone erosions in patients with rheumatoid arthritis [205]. In our experiment Kineret treatment did not affect bone formation after SCI. Etanercept – TNFα inhibitor has been successfully used in treatment of patients with rheumatoid arthritis and ankylosing spondilitis [206-209]. Genovese et al. have demonstrated that treatment with etanercept significantly reduced neuroinflammation as soon as 24 hours after treatment initiation and even stimulated movements in affected limbs in mouse model of spinal cord compression 10 days after injury [185]. Authors have found significant decrease in neutrophil infiltration along with reduced expression of TNFα and IL-1β locally in the spinal cord of mice treated with etanercept together with reduced expression M1 marker iNOS. While it effectively reduced inflammatory markers mediators it has also increased specific bone resorption markers (c-telopeptide-1 and sclerostin) in blood of patients with rheumatoid arthritis treated with etanercept for 12 weeks [210]. It confirms our results of NHO reduction after treatment with high dose of Etanercept or combination of Etanercept and Kineret. Both treatments are associated with a number of potentially serious adverse effects. Etanercept can cause leukopenia with subsequent higher risks of bacterial and viral infection development that has been reported by several authors [211, 212]. Anakinra’s adverse effects include hepatotoxicity [213], neutropenia [214] and injection-site reactions [215]. Therefore it is highly unlikely to use the combination of both in treatment of NHO in patients at this stage.
**MSP/Ron signalling**

We observed dramatic changes in MSP/Ron signalling pathway, where all included genes were highly up-regulated in SCI+CDTX group of mice. MSP/Ron signalling pathway plays an important role in wound healing, tumour growth but what is more important to us - it is strongly associated with macrophage activation [216]. Our findings were very exciting as in the results presented in Chapter 4 we confirmed that macrophages play an important role in SCI-NHO. MSP/Ron signalling pathway plays an important role in wound healing, tumour growth Ron or receptor tyrosine kinase signalling knockout in vivo has been shown to be compatible with life, however inflammatory response in those mice was attenuated [217]. It has also been shown to modulate function of M1 and M2 macrophages by Sharda et al. [218]. The same group of scientists performed in vitro stimulation of macrophages with macrophage stimulating protein (MSP), the only known ligand for Ron, and found upregulation of M2 marker Arginase 1. Interestingly that short exposure to MSP led to increase of both M1 markers, like IL-1β and IL-6, and M2 markers: Mrc1 and Arg1. It is consistent with our microarray results on muscle harvested from SCI-operated mice with CDTX-induced inflammation. It has been suggested that by changing RON activity it would be possible to shift the activation state of macrophages between acute and chronic inflammatory states, which has significant therapeutic potential [216, 219].

**CSF1**

We then decided to test CSF1 kinase inhibitor to reduce NHO. It has previously been published that M-CSF1 up-regulated osteoclast differentiation as osteoclasts are derived from cells of monocyte lineage [220]. Considering our hypothesis of M2 macrophage involvement into NHO after SCI and previous publications that CSF-1 induces polarization towards M2 macrophages and that blocking CSF-1R depletes normal microglia and reduced and M2 polarization [221, 222] it was exciting to observe reduction in bone density of newly formed HO. However we found that CSF-1 inhibition did not change the bone volume suggesting that multiple stimuli are involved in NHO. Thus although our data from Chapters 4 and 5 strongly suggest a driving role for inflammation in NHO it appears that multiple inflammatory factors contribute towards bone formation after SCI and the pathways are redundant in function.

The dosage of inhibitors and agonists was taken from the literature; however we have not tested the efficacy in our mouse model, which is a limitation. Alternative strategies to overcome it would be to use double knock-out mice for receptors to IL-1 and TFN-α. As for
the CSF-1 inhibitor, we have not tested it in the muscles, but our results showed that the same dosage, we took from literature, was sufficient to reduce monocytes and macrophages response to M-CSF stimulation and decrease their proliferation (Fig 5.15).

**Figure 5.15** Effect of CSF1R kinase inhibitor on bone marrow monocytes and macrophages *in vivo*. Mice were treated with saline (Sal), M-CSF, CSF1R kinase inhibitor or combination of M-CSF and CSF1R kinase inhibitor (M+G). Data are mean±SD of 3 mice per group. (R Jacobsen, unpublished data)

**BMP signalling**

It has been documented that in FOP BMP signalling is abnormally activated due to activating mutations of the Acvr1 receptor [48]. Although genetically driven FOP mechanisms might be different from neurologically driven HO, it still does not exclude the possibility of changes in BMP family molecules in response to SCI in genetically normal animals. In order to gain insight into a possible role of BMP signalling molecules in SCI-induced NHO, I followed the mRNA expression of BMP family members in muscle of mice with SCI or SHAM with and without muscular inflammation. TGF-β superfamily role in bone regeneration and formation was most extensively studied to date [223-225]. TGF-β was shown to play an important role in osteogenesis imperfecta – hereditary disease caused by mutation in collagen type I gene [226, 227]. TGF-β caused altered bone formation through inhibition of osteoblast apoptosis [228] and repressing Runx2 thus inhibiting osteoblast differentiation [229]. Inhibition of TGF-α has leaded to increased bone mass and improved bone strength [226].

TGF-β superfamily members like activin A receptor type 1 and BMPs were documented to play important role in osteoblast differentiation and bone formation [225]. As mentioned earlier Acvr1 mutation has a substantial priming effect on HO formation in genetic disease FOP [18, 19, 48]. According to our results Acvr1 was upregulated in mice with SCI in comparison with other groups, however by day 4 upregulation was also observed in group
SCI+CDTX, that usually shows NHO formation. Knock-out models of BMP-2, 4, 7 have shown impaired osteogenesis and mutation in BMP receptor was also shown to increase bone mass and volume in mice [230]. BMP-2 was found to be elevated after muscle injury and released during bone injury [39, 40]. In our experiment BMP-2 and 4 were significantly upregulated by day 4 in mice with SCI, however BMP-4 and 7 expression in SCI group was comparable expression in the muscle of SHAM operated mice. Interestingly that BMP-4, 5 and 7 were downregulated by day 4 in a group of mice that usually develops NHO.

Despite reports of absent increase of BMPs in cerebrospinal fluid from TBI patients suggesting that BMPs are unlikely to be responsible for the osteogenic cell response that triggers HO [42] we have shown an immense and consistent increase of BMP-2, 4 and 7 in the muscle tissue harvested from mice with SCI, that could suggest association with subsequent bone formation. Genetically modified mice overexpressing BMP antagonist Noggin have been shown to cause osteopenia and fractures together with impaired osteoblastic function [231]. In our results Nog was significantly higher expressed in the muscle of SCI operated mice, however its expression markedly decreased by day 4, reaching the level of Nog expression in the other three groups of operated mice. Interestingly microarray analysis has shown that BMP-1 that does not belong to the TGF-β family but is involved in bone and cartilage development though collagen maturation [232, 233] was highly increased exclusively in the SCI+CDTX group. BMP-1 is expressed in osteopblasts but not muscles or macrophages.

As BMP signaling changes may not be reflected in transcriptional changes immediately after SCI with inflammation, it will be necessary to perform assessment of phosphorylation of Smad1, 5 and 8 proteins that are involved in BMP signaling cascade by immunohistochemistry or Western blot analysis.

Early onset of Runx2 expression has been shown to induce mineralization in embryo 13.0. It was sufficient to direct MSCs to osteoblasts and lead to intramembranous bone formation during embryogenesis. Thus it is essential for skeletal development [234]. Interestingly it was substantially upregulated in mice with SCI alone on Day 2, however this effect was diminished to the level of the other three groups of operated mice by the 4th day after surgery.

Increased expression of osteoblastic differentiation genes Runx-2, osterix and cathepsin-K has been reported in TBI patients [41]. Muscle from SCI operated mice expressed higher levels of markers for early osteogenic development: Runx2 as was described earlier and osterix/Sp7, however osterix expression was significantly downregulated in SCI alone group by day 4. This effect was opposite to the groups of mice with CDTX injection without
correlation to SCI as in both groups of mice with inflammation we have shown significant increase of osterix by day 4 after surgery. Among other markers, that are known to be associated with bone formation leptin has been shown to be expressed by human healthy osteoblasts in vitro during mineralization phase [235]. Later the same research group has published that leptin promotes osteoblast differentiation in vitro along with its function of mineralization and collagen deposition [38]. Although leptin is known to be a promoter of bone progenitors and osteoblasts activation and was found also to decrease osteoblastic activity by binding to hypothalamic neurons [29]. Osteocalcin is another biomarker of bone remodelling, which was found to be significantly lower in patients with TBI [45] along with collagen type I, that suggests that patients with neurotrauma are prone to HO development. We did not get convincing data in regards to these markers in our mice at early time points (days 2 and 4) following surgery.

The main finding of this chapter is an increased production of inflammatory cytokines. They can be involved in NHO formation by polarizing macrophages, which instead of fulfilling their roles by dampening inflammation and repairing damaged muscles, can be pushed to contribute to the induction of osteogenic differentiation. Indeed in order to get the full picture of changes in the muscles of operated mice further analysis is required. Therefore we are planning to perform gene microarray on the muscle samples from the same groups of mice at later time points. In view of considerable changes in inflammatory markers and macrophages it is necessary to sort macrophages from muscles of SCI- and SHAM- operated mice to look at their gene expression profile as it may enlighten new potential candidates for target treatment. Furthermore experiments using mice knock-out for genes encoding these cytokine receptors will be designed to test their role in SCI-NHO.
Chapter 6: Characterisation of muscle and bone marrow progenitor cells prone to osteogenic differentiation

6.1 Introduction
Osteoblasts are required to form bone in damaged muscles. Osteoblasts are derived from mesenchymal progenitors that may constitutively reside in muscles in healthy individuals or be recruited to damaged muscles via the blood from remote reservoirs such as the bone marrow. Discovering the “cells of origin” of osteoblasts that accumulate in injured muscles following SCI has potential to improve understanding of the physiopathology of NHO, increase prognostic information, enhance diagnostic abilities as well as provide treatment targets. In this chapter I experimentally evaluated the potential origins of osteoblasts during heterotopic ossification in mice.

6.1.1 Aims and Objectives
The potential cell source of osteoblast for bone formation in heterotopic ossifications are: three types of mesenchymal progenitor cells in muscle and two types of mesenchymal progenitor cells in bone marrow MSCs. The aim of this chapter was to isolate and culture an adult murine bone/bone marrow mesenchymal stromal cell and muscle mesenchymal progenitors cells populations and to define their osteogenic potential in vitro. Osteogenic differentiation potential was evaluated by measuring mineral calcium deposition by Alizarin Red staining, and mineral phosphate deposition by Von Kossa staining. RNA expression was tested via qRT-PCR on cells from the hind limb muscle and BM to identify the cell type that contributes to osteogenic differentiation in vitro.

6.2 Methods

6.2.1 Harvest sort and culture of muscle and bone marrow progenitors
To identify which cells are responsible for making bones in muscles (are they derived from the muscle itself, or are they recruited from the skeleton and bone marrow via the blood) I used hind limb muscles. As control mesenchymal progenitor were isolated from the bone marrow of femurs and tibias from naïve untreated adult C57Bl/6 wild type mice. Sorting and culture methods are described in Chapter 2, section 2.3. In total 10 FACS sorts of muscle mesenchymal progenitor cells and 7 FACS sort for BM MSCs from 47 and 34 mice respectively were performed over the course of the project.
6.2.2 Osteogenic differentiation of muscle and bone marrow progenitors

Methods for osteogenic differentiation are described in Chapter 2, section 2.4.

6.2.3 qRT-PCR analysis

For qRT-PCR analysis the expression was determined relative to β2-microglobulin (β2M). For myogenic markers RNA extracted from whole hamstring group of muscle was used. Level of significance was calculated using the Mann-Whitney test in GraphPad Prism v5.01 (San Diego, California, USA).

Primers used in this chapter are outlined in the Table 6.1.

Table 6.1 Primers used for qRT-PCR

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<th>Primer</th>
<th>Sequence</th>
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<td>75'f CTGGTCTTTTCTGGTGCTTGTC 181'b GTATGGCTGGCTTCCATT</td>
<td>106</td>
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*for TaqMan Gene Expression Array Mix

6.3 Results

6.3.1 FACS-sorting of muscle and bone marrow mesenchymal progenitors

I prepared single cell suspension from hamstring muscles from naïve C57BL/6 mice by digesting muscle fragments with Collagenase I and Dispase II. Then I sorted viable nucleated cells, defined as 7-AAD* (viable cells exclude this non-permeable DNA intercalating fluorochrome) and Hoechst* (cell permeable DNA intercalating fluorochrome).
Erythrocytes were excluded on Ter119 marker, endothelial and leukocytes were also excluded on the basis CD45 and CD31 markers. The resulting 7-AAD−Hoechst+ CD45−Ter119−CD31− cells were sorted and collected as follows:

- muscle satellite cells (SC) as CD45−Ter119−CD31−Sca1−CD34+ cells;
- muscle mesenchymal interstitial cells (IC) as CD45−Ter119−CD31−Sca1+CD34+ cells as previously reported [100, 101];
- CD45−Ter119−CD31−Sca1−CD34+ progenitor cells;
- and CD45−Ter119−CD31−Sca1+CD34+ progenitor cells (Figure 6.1).

**Figure 6.1** Phenotypic characterization of cell populations in mouse skeletal muscle and bone marrow of mice.

This figure represents the sorting strategy to collect satellite cells and interstitial cells from mouse skeletal muscles (top row), and MSCs from mouse bone marrow stromal cells (bottom row). The left dot-plots show gating of viable (7AAD-negative) mononucleated (Hoechst33342+) cells. In the middle dot-plots, viable mononucleated cells are gated after exclusion of CD45+ leukocytes, Ter119+ erythroid cells and CD31+ endothelial cells. In the right dot-plots, non-hematopoietic non endothelial CD45−Ter119−CD31− cells are further gated as Sca1−CD34+ satellite cells, Sca1+CD34+ interstitial cells or Sca1−CD34+ progenitor cells.
Satellite cells and interstitial cells represented a frequency of 0.1-1% and 0.4-3% of all muscle sorted cells, respectively. Sca1+ CD34- progenitors and Sca1- CD34- progenitors represented the smallest and the largest populations with a frequency of 0.1% and 7-15% of all muscle sorted cells, respectively.

We performed the same sort on cell suspension harvested from mouse bone marrow (Figure 6.1). Sca1+ CD34+ and Sca1+ CD34- bone marrow progenitors represented a frequency of 0.031-0.2% and 0.09-0.3% of all sorted bone marrow cells, Sca1- CD34+ bone marrow progenitors represented the smallest group of 0.01-0.08% and Sca1- CD34- progenitors represented the largest population with a frequency of 2-5% of all bone marrow sorted cells. Sorted cells were cultured until passage 2, and were used for mesodermal differentiation assay and RNA extraction. Part of cells was frozen in liquid nitrogen for future experiments.

The morphology of IC, SC and Sca1+ BM MPCs populations was similar throughout the cultures. They were adherent, large elongated cells that exhibited a fibroblast-like morphology (Figure 6.2). Muscle Sca1- CD34- progenitors had similar morphology, however were larger in size. When plated at low densities, all cell populations formed colonies as would be expected of the typical clonal colony forming cell. Muscle Sca1+ CD34- progenitors and bone marrow Sca1- CD34+ progenitors represented the rarest groups, these cell populations showed poor growth and self-renewal abilities and survival and were not included in the next experiments. Mouse osteoblastic cell line MC-3T3 was a kind gift from Dr Allison Pettit’s group. These cells were elongated and smaller in size than previously described sorted cell populations, they formed colonies and looked similar to sorted bone marrow Sca1- CD34- progenitors.
### Figure 6.2 Morphology of cell populations sorted from mouse bone marrow and skeletal muscle after exclusion of erythroid cells, endothelial cells and leucocytes.

Adherent bone marrow (left column) and muscle (right column) cells with fibroblast-like morphology by light microscopy. The following populations were cultured until passage 2: Sca1\(^+\) CD34\(^+\) (first row), Sca1\(^+\) CD34\(^-\) (second row), Sca1\(^-\) CD34\(^+\) (third row) and Sca1\(^-\) CD34\(^-\) (fourth row). White bars 200µm.

### 6.3.2 Osteogenic differentiation of muscle and bone marrow mesenchymal progenitors

For osteogenic differentiation muscle IC, SC, BM Sca1\(^+\) MPCs (further called BM MSC), BM and muscle Sca1\(^-\) CD34\(^-\) progenitors and cell line MC-3T3 was plated into 96 wells
plate in normal culture medium (DMEM supplemented with 20\%FBS, 10\% horse serum and antibiotics) as per Chapter 2, section 2.3. At confluence, expansion medium was replaced by a classic osteogenic medium containing phospho-ascorbic acid, β-glycerophosphate and dexamethasone. Cells were cultured in osteogenic differentiation medium for three weeks; at the end of each week two plates were stained with Alizarin Red to detect mineral calcium deposits (Figure 6.4) and von Kossa to detect mineral phosphate deposits (Figure 6.3). By the end of the first week bmMPCs and muscle IC were capable of depositing mineralized matrix, stained both by von Kossa and Alizarin Red. By the end of second week Alizarin Red staining was positive also in sorted muscle satellite cells, while phosphate deposits were shown in all populations of cells. By the end of the third week all plated populations of cells show mineralized matrix, however muscle interstitial cells had the highest osteogenic potential over all other plated cell types including BM mesenchymal progenitor cells and osteoblastic cell line MC3T3 \textit{in vitro} (Figure 6.5). This experiment suggests that resident progenitors from the muscle (interstitial cells and satellite cells) both have osteogenic potential and could possibly originate osteoblasts responsible for NHO, although muscle IC could differentiate and deposit a mineral matrix faster than satellite cells. I have shown that another type of resident muscle progenitor cells - Sca1\(^{-}\)CD34\(^{-}\) muscle cells – exhibits osteogenic potential similar to interstitial cells and bmMSCs \textit{in vitro}. Therefore in the mouse, resident progenitors located within the muscle have osteogenic potential \textit{in vitro} and could directly contribute to the bone formation after SCI, thus eliminating the need to recruit mesenchymal progenitors from the bone marrow via blood.
Figure 6.3 Von Kossa staining of mouse bone marrow and skeletal muscle cell populations. From top to the bottom: 1 week, 2 weeks, 3 weeks in culture. Columns of wells contained muscle interstitial cells (1), muscle satellite cells (2), Sca1−CD34− muscle progenitor cells (3), Sca1+ bone marrow MPCs (4), bone marrow Sca1-CD34- MPCs (5), and mouse osteoblastic cell line MC3T3 (6) cultured for 3 weeks in osteogenic medium or without osteogenic factor. The black staining shows mineralized phosphate deposited at the bottom of the wells.
Figure 6.4 Alizarin Red staining of mouse bone marrow and skeletal muscle cell populations. From top to the bottom: 1 week, 2 weeks, 3 weeks in culture. Columns of wells contained muscle interstitial cells (1), muscle satellite cells (2), Sca1^−CD34^− muscle progenitor cells (3), Sca1^+^ bone marrow MPCs (4), bone marrow Sca1−CD34− MPCs (5), and mouse osteoblastic cell line MC3T3 (6) cultured for 3 weeks in osteogenic medium or without osteogenic factor. The red staining shows mineralized calcium deposited at the bottom of the wells.
Figure 6.5 Quantification of osteogenic differentiation of mouse bone marrow and skeletal muscle cell populations by Alizarin Red staining. From the top to the bottom 1 week, 2 weeks, 3 weeks in culture.
The histograms show the quantification of the Alizarin Red staining by light absorbance at 562nm of cells cultured in osteogenic conditions (grey bars) or in non-osteogenic expansion medium (empty bars). X axis: muscle interstitial cells (1), muscle satellite cells (2), Sca1^-CD34^- muscle progenitor cells (3), Sca1^+ bone marrow MPCs (4), bone marrow Sca1^-CD34^- MPCs (5), and mouse osteoblastic cell line MC3T3 (6). Data are mean ± SD of four wells of a typical experiment out of 5 independent sorts and experiments.
6.3.3 Osteogenic and tissue specific mRNA expression in cultured muscle and bone marrow mesenchymal progenitors

To further investigate the populations of mesenchymal progenitor cells from muscle and bone marrow, qRT-PCR was performed on mRNA extracted from cells at passage 2 for lineage-specific markers. I found that BM MPCs and osteoblastic cell line MC3T3 expressed higher levels of markers for early osteogenic development, such as RUNX2 and osterix/Sp7, when compared to muscle IC and SC (Figure 6.7 A, B). In addition, BM MPCs and MC3T3 also expressed higher levels of osteocalcin (also known as bone gamma-carboxyglutamate protein), a marker of mature – osteoblasts (Figure 6.7 C). Finally collagen 1 expression was increased in muscle IC and bmMPCs in comparison with muscle SC and MC3T3 cell line (Figure 6.7 D). Muscle specific marker MyoD, and the muscle satellite cell marker Pax7 were below detection in all four cell types, while highly expressed in whole murine hind limb muscle (Figure 6.7 E, F). Muscle interstitial cell marker PW1 [236] was highly expressed in muscle IC, SC and bone marrow MPCs, however was below detection in MC3T3 cell culture (Figure 6.7 G). Finally, the mesenchymal progenitor cell marker Prrx1 was well expressed in all cultured cell types with higher expression in bmMSCs and MC3T3, relative to cultured in muscle progenitor cells (Figure 6.7 H).
Figure 6.6 Osteogenic myogenic and mesenchymal mRNA expression in muscle and bone marrow mesenchymal progenitors.

Quantification of (A) Osterix, (B) Runx2, (C) Osteocalcin, (D) Collagen1, (E) MyoD, (F) Pax7, (G) PW1, (H) Prrx1 mRNA by qRT-PCR on muscle interstitial cells, satellite cells, bone marrow MSCs sorted from wild-type C57BL/6 mice and cell culture MC3T3. mRNA from whole hamstring muscle group was used as a positive control for myogenic markers MyoD(E) and Pax7(F). All expression was compared to the housekeeper gene β2M. Each dot represents one sample of mRNA, 4 samples of cells were harvested and sorted from 4 different experiments.
6.4 Discussion

It is well known that osteoblasts and osteocytes participate in normal bone formation, bone growth and regeneration in a fine balance with osteoclasts [237]. However the source of new bone in abnormal sites in the muscle after spinal cord injury remains a mystery. Potential sources of osteoblasts include mesenchymal progenitor cells, however it is unknown whether they are present locally in the damaged tissue or are recruited to the site of inflammation via blood from the most common source of MPCs – bone marrow. It is also a common knowledge that MPCs reside in different organs and tissues, such as heart muscle, kidney, skin, etc. [85]. A few groups of progenitor cells have been reported to be present in skeletal muscle [109, 238].

Muscle progenitor cells include interstitial cells and satellite cells. Satellite cells were discovered in 1961 by Dr. Mauro [239] and were reported to locate under basal lamina of the muscle fiber, able to self-renew and differentiate towards myogenic lineage in case of muscle damage. Fukada et al. generated monoclonal antibody SM/C-2.6 to simplify isolation of satellite cells [240]. Quiescent SC do not express MyoD, while during differentiation this marker is getting switched-on [240]. Previously SCs were isolated based on negative selection for Sca-1, CD31 and CD45 markers along with positive selection for CD34 and integrin α 7 [96, 241]. I used the same markers in my experiment except for integrin α 7. They’ve also been shown to express M-cadherin and Pax3 [101]. In my experiments qRT-PCR showed that Pax7 gene specific for SC [242] was highly expressed in whole fresh muscle. However it was below detection in all sorted cell populations including SC. This could be associated with changes in cells occurring during the culture by passage 2 as Hashimoto et al. found that muscle MPCs can differentiate in culture and Pax7 was detected in human SC in early passage, but not during subsequent passages [107].

Besson et al. [236] studied PW1 marker (Peg3) for identification the other group of self-renewing stem cells in adult tissues, so called interstitial cells due to their location in the interstitial space between muscle fibers. Previously the same marker was also reported by Mitchell et al. to be present in muscle interstitial cells [100]. According to the authors PW1+ cells were different from SC in terms of location in the muscle and other surface markers (Sca-1) however contributed toward muscle regeneration in vivo at levels comparable to SC. IC have also been reported to have the ability to self-renew and segregate SC population [100]. Surprisingly PW1 mRNA was well expressed in all sorted muscle MPC and BM Sca-1+ progenitors in my experiment which could again be associated with changes in cells in culture.
IC also have been shown to express mesenchymal marker PDGFRα while freshly isolated [111] and have adipogenic potential in vitro, which is consistent with my results of adipogenic differentiation. This cell population was Sca-1+ and part of them expressed CD34, similar to IC and BM Sca-1+ progenitors that were isolated in my project. Unlike SC, PDGFRα+ IC did not express MyoD and Pax7, unless they acquire a myogenic fate [100, 111]. These IC have also been reported to show multiple mesodermal differentiation potential (adipogenic, osteogenic and minimal myogenic capacity) [238, 243]. Further to this PDGFRα+ IC in degenerative muscle also showed higher expression of collagen type 1a gene [244] which makes them possibly responsible for excessive fibrosis in muscular dystrophy. Interestingly, in my experiments collagen type 1a was highly expressed in IC and BM Sca-1+ progenitors.

Sorting of bone marrow Sca-1+ progenitors (BM MSCs) was similar to that published previously [123]. Sorted population negative for Sca-1 and CD34 markers in bone marrow could represent osteochondroprogenitors as have been suggested before [124, 245] and therefore it can be already prone to osteogenic fate which explains their high osteogenic differentiation rate and expression of Runx2, Osteocalcin and Osterix, similar to BM Sca-1+ progenitors and MC3T3 cell line population. MC3T3 cell line consists of mouse preosteoblasts, thus its osteogenic fate is predisposed. For this reason I included these cells in my experiments as a positive control. Prrx-1 is mesenchymal marker, essential for the normal osteogenesis and its depletion causes severe defects in skull, limbs, spinal column and inner ear [246]. It has been shown to be present on MC3T3 cells and primary mesenchymal stromal cells before [247] which is consistent with my qRT-PCR results.

I have shown that all populations of cells differentiated towards osteoblasts by the third week of culture in osteogenic conditions in vitro. Previously aforementioned cell populations have been shown to differentiate towards this mesodermal lineage under different conditions:

a) Muscle SC exhibited osteogenic potential following treatment with BMPs [109].

b) Human SC expressing Pax-7 also displayed osteoblastic properties in Duchenne muscular dystrophy patients [107]. In vitro these cells also expressed osteogenic lineage markers Runx2 and ALP without additional BMP-2 stimulation.

c) CD44+Sca-1+ cells isolated from mouse muscles showed osteogenic potential [248] after 4 week culture in osteogenic conditions.

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d) Muscle-derived progenitors (both Sca-1⁺ and Sca-1⁻) retrovirally transduced to express BMP-4 and non-transduced muscle-derived progenitors with prolonged exposure to BMP2 showed heterotopic ossification in vivo [249] as soon as 2 weeks after transplantation.

These findings demonstrate that all the progenitor cell populations isolated from the naïve muscle can potentially differentiate towards one of the mesodermal lineage - osteogenic, and these differentiation capabilities can be further amplified by culture in lineage-specific induction conditions as they do express Osteocalcin, Runx2, collagen type 1a and Prrx1 and can form mineralized matrix in vitro.

We investigated a few populations of muscle progenitor cells along with bmMSCs and BM Sca1⁻ CD34⁻ progenitors and found that a few populations of cells in the muscle have osteogenic potential in vitro, moreover muscle interstitial cells are prone to deposit mineralized matrix required for the bone formation even earlier bone marrow MSCs. Thus we confirmed the possible source of HO locally in the damaged muscle.

Further to confirm the source of osteoblasts in muscle during the HO formation we are planning to establish in vivo experiment where we would be able to track specific cells and test their role in bone formation after SCI. We are working on the mouse model for lineage tracking experiments. These include: B6.Cg-Pax7tm1(cre/ERT2)Gakd/J x B6.Cg-Gt(ROSA)26Sor tm6(CAG-ZsGreen1)Hze/J to track muscle progenitor cells and B6.Cg-Tg(Prrx1-cre)1Cjt/J x B6.Cg-Gt(ROSA)26Sor tm6(CAG-ZsGreen1)Hze/J to track mesenchymal progenitor cells.

Finally, our model suggests that the osteoblasts responsible for NHO in muscles are not necessarily derived from mesenchymal progenitor recruited from the bone marrow. Although this remains to be demonstrated genetically with lineage tracking experiments, muscle satellite cell, interstitial cells or MPCs sorted from the muscle of naïve mice, which all have the potential to regenerate myofibers in vivo following muscle injury [101], were all capable of osteogenic differentiation in vitro as previously reported in mouse [109] and human [250].
Chapter 7: Evidence of Systemic Factors Driving NHO following SCI

7.1 Introduction

Our model of NHO after SCI clearly suggests that two factors are required in order to cause abnormal bone formation: spinal trauma and local muscle inflammation. While role of inflammation was extensively discussed in the previous chapters, in this chapter I focus on the neurogenic part of NHO. Our hypothesis is that systemic/endocrine factors are released in blood in response to SCI, which prime mesenchymal progenitor cells to osteogenic differentiation upon muscle injury. It is unknown what factors released after spinal cord disruption locally or systemically can stimulate osteogenic differentiation of progenitors in some patients that develop HO. Discovering the systemic or local factors released in response to major nerve disruption has a potential to improve understanding of the pathogenesis, increase prognostic information and diagnostic abilities as well as provide potential treatment targets. To date very little is known about release of osteoinductive biochemical factors and their influence on osteogenic differentiation of MPCs. It has been previously shown that SCI stimulates systemic release of biologically active factors and hormones in the plasma such as calcium, leptin and [38, 251, 252]. It has been proposed that substance P might be one of systemic factors responsible for HO in mouse models of FOP [36, 126]. Salisbury et al. have shown that BMP-2 stimulation of substance P and calcitonin gene related peptide increased concentration of substance P in muscle [36]. They suggested that substance P is released from the sensory neurons as they did not observe substance P concentration increase in animals lacking functional sensory neurons. Nerve fibres staining positive for substance P, a well-known nociceptive signalling molecule typically associated with sensory nerves, enter the bone in association with vessels innervating medullary tissue of bones [253], and then dissociate and terminate as free nerve endings. Salisbury et al. have also found decrease in HO formation in mice with altered sensory neurons. Kan et al. have also investigated role of substance P in their Nse-BMP4 transgenic mouse model of FOP [126]. They found increased concentration of substance P in bone masses from FOP patient and in mouse model after CDTX-induced injury. Also the authors observed less bone formation in NK-1 knockout mice. Substance P is a neuropeptide mediating response to pain [254] and produced by neurons, osteoblasts and macrophages in injured and diseased tissues [36, 255]. Three types of SP receptors were previously identified, that are called neurokinin receptors type
1, 2 and 3 [256]. SP binds more strongly to the NK1 receptors however it binds with a lower affinity to NK2R and NK3R [257, 258]. Substance P has been shown to increase chondrocyte differentiation [259], osteoblast differentiation through upregulation of osterix expression [260], bone colony formation, and osteoblast cyclic AMP production. It stimulated osteoblasts specific markers production such as alkaline phosphatase, Runx2 and osteocalcin in bone marrow mesenchymal progenitor cells *in vitro* [261]. At least one of its receptors, neurokinin-1 is expressed by osteoclasts. Neurokinin-1 drives osteoclast resorption activity *in vitro* when osteoclasts are exposed to substance P [262]. A non-peptide NK1-receptor antagonist RP67580 has been reported over a decade ago [263]. It was shown to be effective in reducing neurogenic inflammation and in antinociceptive test its efficacy was comparable to the effect of morphin with just 1.5 fold less [264]. Substance P has proinflammatory effects or augments inflammatory responses in the respiratory, gastrointestinal, and musculoskeletal systems [265]. Indeed substance P receptor antagonist RP67580 has been reported to reduce leucocyte, neutrophil and monocyte accumulation in the lungs of mice with delayed-type hypersensitivity, thus confirming its ability to decrease inflammatory response [266].

Although substance P has been reported to promote HO in genetic mouse models of FOP induced by overexpression of BMP2/4, it is unknown whether substance P is involved in our model of NHO after SCI, where there is no genetically enforced expression of BMPs.

### 7.1.1 Aims and Objectives

This chapter is to test the hypothesis that factors are released in the circulation following SCI that prime the formation of HO in inflamed muscles.

The aim of this chapter is to determine whether SCI causes the release of systemic factors that could affect the osteogenic differentiation of muscle progenitor cells *in vitro* and promote the development of HO *in vivo*.

In the first part, I tested the hypothesis that endocrine factors are released in the circulation following SCI that prime the formation of HO in inflamed muscles by causing muscle inflammation in non-paralysed front limb above the level of SCI. In a second part, I tested whether the plasma of mice with SCI could promote mineralization of culture muscle progenitor cells *in vitro*. In a third part, I tested the effect of substance P *in vitro* in cultures of sorted adult murine bone/bone marrow mesenchymal stromal cells and muscle mesenchymal progenitor cells. Finally the effect of a substance P receptor antagonist on the development of NHO following SCI was tested *in vivo*.
7.2 Methods

7.2.1 Mouse surgery and treatment
Mouse spinal cord injury surgery was performed as described in Chapter 2, section 2.2. To assess the possible effect of spinal cord injury on NHO cardiotoxin was injected intramuscularly in the right front limbs of the mice in the usual dose 0.256mg/kg. To assess the substance P receptor antagonist effect we used our usual model of NHO with CDTX injected in the hind limb. RP67580, a selective antagonist of the substance P receptor NK1R [264], was injected intraperitoneally twice daily at a dose of 2 mg/kg in 2% ethanol diluted with saline. Control mice were injected in parallel with an equivalent volume of vehicle – 2% ethanol in saline.

7.2.2 Harvest sort and culture of muscle and bone marrow progenitors
To identify which cells within the muscle are capable of mineral deposition are responsible for making bones in muscles (are they derived from the muscle itself, or are they recruited from the skeleton and bone marrow via the blood) I used hind limb muscles from naïve adult C57BL/6 mice and I sorted by flow cytometry the following populations:
- muscle satellite cells (SC) as CD45⁻ Ter119⁻ CD31⁻ Sca1⁻ CD34⁺ cells;
- muscle mesenchymal interstitial cells (IC) as CD45⁻ Ter119⁻ CD31⁻ Sca1⁺ CD34⁺ cells as previously reported [100, 101]; and
- CD45⁻ Ter119⁻ CD31⁻ Sca1⁻ CD34⁻ progenitor cells as per Chapter 6.

As positive controls for osteogenic differentiation and mineral deposition, I sorted CD45⁻ Ter119⁻ CD31⁻ Sca1⁺ mesenchymal progenitor cells from femurs and tibias from naïve adult C57Bl/6 wild type mice and CD45⁻ Ter119⁻ CD31⁻ Sca1⁻ CD34⁻ bone marrow progenitor cells. Sorting and culture methods are described in Chapter 2, section 2.4.1 and Chapter 6, Methods.

7.2.3 Osteogenic differentiation of muscle and bone marrow progenitors
Methods for osteogenic differentiation and quantification of calcium and phosphate mineral deposition (Alizarin Red staining and von Kossa staining) are described in Chapter 2, section 2.5. Briefly sorted populations of cells were seeded in 96 wells plates at a density 5000 cells/cm². All cell populations were seeded into two columns of wells, so that there would be two replicates of each cell type for each concentration of substance P/substance P receptor antagonist/vehicle control. The tissue culture medium (1X high glucose DMEM
high glucose containing 20 % (v/v) FCS, 10% horse serum, 10 U penicillin/mL, 10ug/ml streptomycin and 2mM glutamine) was replaced by osteogenic differentiation media 48 hours after. Osteogenic differentiation medium consisted of 1x \(\alpha\)-MEM supplemented with dexamethasone (0.2 \(\mu\)M), \(\beta\)-glycerol phosphate (10 mM), L-ascorbate-2-phosphate (200 \(\mu\)M), calcium chloride (2 mM), 10\% (v/v) FBS and 10U Pen/mL;10ug Strep/ml; 2mM Gln. Mouse osteoblastic cell line MC-3T3 (a kind gift from Allison Pettit group) was used as a positive control for mineral deposition.

R67580, a selective antagonist of the substance P receptor NK1R (catalogue # 1635, TOCRIS) was diluted in 10% ethanol at a concentration 20mM (8.86mg/mL) and stored in -70°C. Substance P (1156, TOCRIS) was reconstituted from powder with water as per manufacturer’s instructions at a concentration 1mM and stored in -70°C.

The outline of substance P and substance P receptor antagonist (RP67580) used in the cultures concentrations is presented in the Table 7.1. Substance P receptor antagonist concentration was used as described previously with a wider range of doses [265].

**Table 7.1** Substance P, substance P receptor antagonist concentrations.

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10% ethanol was used as a negative control in the following concentrations: 0 \(\mu\)M, 1 \(\mu\)M, 3 \(\mu\)M, 5 \(\mu\)M and 10 \(\mu\)M.

7.2.4 Quantification of substance P in mouse plasma and muscle protein extract

Plasma and muscle protein extract were collected and prepared as described in Chapter 2, section 2.7.1, 2.11. Concentration of protein in muscle extracts was determined using Pierce™ BCA Protein Assay Kit (23227; Thermo Fisher Scientific) in \(\mu\)g/mL according to the manufacturer’s instructions. Briefly all samples were diluted 1/10 with calibration diluent, standard diluted bovine serum albumin standards (BSA) were prepared and 25\(\mu\)L of sample or standard dilution were transferred into 96-wells plates. Working solution was added next (50 parts of reagent A and 1 part of reagent B from the kit) and the plate was covered and incubated on the shaker for 30 min at 37°C. Absorbance was then measured at 562nm on a plate reader and results were calculated and adjusted to the calibration curve with BSA.
To quantify substance P concentration we used the Substance P Parameter Assay Kit (KGE007; R&D Systems, Inc., Minneapolis, MN, USA). This assay is a competitive enzyme immunoassay in which an antibody specific for substance P is bound to a plastic plate. Then sample containing unlabelled substance P is combined with substance P linked horse radish peroxidase for binding to the unmobilised antibody. Therefore, a decreasing signal indicates higher concentrations of substance P present in the sample to test. This assay was performed with a change to the kit instructions as the plate is coated with a goat anti-mouse polyclonal antibody and could be blocked by mouse IgG contained in the mouse plasma samples. To overcome this: 50μL of Primary Antibody Solution (mouse monoclonal antibody for Substance P) was added to the plate first and incubated overnight at 4°C. Calibrator Diluent RD5-45 was added to the first 2 wells (non-specific binding wells). The next morning 50 μL of samples were mixed with Calibrator Diluent RD5-45 in proportion 1:2. Standard concentrations of 2500, 1250, 625, 312, 156, 78 and 39pg/mL were prepared. Diluted samples or standard dilutions were then mixed with Substance P Conjugate in proportion 1:1 before adding to the plate. 100μL of sample or standard dilution mix was then added to the wells coated with the mouse anti-substance P antibody and incubated for 3 hours at room temperature on a shaker. Each well was then aspirated and washed four times with Wash buffer from the kit. The rest of the protocol was followed as per manufacturer’s instructions. Briefly 200μL of substrate solution were added into each well and the plate was covered and incubated at the room temperature for 30 min. Then 50μL of Stop Solution from the kit was added into each well and the plate was scanned at 450nm on a plate-reader.

The calibration of the assay was performed using GraphPad Prism 5 software (GraphPad Sofwares, La Jolla, CA) by plotting standard dilution concentrations in log10 value versus optical density. The best curve fit model employed was a non-linear regression with the equation of dose-response – inhibition fit. The equation for the standard curve is represented below:

\[
OD = OD_\alpha + \frac{(OD_\alpha - OD_0)}{(1 + \text{Slope} \times 10^{(\text{LogIC50-[SP]})})},
\]

where \(OD\) is optic density; \(OD_\alpha\) is optic density of highest concentration of substance P, \(OD_0\) is optic density of lowest concentration of substance P; \([SP]\) is concentration of substance P.

Interpolated x-values that represent log-values of concentration were calculated, converted to numbers and corrected according to dilution factor. The substance P concentration for plasma and muscle protein extract was then calculated from this best fit as ng/mL of plasma or and pg/μg of muscle protein, respectively.
7.3 Results

7.3.1 Limb paralysis is not necessary for NHO development

To test whether the paralysis of the limb was necessary to NHO development after SCI, cardiotoxin was injected intramuscularly in the front limb muscles of mice that underwent spinal cord injury between T7 and T8. Front legs remain functional and active in these mice as the spinal cord transection between these two vertebrae results in the paralysis of the bottom part of the body (both hind limbs and bladder) without affecting motor or sensitive innervation of the upper part of the body. Micro CT scans at day 10 after SCI and CDTX injection showed the presence of heterotopic ossification in the CDTX injected mobile front limb (Figure 7.1A). HO developed in the CDTX-injected front limb whereas there was no HO in the non-injected limbs (front or back) (p-value: 0.008** by Mann-Whitney test) (Figure 7.1B). However the amount of bone formed above the level of spinal cord injury was significantly less in comparison with mice where inflammation was induced below the level of injury (hind limb muscles) (1-3mm³ vs 20-30mm³) (p-value: 0.0012** by Mann-Whitney test). The smaller volume of HO in the front limb is however to be considered relative to the muscle mass of the mouse front limb, which is much smaller than the hamstring muscle of the hind limb.

![Figure 7.1](image)

**Figure 7.1** Evidence of systemic factors contributing to NHO. (A) Illustrative μCT of right front limbs after SCI with injection of CDTX; (B) Quantification of HO volumes by 3D μCT reconstitution following SCI and intramuscular CDTX injection into front limb. Data presented as mean±SD of 5 mice per group.
From these findings we conclude that limb paralysis is not necessary to support NHO following SCI. An additional muscle inflammation is sufficient provided that the mouse has a spinal cord injury.

Further to explain this phenomenon, we hypothesized that systemic factors may be released into the blood in response to SCI and these systemic factors may prime muscle cells to form an HO following muscle inflammation as CDTX-mediated muscle inflammation alone is not sufficient to induce HO development. This is explored with following experiments.

7.3.2 Plasma harvested from mice with HO promotes osteogenic differentiation of muscle progenitor cells

To further test the 2\textsuperscript{nd} conclusion above (that is whether systemic osteogenic factors are released in the blood following SCI), muscle interstitial cells, satellite cells and bone marrow Sca-1\textsuperscript{+} mesenchymal progenitor cells were isolated from hamstring muscles of naïve untreated wild-type C57BL/6 mice and sorted by flow cytometry based on the CD45-Ter119- CD31- Sca-1\textsuperscript{+} CD34+-/\textsuperscript{-} phenotype (as described in Chapter 6). They were then expanded in culture until confluent and then cultured for three weeks in the presence of 10\% blood plasma from SCI or SHAM-operated mice, without addition of any osteogenic factor. Cells were then fixed, washed and stained with Alizarin red to quantify mineral calcium deposition (Fig.7.2A). I had 2 mice in each treatment group and plasma from each mouse was tested individually and in separate well. Thus all the data are from two different biological replicates per treatment group. Plasma from mice with SCI alone did not cause mineralized matrix formation in all population of cells: muscle IC, muscle SC and bone marrow Sca-1\textsuperscript{+} mesenchymal progenitor cells, relative to control wells cultured with 10\% plasma from SHAM-operated mice or with wells without mouse plasma. However plasma from mice with combination of SCI and intramuscular injection of CDTX significantly enhanced mineral deposition by muscle interstitial cells (p-value <0.05*, one-way ANOVA with Bonferroni’s multiple comparison test) (Fig 7.2B,C). Experiment was repeated three times with similar results.
Figure 7.2 Effect of plasma from SCI and SHAM-operated mice on osteogenic differentiation of interstitial cells, satellite cells and bone marrow MPCs. (A) Alizarin Red staining of mouse bone marrow and skeletal muscle cell populations. Rows of wells contained muscle interstitial cells (top), muscle satellite cells (middle), Sca1+ bone marrow progenitor cells (bottom) cultured for 3 weeks in tissue culture medium supplemented with plasma harvested from mice with SHAM surgery, SCI with saline injection and SCI with CDTX injection. The red staining shows mineralized calcium deposited at the bottom of the wells.
(B) Quantification of mouse plasma effect on osteogenic differentiation of mouse bone marrow and skeletal muscle cell populations by Alizarin Red staining for each cell population. The histograms show the quantification of the Alizarin Red staining by light absorbance at 562nm of cells cultured in tissue culture media supplemented with mouse plasma. Data are mean ± SD of four wells of a typical experiment.

(C) Quantification of mouse plasma effect on osteogenic differentiation of mouse bone marrow and skeletal muscle cell populations by Alizarin Red staining for each condition for plasma harvest. Grey bars – muscle interstitial cells, dotted bars – muscle satellite cells, bars with diagonal lines - Sca1+ bone marrow progenitor cells. Data are mean ± SD of four wells of a typical experiment.

As the volume of plasma collected per mouse was limiting factor I repeated this experiment with 8 plasma samples from 8 mice per group only on muscle mesenchymal progenitor cells – interstitial cells as they mineralised the best and satellite cells as internal muscle control (Fig.7.3).

Figure 7.3 Effect of plasma from SCI and SHAM-operated mice on osteogenic differentiation of interstitial and satellite cells. (A) Alizarin Red staining of mouse bone marrow and skeletal muscle cell populations. Rows of wells contained muscle interstitial cells (top), muscle satellite cells (bottom) cultured for 3 weeks in tissue culture medium supplemented with plasma harvested from mice with SHAM surgery and SCI with CDTX injection. The red staining shows mineralized calcium deposited at the bottom of the wells.
(B) Quantification of mouse plasma effect on osteogenic differentiation of mouse bone marrow and skeletal muscle cell populations by Alizarin Red staining for each cell population. The histograms show the quantification of the Alizarin Red staining by light absorbance at 562nm of cells cultured in tissue culture media supplemented with mouse plasma. Data are mean ± SD of eight wells of a typical experiment. Plasma used in each well was harvested from different mice. *** p<0.001

This result suggests that systemic factors that may promote mineral deposition by muscle derived progenitor cells are released in the circulation following SCI and intramuscular inflammation.

7.3.3 Substance P enhances osteogenic differentiation of muscle and bone marrow mesenchymal progenitors

One of the possible endocrine factors released in response to spinal cord injury is substance P, an 11 amino acid residues peptide (Arg Pro Lys Pro Gln Gln Phe Phe Gly Leu Met). It is known as neuromediator of nociception and physiological and immune responses to pain. Substance P is produced by neurons and macrophages in injured and diseased tissues [255]. Three types of SP receptors were previously identified, that are called neurokinin receptors type 1, 2 and 3 [256]. SP binds more strongly to the NK1 receptors however it binds with a lower affinity to NK2R and NK3R [258].

It has been previously reported that the highly selective NK1R antagonist RP67580 [267] or the deletion of the *NK1r* gene encoding substance P precursor significantly reduces HO in transgenic Nse-BMP4 mouse model of FOP [126].

To determine whether substance P could also be involved in HO development following SCI, we performed in vitro experiments with muscle mesenchymal progenitors and bone marrow MPCs, where we added substance P and Substance P receptor antagonist (RP67580) in culture media together with osteogenic factors to assess their effect on mineral deposition. Cell groups were sorted from hamstring muscles and bone marrow of naïve C57BL/6 mice as described in methods. Sorted cells were cultured until passage 2, and then were used for osteogenic differentiation assay. Mouse osteoblastic cell line MC-3T3 was used as a positive control for mineral deposition. Formation of calcium matrix was induced by substance P in a dose-dependent manner in all cell populations tested (Fig. 7.4A, 7.5A).

As substance P receptor antagonist was diluted in 10% ethanol, control wells were incubated with ethanol concentrations matching those substance P receptor antagonist
dilutions. As shown in Fig. 7.4C, ethanol did not affect calcium matrix formation stained by Alizarin Red. Substance P receptor antagonist decreased mineral deposition at concentrations between 1 and 5μM in a dose dependent manner (Fig. 7.4B, 7.5B). This effect was more profound with muscle interstitial cells, which have also shown the highest potential of mineral deposition in medium supplemented with mouse plasma. However increasing concentrations of RP67580 added to the osteogenic differentiation media caused an increase of calcium matrix formation in all cell populations.

**Figure 7.4** Effect of substance P and substance P receptor antagonist on osteogenic differentiation of muscle and bone marrow mesenchymal progenitor cells. Alizarin Red staining of muscle interstitial cells (1), muscle satellite cells (2), Sca1-CD34- muscle progenitor cells (3), Sca1+ bone marrow MPCs (4), bone marrow Sca1-CD34+ MPCs (5), and mouse osteoblastic cell line MC3T3 (6) cultured for 3 weeks in osteogenic medium supplemented with substance P (A), substance P receptor antagonist (B) or vehicle control (C). The red staining shows mineralized calcium deposited at the bottom of the wells. This experiment was performed twice on different batches of cells and showed similar results.
Figure 7.5 Quantification of osteogenic differentiation of murine bone marrow and skeletal muscle cell populations supplemented with substance P or substance P receptor antagonist by Alizarin Red staining. The histograms show the quantification of the Alizarin Red staining by light absorbance at 562nm of cells cultured for 3 weeks in osteogenic medium supplemented with substance P (A), substance P receptor antagonist (B). Data are mean ± SD of four wells of a typical experiment out of 5 independent sorts and experiments. X axis: muscle interstitial cells (1), muscle satellite cells (2), Sca1−CD34− muscle progenitor cells (3), Sca1+ bone marrow MPCs (4), bone marrow Sca1−CD34− MPCs (5), and mouse osteoblastic cell line MC3T3(6)
7.3.4 Substance P receptor antagonist RP67580 reduced SCI-HO in vivo

To test substance P involvement in SCI-induced NHO, mice that had undergone SCI and intramuscular injection of CDTX, were injected twice daily with the selective substance P receptor NK1R antagonist RP67580 at 2mg/kg per dose or vehicle alone from immediately after SCI surgery and CDTX injection. Mice were sacrificed 10 days after surgery, and microCT was performed. The heterotopic bone mass was moderately but significantly reduced by 27.6% in the group where treatment was performed (p-value: 0.0418*, Mann-Whitney test) (Fig.7.6). The bone density did not differ between the groups.

Figure 7.6 Effect of substance P receptor antagonist on NHO. (A) Illustrative μCT of right hind limbs after SCI with injection of CDT and treatment with Substance P receptor antagonist; Quantification of HO volume (B) and density (C) by 3D μCT reconstitution following SCI, intramuscular CDT injection and treatment with Substance P receptor antagonist. Data are mean±SD of 6 mice per group pooled from two different experiments.
7.3.5 Spinal cord injury and inflammation do not affect plasma and muscle concentration of substance P

Substance P was measured in plasma of wild-type C57BL/6 mice with SCI and inflammation induced by CDTX injection and compared with plasma harvested from SHAM operated mice with and without inflammation and plasma harvested from mice with SCI alone. The measurements were also performed on protein extracted from the hind limbs of the same groups of mice. Time points included day 1, 2, 3 and 4 after surgery. Plasma and muscle protein standard curves and equation are presented on Fig.7.7 A,B.

![Standard curves for plasma (A) and muscle extracted protein (B) for the substance P quantification assay.](image)

Upon plasma analysis we noticed significant decrease in the substance P concentration at Day 2 time point in all 4 groups (Fig.7.8E). However by the day 3 subsequent increase of substance P concentration was found in groups of mice where local inflammation was caused by CDTX injection which then plateaus between Day3 and 4 (Fig.7.8A,B,D). Surprisingly this increase was also seen in SHAM operated mice. Substance P concentration in plasma harvested from mice with SCI did not change drastically within first 4 days after surgery apart from the aforementioned decrease at the day 2 (Fig.7.8C).
Figure 7.8 Substance P quantification in plasma harvested from mice with SCI+CDT (A), CDT alone (B), SCI alone (C) and SHAM-operated mice (D). Each dot represent single animal. Data are mean ± SD. Combined time course for all four experimental groups of animals (E); p-value: <0.05*; 0.01-0.001**, <0.001*** Two-way ANOVA with Bonferroni posttest.
Interestingly, substance P concentration in mouse protein extract showed similar drop to plasma substance P concentration (Fig.7.9E) at Day 2. However this drop was followed by an increase by the Day 3, similarly to plasma concentration amount of substance P in the muscle extract drastically decreased by the Day 4 time point in all four groups, reaching the same values. The decrease at Day 2 also was not as significant between the groups as we have seen in plasma substance P concentration (Fig.7.9A,B,C,D). Substance P concentration in muscles from SCI-operated mice has shown minimal changes throughout the first 4 days (Fig,7,8C) in comparison with the other three groups of operated mice. No significant differences between the groups were found at any particular time points (Day1, Day2, Day3 and Day4).
Figure 7.9 Substance P quantification in protein extracted from hind limb muscles in pg/μg of protein, harvested from mice with SCI+CDT (A), CDT alone (B), SCI alone (C) and SHAM-operated mice (D). Each dot represents a single animal. Data are mean ± SD. Combined time course for all four experimental groups of animals (E); p-value: <0.05*; 0.01-0.001**, <0.001*** Two-way ANOVA with Bonferroni posttest.
However despite of minor differences in concentration of substance P both in muscle protein extract and plasma, in all groups substance P concentration was below mouse normal range (60.6±2.1ng/mL [268]).

7.4 Discussion

Our observation that muscular inflammation in the front limb, above the SCI, still promotes NHO suggests that factors priming resident muscle progenitor cells to osteogenic fate could be either released in the circulation or locally induced by a feed-back mechanism from the injured central nervous system. These data in our mouse model are consistent with similar occurrence of NHO in non-paralyzed shoulder or elbow in NHO patients [5]. However the NHO volume in the non-paralyzed front limb was considerably smaller than in the hind limb injected with CDTX. In a normal mouse hind limb/front limb ratio is 6/1. However there is a reduction in muscle volume of the hind and front limbs after SCI [188]. By the end of second week the hind limb muscle volume is reduced by 28% and the front limb muscle volume is reduced by 21%, thus decreasing the hind limb/front limb ratio to 3/1. Interestingly the NHO volumes ratio between hind and front limbs is around 10/1, which is not compatible with the amount of muscles. This suggests that despite that SCI stimulates release of systemic factors contributing to NHO, the biggest effect is seen in the immobilized limbs.

The fact that the blood plasma of mice that underwent both SCI and CDTX intramuscular injury accelerated mineralization of cultured muscle interstitial cells supports the hypothesis that osteogenic factors may be released after SCI and muscular inflammation which prime NHO formation from muscle progenitors. Consistent with this, blood serum from TBI patients has been reported to enhance proliferation and induce expression of osteoblast markers: osterix in cultures of primary skeletal muscle cells [269], and osterix, Runx2 and alkaline phosphatase in the human osteoblastic cell line FOB1.19 [41]. It has been also published earlier that plasma from rodents with severe burns also induced osteogenic differentiation of muscle MPCs [270]. This is an interesting fact, as severe burns also have a potential to cause HO formation in patients [271]. Considering that substance P is expressed by neurons and macrophages [254] and stimulates osteoblast differentiation in mice and human osteoblastic cell line [260, 261, 272] and that NK1R antagonists or deletion of the NK1r gene encoding substance P precursor significantly reduces HO in transgenic Nse-BMP4 mouse models of FOP [126], we decided to investigate first whether substance P could be one of the priming factors released in response to SCI. We found that substance P concentration was below the
normal mouse range in plasma (60.6±2.1ng/mL [268]) of all operated mice, despite differences between the groups. This could be due to the differences in methods of quantification of substance P. At Day 2 post-surgery we have noticed significant decrease in the substance P concentration both in plasma and muscle protein, which is consistent with results described by Salisbury et al. in their model of FOP in which HO are caused by injection of mouse fibroblasts transduced with an adenovirus carrying BMP-2 [36]. Administration of RP67580, a selective antagonist of the substance P receptor NK1R reduced NHO by approximately 30% in our mouse model. Although this effect was statistically significant, this reduction was more modest than that observed in mouse genetic models of FOP where transgenic Nse-BMP4 mice were injected with CDTX, which was around 70% [126]. RP67580 has been shown to be highly selective for NK1 receptors (with K_i value of 4.16±0.59nM) which are the main receptors for Substance P binding and did not show any affinity to other neuropeptide receptors [273]. RP67580 was not active on NK2 and NK3 receptors as seen in binding assays and in isolated preparations on rabbit and rat tissues [264, 273]. It has also shown poor affinity for dopamine, serotonin, adrenergic and a variety of peptide receptors. However while RP67580 does not interact with other two types of neurokinin receptors, substance P still has low affinity to these receptors, and may still activate them which was a limitation of our experiment and could be associated with partial block of NHO formation [263, 264]. An alternative explanation is that substance P is only one of several pro-osteogenic factors released in the circulation in response to SCI and muscular inflammation. Proteomics studies comparing protein content of plasma from mice that underwent SCI alone or SCI plus intramuscular CDTX may provide additional clues to this question. It is unknown at this stage whether NHO reduction after RP67580 treatment was associated with dampened neuroinflammation or decrease in local monocyte infiltration in the muscles or delay in osteoblast differentiation as substance P has been reported to be involved in all of aforementioned processes.

Several markers were found in human serum from patients with central nervous system injury, such as C-reactive protein, erythrocyte sedimentation rate, Interleukin-6, Parathyroid hormone, Alkaline Phosphatase, tumour necrosis factor-alpha (TNF-α), each of them could be a potential biomarker for HO [38]. Obviously it is still unclear from the literature which factors are released in the blood as a result of SCI or as the result on the combination of neurodamage and muscular inflammation that both are required for the heterotopic bone formation. More work is required to identify these factors and establish firm mechanistic connections between
plasma markers and osteogenic differentiation of muscle mesenchymal progenitors to develop NHO.
Chapter 8: General Conclusion
The etiopathology of NHO is poorly understood for two reasons. Firstly, most research in patients is retrospective, no studies on the earliest stages of NHO have occurred to aid identification of the initiating cellular and molecular events underlying HO in muscles and joints. Secondly, there is no clinically-relevant animal model of NHO after central nervous system (CNS) lesions [125]. The only existing animal models of HO are genetically modified mouse models of progressive osseous heteroplasia [127], and FOP [18, 46, 48, 125]. Development of a non-genetically modified animal model of NHO would allow investigation of the mechanism linking the original neurological lesion to HO, and ultimately provide a model for pre-clinical testing of treatments to prevent or reduce NHO. I have addressed a number of questions in this project – 1) what is the source of the muscle osteoblasts responsible for making bones in muscles (are they derived from the muscle itself, or are they recruited from the skeleton and bone marrow via the blood); 2) which factors are produced in response to spine disruption that predispose bone formation in muscles; 3) whether inflammation in the muscle is the event triggering this abnormal ossification; 4) is there another agent other than cardiotoxin that can instigate heterotopic ossification in murine model of spinal cord injury? These questions were answered to some extent during the course of my PhD. Overall there are five key findings of my project:

- SCI-NHO requires a combination of spinal cord injury and local muscle inflammation, which is induced by cardiotoxin in our mouse model;
- Inflammatory macrophages play an important role in bone formation in our model of SCI-NHO;
- In the presence of SCI macrophages are activated differently in comparison with mice that have got CDTX-induced inflammation combined with SHAM surgery;
- SCI promotes release of systemic factors that prime osteogenic differentiation of mesenchymal progenitor cells;
- Mesenchymal progenitor cells which are capable of osteogenic differentiation are presented locally in the muscles.
While transgenic mouse models of HO in which BMP signaling is increased by means of introduction of transgenes or constitutively active mutants of BMP receptors are physiologically relevant models of FOP (a very rare genetic disease involving activating mutations of \textit{ACVR1}) they are irrelevant to model NHO in otherwise genetically normal patients.

Therefore we have developed a clinically-relevant mouse model of NHO following SCI which displays the pathophysiology seen in patients with NHO. In most cases we are assessing NHO by bone volume and bone density by microCT. NHO in our mouse model and patients have the same pattern of growing where NHO forms as small foci of ossifications that grow with time merging into a big mass. The involvement of local inflammation and macrophages is also common to our model and NHO in patients. Our mouse model clearly demonstrates that it is the combination of SCI and local muscle inflammation that is the underlying mechanism in NHO development with either insult alone being insufficient. This suggests that SCI could prime mesenchymal or muscle progenitor cells towards osteogenic differentiation, by releasing factors such as substance P, whilst local macrophage-dependent inflammation triggers and drives site-specific osteogenic differentiation leading to heterotopic ossification. Our mouse model is highly consistent with the clinical observations as SCI and TBI patients that develop NHO as the incidence of NHO in patients is higher in cases of concomitant infection or severe trauma [5], including much higher incidence in war casualties who are more likely to have these co-morbidities than civilians [35].

Our model is consistent with the clinical observation that ossification can be reduced by NSAID in NHO patients [49, 52, 53], and with our previous observations that specific subsets of resident macrophages are critical to the maturation of osteoblasts and bone formation in skeletal bones [114, 120]. In long bones, macrophage depletion induces a rapid loss of mature osteoblasts on bone surface, arrests bone formation and mineralization in adult mice [114, 120], and impairs bone repair following fracture [147]. Emerging data also suggest that inflammatory macrophages can promote osteoblast and bone formation [274]. We have excluded potential involvement of splenic population of monocytes into NHO formation after SCI by performing a set of experiments with splenectomised mice. The results of experiments on CCR-2 knockout mice suggest that local muscle tissue macrophages are involved in the process of heterotopic bone formation. Alternatively macrophages could be recruited from distant sited independently of CCR-2. Whether macrophages directly or indirectly support ossification in NHO, their
potential molecular mediators and which population of macrophages is driving NHO are subjects of ongoing research. The finding that muscular inflammation in the front limb, above the SCI, also promoted NHO, suggests that factors priming resident muscle progenitor cells to osteogenic fate could be released in the circulation or induced locally by a feed-back mechanism from the injured CNS. The ability of plasma from mice with SCI to facilitate mineralization of cultured muscle interstitial cells supports the former hypothesis but does not exclude the latter. Consistent with this, blood serum from TBI patients induces expression of osterix in primary skeletal muscle cells [269], and osterix, Runx2 and alkaline phosphatase in the human osteoblastic cell line FOB1.19 [41]. We have performed gene microarray analysis that revealed up-regulation of inflammatory cytokines IL-1β, TNF-α, IL-6 and CSF-1 together with up-regulation of M2 macrophage markers in SCI-operated mice with CDTX-induced inflammation – the only group that develops NHO. Along with these results, we compared mice with CDTX-induced inflammation with and without SCI. Despite that both groups of mice were injected with CDTX they had differences in inflammatory signaling process that were up-regulated in SCI+CDTX group. Interestingly the group of mice with SCI alone had increased up-regulation of inflammatory markers in comparison with SHAM-operated mice confirming previously published results about SCI-mediated inflammatory response [142, 143, 275].

In our mouse model, development of NHO was accompanied by an osteoporotic phenotype with degradation of skeletal bones, similar to osteoporosis in NHO patients [276, 277] suggesting that hyperphosphatemia and hyperparathyroidism could fuel developing HO. However, phosphatemia and blood PTH concentrations remained within the normal range in our mice developing HO following SCI and CDTX injection. We finally found that substance P is a possible candidate as one of the osteogenic priming factors released in response to SCI. Together with osteogenic potential substance P has been reported to reduce neuroinflammation after injury. By blocking substance P signaling we managed to partially but significantly reduce NHO in our model by 30%. The incomplete reduction in NHO after NK1R antagonism might be associated with low affinity of substance P to NK2R and NK3R and suggests that substance P may play a mediatory role and that alternative systemic mediators of NHO which remain to be identified, will represent more effective pharmacologic targets.

Finally, our model suggests that the osteoblasts responsible for NHO in muscles are not necessarily derived from mesenchymal progenitors recruited from the bone marrow. Two types of mesenchymal progenitor cells were found locally in the muscles: satellite cells and...
interstitial cells [100]. In the series of experiment we have shown that muscle interstitial cells are prone to mineralization and osteogenic differentiation in vitro in shorter time frames in comparison with SC and bone marrow mesenchymal progenitor cells. Also interstitial cells have readily formed mineralized matrix upon culture in media supplemented with plasma from mice developing NHO. Although this remains to be demonstrated genetically with lineage tracking experiments, muscle satellite cells, interstitial cells or MPCs sorted from the muscle of naïve mice, which all have the potential to regenerate myofibers in vivo following muscle injury [100, 278], were all capable of osteogenic differentiation in vitro as previously reported in mouse [109] and human [250].

In conclusion, a significant advantage of our model over the only other existing HO models [18, 26, 46, 48] is that it occurs in non-genetically modified mice. We have developed the first pre-clinical model of NHO that reliably reproduces disease pathophysiology without the necessity of creating genetic susceptibilities (e.g. introduction of a mutant gene or overexpression of a gene involved in BMP signaling) that are not clinically relevant to a large proportion of patients that suffer from NHO. This unique model could be used to understand the mechanisms causing this complication and test treatments that could prevent it. Importantly it provides a clinically relevant animal model to test drugs for their ability to reduce or prevent NHO in patients that include a high proportion of injured soldiers, with SCI. Finally the ramifications of our findings to basic stem cell biology are important as it illustrates how the cross regulation between CNS and innate immunity can lead to pathological osteogenic differentiation of muscle cells instead of myogenic repair.

I have also improved our mouse model during the course of my PhD. Considering the CDTX affects muscle tissue very intensively causing massive necrosis of the underlying tissues, these stimuli may be different to what happens clinically. I found that combination of SCI with crush-injury and LPS-mimicking bacterial infection may be more adequate model to test drug efficacy and will be used in further testing of potential treatment options. Thus our future directions include testing Etanercept and Substance P receptor antagonist that have shown partial benefit in SCI+CDTX model on our new physiological mouse model of NHO after SCI. We consider this new model more adequate and close to what happens clinically as it has been reported earlier that NHO develops more often in patients with severe concomitant trauma or opportunistic infection. Therefore a combination of both together with SCI-injury should reveal mechanisms that are involved in this debilitating process.
Summarizing all the findings together, our model suggests that NHO requires SCI that stimulates the release of factors that sensitize muscle mesenchymal progenitor cells to abnormal osteogenic differentiation in muscles, however this process doesn’t happen without inflammatory macrophages accumulating in inflamed muscles then triggering abnormal osteogenic differentiation of muscle MPC. The potential impact of the project is four-fold: scientific, medical, social and financial. Scientifically the novelty of this project lies in the generation of new mouse model of heterotopic ossification after spinal cord injury and the enlightening of mechanisms leading to that. Insights gained from this project could lead to new research with the aim to supress new bone formation in damaged muscles and to changes in heterotopic ossification treatment. This may lead to better repair of tissue damage, reduction of new bone formation, and improvement of quality of life. The project social significance consists in changing demographic parameters of mortality and morbidity and increasing the rehabilitation success, leading to financial benefits to the health care system.
Bibliography

85. Pelekanos, R.A., et al., Comprehensive transcriptome and immunophenotype analysis of renal and cardiac MSC-like populations supports strong congruence with


91. Yin H Fau - Price, F., M.A. Price F Fau - Rudnicki, and M.A. Rudnicki, Satellite cells and the muscle stem cell niche. (1522-1210 (Electronic)).


Appendix A: Animal Ethics Approval Certificate

Dear Associate Professor Jean Pierre Lusasque

The following project: Development model of acquired heterotopic ossification in mice to determine the role of the mesenchyme and inflammatory factors

Requesting funding from (Grant Awarding Body) - involves animal experimentation. It has been reviewed and ethical clearance obtained from the University Animal Ethics Committee (Health Sciences).

AFC Approval Number: MIR147011

Previous AFC Numbers:

Approval Duration: 02 Mar 2012 to 01 Mar 2014

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Proviso(s):

Please note the animal numbers supplied on this certificate are the total allocated for the approval duration.

Please use this Approval Number:
1. When ordering animals from Animal Breeding Houses
2. For labeling of all animal cages or holding areas. In addition please include on the label, Chief Investigator's name and contact phone number.
3. When you need to communicate with this office about the project.

It is a condition of this approval that all animal usage details be made available to Animal House OIC.

(LKEC ruling 14/12/2001)

This certificate supersedes all preceding certificates for this project (i.e. those certificates dated before 01 Mar 2012)
Appendix B: Animal Ethics Approval Certificate

Animal Ethics Approval Certificate
Please check all details below and inform the Animal Welfare Unit within 10 working days if anything is incorrect.

Activity Details
Chief Investigator: Associate Professor Jean-Pierre Levesque, Mater Research Institute-UQ
Title: Model of acquired heterotopic ossification following spinal cord injuries in mice
AEC Approval Number: TRI/MATER/054/14/MATER/NHMRC
Previous AEC Number: MMRI/470/11/
Approval Duration: 08-Apr-2014 to 08-Apr-2017
Funding Body: Mater Trust, NHMRC
Group: Health Sciences
Other Staff/Students: Ingrid Winkler, Valerie Barbier, Thibaud Honore, Thomas Kecch, Bianca Nowlan, Joshua Tay, Roland Steck, Mel-Fong Ho, Francois Genet, Irina Kulina, Rebecca Jacobsen, Siamak Safi-zadeh, Marc Ruikenberg, Lisa Craig
Location(s): PA Hospital Translational Research Institute (TRI)
Princess Alexandra Hospital (PAH)

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<td>16</td>
</tr>
<tr>
<td>20 Mar 2014 Initial approval</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mice - genetically modified (LynMCre+/IL-4Ralpha-/-, Mix, Adults, Institutional Breeding Colony)</td>
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<td>16</td>
</tr>
<tr>
<td>20 Mar 2014 Initial approval</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mice - genetically modified (LynMCre/IL-4Ralpha-flox, Mix, Adults, Institutional Breeding Colony)</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>20 Mar 2014 Initial approval</td>
<td></td>
<td></td>
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<tr>
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<td>16</td>
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</tr>
<tr>
<td>Mice - genetically modified (Pax7Cre Rosa26R-iDTR, Mix, Adults, Institutional Breeding Colony)</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td>20 Mar 2014 Initial approval</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mice - genetically modified (Pax7Cre Rosa26R-Zsgreen, Mix, Adults, Institutional Breeding Colony)</td>
<td>130</td>
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</tr>
<tr>
<td>20 Mar 2014 Initial approval</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mice - genetically modified (Pax7CreER, Mix, Adults, Institutional Breeding Colony)</td>
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</tr>
<tr>
<td>21 Aug 2014 Mod #2</td>
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</tr>
<tr>
<td>Mice - genetically modified (Pax7CreER x Hif1aflox/flox, Mix, Adults, Institutional Breeding Colony)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Page 2 of 3
<table>
<thead>
<tr>
<th>Date</th>
<th>Approval Number</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>21 Aug 2014</td>
<td>Mod #2</td>
<td>Mice - genetically modified (Prrx1Cre Rosa26R iDTR, Mix, Adults, Institutional Breeding Colony)</td>
</tr>
<tr>
<td>20 Mar 2014</td>
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<td>Initial approval</td>
</tr>
<tr>
<td>20 Mar 2014</td>
<td>32</td>
<td>Initial approval</td>
</tr>
<tr>
<td>23 Oct 2014</td>
<td>16</td>
<td>Mice - genetically modified (RAG1 +/-, Mix, Adults, Institutional Breeding Colony)</td>
</tr>
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<td>16</td>
<td>Mod #3</td>
</tr>
<tr>
<td>20 Mar 2014</td>
<td>976</td>
<td>Mice - non genetically modified (C57BL/6, Mix, Adults, Institutional Breeding Colony)</td>
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<td>104</td>
<td>Mod #2</td>
</tr>
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<td>23 Oct 2014</td>
<td>80</td>
<td>Mod #3</td>
</tr>
</tbody>
</table>

Please note the animal numbers supplied on this certificate are the total allocated for the approval duration.

Please use this Approval Number:
1. When ordering animals from Animal Breeding Houses
2. For labelling of all animal cages or holding areas. In addition please include on the label, Chief Investigator's name and contact number.
3. When you need to communicate with this office about the project.

It is a condition of this approval that all project animal details be made available to Animal House OIC.
(UAEC Ruling 14/12/2001)

The Chief Investigator takes responsibility for ensuring all legislative, regulatory and compliance objectives are satisfied for this project.

This certificate supercedes all preceeding certificates for this project (i.e. those certificates dated before 30-Oct-2014)
# Appendix C: Mice Score Sheet

## SCORE SHEET FOR SCORING ENDPOINTS IN MICE

*(Scoring to commence on the day of surgery)*

<table>
<thead>
<tr>
<th>Indicators</th>
<th>Scoring of independent variables:</th>
<th>After hours:</th>
<th>Score of each animal in group</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>General Health</strong></td>
<td></td>
<td></td>
<td>Day1</td>
</tr>
<tr>
<td><strong>Eating</strong></td>
<td>0. Drinking and eating well</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1. Change in eating or drinking habit</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2. Inappetence</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3. Not eating/drinking, severely dehydrated</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Locomotion</strong></td>
<td>0. Full extension of hind legs away from lateral midline when mouse is suspended by its tail. The mouse is able to sustain extension for 2 seconds, suspended 3 times.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1. Collapse of partial collapse of leg extension toward lateral midline or trembling of hindlegs during tail suspension</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2. Rigid paralysis of hindlimbs at least one foot not being used for forward movement.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3. Mouse is unable to right itself within 30 seconds after being placed on its back.</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Behaviour</strong></td>
<td>0. Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1. Away from littermates</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2. Aggressive or huddled in corner</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3. Severe distress</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Appearance</strong></td>
<td>0. Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1. Ruffled fur</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2. Weeping eyes</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3. Weeping/closed eyes, urine staining, difficulty defecating</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Weight loss</strong></td>
<td>0. Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(scored every 2nd day)</td>
<td>1. 5-10%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2. 10-15%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3. &gt;15% over 3 days</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

## TOTAL SCORE

For total scores

<table>
<thead>
<tr>
<th>Score</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-4</td>
<td>Normal: No action</td>
</tr>
<tr>
<td>5-10</td>
<td>Moderate changes: Should be monitored daily</td>
</tr>
<tr>
<td>&gt;10</td>
<td>Significant changes: Monitor twice daily</td>
</tr>
</tbody>
</table>

**Immediate euthanasia**

1. A score of 3 in any one category
2. Loss of the righting reflex (unable to right within 30 seconds of being placed on their back). Any persistent signs of undue health (eye infections etc). Excessive weight loss (greater than 10% from the previous day). Complete paralysis of any hindlimb that renders the animal incapable of reaching food and water.

Signature of person scoring: ..........................................................
Appendix D: Abstracts and presentations

Oral:

2015
- 05/2015 – Australian Health and Medical Research Postgraduate Student Conference (oral presentation selected from abstract) – won People’s Choice Award in Oral Presentations

2014
- 11/2014 – Australian Health and Medical Research Congress 2014 (oral presentation selected from abstract for Australian Society for Medical Research)
- 11/2014 – Australasian Stem Cell Science And Therapy Meeting 2014 (oral presentation selected from abstract) – won National Stem Cell Foundation of Australia travel award
- 10/2014 – TRI Poster Symposium 2014 (oral presentation selected from abstract)
- 09/2014 – Australian and New Zealand Bone and Mineral Society, 2014 Annual Scientific meeting (oral presentation selected from abstract) – won travel award, Roger Melick Young Investigator Award
- 06/2014 – Mater Research 3 Minute Thesis Competition, TRI, Brisbane (public talk)

2013
- 09/2013 – TRI Friday Seminar Series, Brisbane (invited seminar)

Poster:

2014
- 09/2014 – Australian and New Zealand Bone and Mineral Society, 2014 Annual Scientific meeting (poster selected from abstract) – won travel award
- 05/2014 – Postgraduate Medical Research Student Conference, the Australian Society for Medical Research, Brisbane

2013
- 10/2013 – 6th Australian Society for Stem Cell Research Annual Meeting, Brisbane (poster selected from abstract) – won the best student poster prize, travel award
- 05/2013 – Postgraduate Medical Research Student Conference, The Australian Society for Medical Research, Brisbane (poster selected from abstract) – won travel award
2012

- 11/2012 – 5th Australian Society for Stem Cell Research Meeting, Leura, NSW (poster selected from abstract) – won the best student poster prize, travel award
- 11/2012 – Early Career Researcher Poster Symposium, Brisbane
- 05/2012 – Mater Medical Research Institute 2012 Stem Cell Symposium, Brisbane
- 05/2012 – Australian Society for Medical Research Postgraduate Medical Research Student Conference, University of Queensland, Brisbane
- 04/2012 – The TRI Student Retreat, Brisbane

2011

- 10/2011 – 4th Australian Society for Stem Cell Research Annual Meeting, Leura, NSW (poster selected from abstract)

**Awards:**

2015

- 2015 – People's Choice Award in Oral Presentations, Australian Society for Medical Research, Postgraduate Student Conference

2014

- 2014 – Mater Research Higher Degree Award
- 2014 – National Stem Cell Foundation of Australia travel award to attend the Australasian Stem Cell Science and Therapy meeting 2014
- 2014 – Roger Melick Young Investigator Award
- 2014 – Winner of Australian and New Zealand Bone and Mineral Society Travel Grant
- 2014 – Winner of a Mater Student Travel Award

2013

- 2013 – Winner of a Poster Prize at the 6th Australasian Society for Stem Cell Research Meeting
- 2013 – National Stem Cell Foundation of Australia Conference Education Award

2012

- 2012 – Winner of a Student Poster Prize at the 5th Australasian Society for Stem Cell Research Meeting
- 2012 – Australian Society for Medical Research Student Travel Subsidy to attend Congress in Adelaide
- 2012 – Travel Award to attend the Australasian Society for Stem Cell Research 2012 meeting
2011

- 2011 – Mater Medical Research Institute Top-up scholarship. This scholarship commenced in July 2011
- 2011 – University of Queensland International Scholarship (tuition fees award and living allowance). This scholarship commenced in July 2011